Long-term monitoring of ten selected pathogens in wild boar (*Sus scrofa*) in
Sierra Nevada National Park, southern Spain

Francisco J. Cano-Manuel ^a, Jorge López-Olvera ^b, Paulino Fandos ^c, Ramón C.
Soriguer ^d, Jesús M. Pérez ^e, José E. Granados ^a

^a Espacio Natural Sierra Nevada, Carretera Antigua de Sierra Nevada, Km 7, E-
18071, Pinos Genil, Granada, Spain.

franciscoj.canomanuel@juntadeandalucia.es,

josee.granados.ext@juntadeandalucia.es

^b Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina
i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), Bellaterra,
Barcelona E-08193, Spain. elrebeco@yahoo.es

^c Agencia de Medio Ambiente y Agua, Isla de la Cartuja, E-41092, Sevilla, Spain
pfandos@agenciamedioambienteyagua.es

^d Estación Biológica de Doñana (CSIC), Av. Américo Vespucio, s.n., E-41092
Sevilla, Spain. soriguer@ebd.csic.es

^e Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad
de Jaén, Campus Las Lagunillas, s.n., E-23071, Jaén, Spain. jperez@ujaen.es

ABSTRACT

Wild boar (*Sus scrofa*) populations are increasing in the Iberian Peninsula, and population management must include disease management and control. In this study, the epidemiology of ten selected pathogens (Aujeszky's disease virus - ADV-, porcine reproductive and respiratory syndrome virus -PRRSV-, porcine influenza virus, porcine circovirus, porcine parvovirus, *Erysipelotrix rhusiopathiae*, *Leptospira pomona*, *Chlamydia/Chlamydiaceae* sp., *Salmonella* sp. and *Mycobacterium bovis*) in the wild boar population in Sierra Nevada National Park (SNNP), an open unfenced area, is reported, taking into account wild boar population abundance variation in space and time in an open unfenced environment. A total of 1,103 wild boar were sampled in 141 hunting events randomly carried out for sampling in seven hunting seasons (October to February from 2002-2003 to 2009-2010, (except 2007-2008). Prevalence was overall lower than those previously reported for fenced wild boar populations in Spain, but all the pathogens analyzed except PRRSV were considered endemic in the SNNP. ADV, *E. rhusiopathiae* and total pathogen prevalence were positively correlated to wild boar density. Prevalence in the positive areas was significantly higher in females for ADV, *E. rhusiopathiae*, *L. pomona*, *Chlamydia/Chlamydiaceae* sp. and *Salmonella* sp., and in males for *M. bovis*. This longitudinal study provides the first data on the health status of the relatively unmanaged and low density wild boar population of SNNP. It is concluded that non-intensively managed wild boar populations are able to maintain the circulation of several pathogens, even in low prevalences and in open unfenced areas with natural density variation both in time and space.

Keywords: *Sus scrofa*, density, pathogens, prevalence

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49 **Introduction**

50 During the last decades, wild boar has experienced a population and
51 distribution range increase in Europe, including the Iberian Peninsula (Fruzinski,
52 1995; Leránoz and Castién, 1996), although **population oscillation and decrease**
53 **in open mountain areas have been reported** (Sarasa and Sarasa, 2013). Wild
54 boar can act as a reservoir species for a number of pathogens (Parra et al.,
55 2006), and population management must include health **issues in** order to
56 improve disease management and control (Naranjo et al., 2008). Intensive
57 management of wild boar for hunting purposes in south-central Spain, which
58 usually includes estate fencing and supplementary feeding, increases the
59 prevalence and transmission of diseases such as bovine tuberculosis,
60 Aujeszky's disease, and type 2 porcine circovirus (Gortázar et al., 2006; Vicente
61 et al., 2004, 2005 and 2006).

62

63 Wild boar home range is very variable, and depends on season, food
64 availability, reproductive status, presence of refuge areas and risk avoidance
65 (Thurfjell et al., 2009). **Spatial** distribution may **also** be affected by forest
66 fragmentation (Virgós, 2002). Wild boars are mainly sedentary (Spitz et al.,
67 1984), but they may travel long distances sporadically (Andrezejewski and
68 Jezierski, 1978). Population and migratory movements are highly variable
69 depending on the location (Sarasa and Sarasa, 2013), which may have an
70 effect on the spread of **diseases (Vieira-Pinto** et al., 2011). Wild boars may
71 aggregate depending on refuge search, food availability, and social behaviour

within their matriarchal social structure, and this aggregation has a determining role in the epidemiology of several diseases (Vicente et al., 2005).

The objective of this study is to describe the spatial distribution and temporal evolution of the prevalence of antibodies against nine selected pathogens and *M. bovis* detection in the wild boar population in Sierra Nevada National Park (SNNP), taking into account wild boar density variation in space and time in an open unfenced environment.

Material and Methods

Study area

The SNNP is an 86,210 hectares protected area, belonging to 44 municipalities and surrounded by the 88,965 hectares of the Sierra Nevada Natural Park, accounting for a total of 175,175 hectares which form the Sierra Nevada Natural Space (SNNS). For the purpose of this study, only the 26,588 hectares of continuous forest within the SNNP, composed of large continuous pine tree (*Pinus* sp.) reforestations, dense scrubland areas and oak patches (*Quercus* sp.), were considered. The SNNS is an open unfenced area with a Spanish ibex (*Capra pyrenaica*) population of around 17,500 individuals, which move in the SNNP and the surrounding Natural Park. In the 44 municipalities of the SNNP, more than 76,000 domestic ruminants (38,927 sheep, 32,047 goats and 5,985 cattle) graze extensively in spring and summer (three to five months). Conversely, there is practically no domestic pig in the SNNS. Cattle herds without the officially tuberculosis-free status (as defined in the EU Directive 64/432/EEC) are present in 13 out of the 44 municipalities of the SNNP. There

is no supplementary feeding for wild boar, and hunting is permitted only for management purposes, which allowed sample collection for this study.

Sample collection

A total of 1,103 wild boars (395 males and 708 females) were sampled in 141 hunting events carried out in seven hunting seasons (October to February 2002-2003, 2003-2004, 2004-2005, 2005-2006, 2006-2007, 2008-2009 and 2009-2010), in areas with known wild boar high density. Mean wild boar age, assessed according to Borgo et al., 2007, was over 24 months, ranging from 6 to 60 months and sex was biased toward females (males 35.81%, females 64.19%). Blood was collected by heart puncture, placed in tubes without anticoagulant and allowed to clot at room temperature until its arrival to the laboratory. Then it was centrifuged at 1,200 g for 15 minutes within 24 hours from its collection, and sera frozen at -18 °C until analysis. For *M. bovis* analyses, tonsils and submandibular and retropharyngeal-parotid lymph nodes were collected and frozen also at -18 °C. Pulmonary parenchyma was also collected when tuberculosis-like lesions were observed. Sample size allows a good (3 to 5%) or moderate (5 to 10%) accuracy for an expected prevalence lower and higher than 10%, respectively (Epidat 3.1, Xunta de Galicia, Spain).

Sample analysis

Table 1 shows the serological analyses performed to detect antibodies against nine of the selected pathogens included in this study. The diagnosis of *M. bovis* infection was based on gross pathology and bacteria isolation and identification, as well as molecular detection (PCR).

Population density assessment

Population density was estimated for each hunting event, from the information obtained in the hunting events, as previously reported by Tellería and Saéz-Royuela (1985). Briefly, the number of wild boars observed during a hunting event was related to the known surface of the hunted area, in order to obtain a minimum density value for each area, expressed as observed wild boars by square kilometre. This methodology is adequate and reliable for areas with a continuous structure or forests (Mauget et al., 1984), provided it is properly designed in space and time to ensure representativeness (Pucet et al., 1975).

Epidemiological indexes

For each pathogen, prevalence was determined both for all the studied areas altogether (total prevalence or TP) and taking into account only the areas where each pathogen was detected (prevalence in positive areas or PPA).

Each pathogen was considered to be actively circulating when PPA was over 35% and mean optical density (MOD) or mean antibody titer (MAT) was as indicated for ADV (MOD<0.299), PRRSV (MOD<0.249), porcine influenza virus (MAT>1:80), porcine parvovirus (MAT>1:1,280), *Erysipelothrix rhusiopathiae* (MAT>1:300), *Leptospira pomona* (MAT>1:1,600), and *Salmonella* sp. (MAT>1:160). For porcine circovirus, *Chlamydia/Chlamydiaceae* sp., and *M. bovis*, where MOD or MAT could not be calculated, PPA>35% was the only criteria. The presence of a pathogen was considered endemic when active circulation (as defined above) was present more than half of the seasons

147 studied (Thrusfield, 1990). Finally, the presence of a pathogen has been
148 considered localized when there was territorial association in the active
149 infections detected (Thrusfield, 1990; White and Harris, 1995).

151 **Statistical analysis**

152 *Pathogen presence*

153 The effects of game season and spatial evolution on the presence (categorical
154 response variable, 1 infected and 0 for uninfected) of the analyzed pathogens
155 were explored by generalised lineal models (GLM) with binomial errors and logit
156 link function.

157
158 Similarly, the effects of sex, and age (categorical predictive variable, age
159 classes 6-9 months, 10-12, 13-24 and > 24 months) and wild boar density
160 (using the values obtained from the 141 hunting events) on the presence of the
161 analyzed pathogens were explored in this case by mixed generalised lineal
162 models (GLMM), also with binomial errors and logit link function. Sampling
163 season was included in the model as random factor in the density analysis.

165 *Number of pathogens*

166 The number of pathogens detected in the same individual was defined as
167 dependent variable, and the effects of sex, age and wild boar density (as
168 defined for pathogen presence) explored with GLMM, in this case with Poisson
169 error and logit link function. As for pathogen presence, sampling season was
170 included in the model as random factor in the density analysis.

Coinfection

The relationship between positivity to more than one pathogen in an individual, which could indicate interaction (either positive or negative) between pathogens, was analyzed. Briefly, the ratio of wild boars positive to one pathogen (dependent variable) which were also positive to each one of the remaining pathogens (predictive variable) was calculated and classified in four categories: up to 24.9%, 25.0 to 49.9%, 50.0 to 74.9% and over 75.0%. GLM with binomial errors and logit link function were used to assess the signification of all the associations among pathogens.

All the statistical analyses were performed in SAS 9.2 with PROC GLIMMIX (SAS Institute, Cary, NC, USA). R Package V.2.15.1 was applied to test the statistical significance.

Results

From all the wild boars analyzed, 67% showed evidences of contact with at least one of the studied pathogens. Table 2 shows TP and PPA values. TP did not differ significantly according to sex ($F_{1,1019}=0.12$; $p=0.730$), age ($F_{3,1019}=0.40$; $p=0.755$) or their interaction ($F_{3,1019}=1.46$; $p=0.225$). However, PPA was significantly higher in females for Aujeszky's disease virus (ADV) ($\chi^2=35.34$; 6 d.f.; $p<0.001$), *Erysipelothrix rhusiopathiae* ($\chi^2=47.72$; 6 d.f.; $p<0.001$), *Leptospira pomona* ($\chi^2=76.52$; 6 d.f.; $p<0.001$), *Chlamydia/Chlamydiaceae* sp. ($\chi^2=19.76$; 3 d.f.; $p<0.001$) and *Salmonella* sp. (serovar C) ($\chi^2=119.92$; 5 d.f.; $p<0.001$), and in males for *Mycobacterium bovis* ($\chi^2=18.69$; 6 d.f. $p=0.002$) (Table 2). *M. bovis*-positive wild boars were detected

in 19 out of the 44 municipalities of the SNNP. In six out of these 19 municipalities cattle herds without the officially tuberculosis-free status were present.

Mean wild boar density in the study areas reached 16.35 individuals/Km² (range: 0.20 - 81.50; Figure 1). Figure 2 shows the wild boar density values obtained for the SNNP throughout the study years, which varied significantly among years ($\chi^2=18.16$; d.f.=6; p=0.006) (Kruskal-Wallis $\chi^2= 34.53$; d.f.=6; p<0.001; range: 5.4 to 21.7 wild boars/Km²). Wild boar density was significantly and positively correlated with prevalence of ADV ($\chi^2=10.10$; d.f.=1; p=0.001; n=1,100), *E. rhusiopathiae* ($\chi^2=4.09$; d.f.=1; p=0.043; n=1,100), and for all the pathogens altogether ($\chi^2=8.48$; d.f.=1; p=0.003; n=1,103) (Figure 3).

Table 3 summarizes the epidemiological results and the classification for each pathogen (as defined in Table 2).

Among the significant associations found amongst the pathogens analyzed, only those of porcine parvovirus with ADV, porcine circovirus, and *Erysipelothrix rhusiopathiae* showed a relatively high value with an acceptable sample size. No spatial relationship between pathogen prevalence was observed.

Discussion

This longitudinal study provides the first data on the health status of the relatively unmanaged and low density wild boar population of SNNP, an open unfenced area. In spite of the low density of the wild boar population and the

relatively low prevalence of several of the pathogens studied, as compared with previous publications on fenced areas in Spain (Ruiz-Fons et al., 2006), eight out of the ten pathogens analysed were present throughout the whole study period and are considered endemic in the wild boar population of the SNNP (Tables 2 and 3). Since there is not a relevant domestic pig population in the SNNP and its surroundings, this finding suggests that wild boar act as true host for these pathogens, maintaining active infection focuses and pathogen circulation. The natural aggregation and unrestricted movements in unfenced areas of wild boar populations (Andrezejewski and Jezierski, 1978; Sarasa and Sarasa, 2013) may favour the maintenance and spread of pathogens. However, the low prevalences found in the SNNP seems opposite to the general increasing pathogen prevalence trend reported in wild boars populations elsewhere in the Iberian Peninsula (Vicente et al., 2002; Acevedo et al., 2006; Ruiz-Fons et al., 2006 and 2008).

It is widely accepted that for most diseases the effect and prevalence of pathogens increases with host population density (Ewald, 1993). The relationship between local population density and pathogen prevalence (ADV, *E. rhusiopathiae*, and total number of pathogens) agrees with previous reports for ADV in high density wild boar populations in estates fenced for game purposes (Ruiz-Fons et al., 2008).

The heterogeneous distribution in space and time of the pathogens analyzed in the SNNP are probably determined by several factors which interact, such as local population density, species behaviour, pathogen specific transmission

route, population management and environmental aspects, including climatology (Vicente et al., 2002). Natural reservoirs maintain pathogens in geographically restricted areas (White and Harris 1995) due to ecosystem features (Collins et al., 1986; Fulford et al., 2002). In wild boar, this has also been reported for porcine parvovirus in Croatia (Roic et al., 2005) and for tuberculosis in Spain (Vicente et al., 2006). The finding of *M. bovis* in wild boars in six municipalities with cattle herds without the officially tuberculosis-free status leaves a door open to the possibility that tuberculosis could circulate in the interface between domestic livestock and the wild boar population of SNNP. However, a more thorough study focused only in *M. bovis*, including cattle sampling as well as wild boar sampling and *M. bovis* strain identification and characterisation should be carried out to assess this possibility.

The absence of sex- and age-related differences in population prevalence agrees with previous reports for ADV, porcine reproductive and respiratory syndrome virus, porcine influenza virus, porcine circovirus, porcine parvovirus, *E. rhusiopathiae*, and *Salmonella* (Closa-Sebastià et al., 2011). Conversely, age-related differences in the seroprevalence porcine influenza virus, *Salmonella*, (Closa-Sebastià et al. 2011), porcine parvovirus (Roic et al., 2005; Ruiz-Fons et al., 2006), ADV and porcine circovirus (Vicente et al., 2005; Ruiz-Fons et al., 2006) have been previously reported.

The higher prevalence observed in the female wild boars for ADV, *E. rhusiopathiae*, *L. pomona*, *Salmonella* sp. and *Chlamydia/Chlamydiaceae* sp. is similar to those previously reported (Jridi et al., 1996; Lutz et al., 2003; Vicente

et al., 2005), and is probably related to higher intraspecific contacts and/or earlier breeding age in females as compared to males (Mauget and Pepin, 1985; Rosell et al., 2001). Conversely, the higher *M. bovis* prevalence observed in male wild boars could be related either to age, since there were more males than females younger than 24 months in matriarchal and mixed groups, and intraspecific transmission of tuberculosis has been reported to be high at early ages in wild boar from Spain (Vicente et al., 2006); or to the higher home range of males as compared to females (Massei et al., 1997), which would make males more likely to get in contact with *M. bovis*.

Regarding coinfection, porcine circovirus has been reported to increase susceptibility and lesion extension for bovine tuberculosis in wild boar, therefore discarding a mere exposure effect but suggesting a favouring pathological mechanism (Risco et al., 2013). Further research, including thorough post-mortem and analytical studies and even experimental consecutive infection should be carried out in order to clarify whether the relationships found in the present study are causal, consequential or related to exposure risk.

To summarize, this study shows non-intensively managed (i.e. free-ranging, not overcrowded, not artificially fed) wild boar populations are able to maintain the circulation of several pathogens in natural undisturbed open unfenced areas, with natural density variation both in time and space. Although badger (*Meles meles*) culling for tuberculosis management has been reported to contribute to disease dispersal due to increased movements and susceptible population (Jenkins et al., 2010; Riordan et al., 2011), this effect has not been observed in

wild boar (Mentaberre et al., 2014). Therefore, and since population density is statistically related with ADV, *E. rhusiopathiae*, and total pathogen prevalence, and since intensively managed high-density populations report higher prevalences than those found in the SNNP, keeping population density low seems an efficient management tool to control pathogen spread in wild boar populations. Maintaining a low wild boar population density should decrease contact possibility among populations and intraspecific transmission, but managers must be aware of the possibility of disease spread by high hunting pressure.

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Figure captions

Figure 1. Wild boar density in the municipalities from the SNNP. Low density below 2 wild boar/Km²; medium density between 2 and 4 wild boar/Km²; high density between 5 and 10 wild boar/Km²; and very high density over 10 wild boar/Km².

Figure 2. Wild boar density trend throughout the study years in the SNNP.

Figure 3. Statistically significant ($p < 0.05$) correlations found between wild boar density (wild boars/Km²) and pathogen prevalence.

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Francisco J. Cano-Manuel ^a, Jorge López-Olvera ^b, Paulino Fandos ^c, Ramón C.
Soriguer ^d, Jesús M. Pérez ^e, José E. Granados ^a

^a Espacio Natural Sierra Nevada, Carretera Antigua de Sierra Nevada, Km 7, E-
18071, Pinos Genil, Granada, Spain.

franciscoj.canomanuel@juntadeandalucia.es,

josee.granados.ext@juntadeandalucia.es

^b Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina
i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), Bellaterra,
Barcelona E-08193, Spain. elrebeco@yahoo.es

^c Agencia de Medio Ambiente y Agua, Isla de la Cartuja, E-41092, Sevilla, Spain
pfandos@agenciamedioambienteyagua.es

^d Estación Biológica de Doñana (CSIC), Av. Américo Vespucio, s.n., E-41092
Sevilla, Spain. soriguer@ebd.csic.es

^e Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad
de Jaén, Campus Las Lagunillas, s.n., E-23071, Jaén, Spain. jperez@ujaen.es

ABSTRACT

Wild boar (*Sus scrofa*) populations are increasing in the Iberian Peninsula, and population management must include disease management and control. In this study, the epidemiology of ten selected pathogens (Aujeszky's disease virus - ADV-, porcine reproductive and respiratory syndrome virus -PRRSV-, porcine influenza virus, porcine circovirus, porcine parvovirus, *Erysipelotrix rhusiopathiae*, *Leptospira pomona*, *Chlamydia/Chlamydiaceae* sp., *Salmonella* sp. and *Mycobacterium bovis*) in the wild boar population in Sierra Nevada National Park (SNNP), an open unfenced area, is reported, taking into account wild boar population abundance variation in space and time in an open unfenced environment. A total of 1,103 wild boar were sampled in 141 hunting events randomly carried out for sampling in seven hunting seasons (October to February from 2002-2003 to 2009-2010, (except 2007-2008). Prevalence was overall lower than those previously reported for fenced wild boar populations in Spain, but all the pathogens analyzed except PRRSV were considered endemic in the SNNP. ADV, *E. rhusiopathiae* and total pathogen prevalence were positively correlated to wild boar density. Prevalence in the positive areas was significantly higher in females for ADV, *E. rhusiopathiae*, *L. pomona*, *Chlamydia/Chlamydiaceae* sp. and *Salmonella* sp., and in males for *M. bovis*. This longitudinal study provides the first data on the health status of the relatively unmanaged and low density wild boar population of SNNP. It is concluded that non-intensively managed wild boar populations are able to maintain the circulation of several pathogens, even in low prevalences and in open unfenced areas with natural density variation both in time and space.

Keywords: *Sus scrofa*, density, pathogens, prevalence

48

49 **Introduction**

50 During the last decades, wild boar has experienced a population and
51 distribution range increase in Europe, including the Iberian Peninsula (Fruzinski,
52 1995; Leránoz and Castién, 1996), although population oscillation and decrease
53 in open mountain areas have been reported (Sarasa and Sarasa, 2013). Wild
54 boar can act as a reservoir species for a number of pathogens (Parra et al.,
55 2006), and population management must include health issues in order to
56 improve disease management and control (Naranjo et al., 2008). Intensive
57 management of wild boar for hunting purposes in south-central Spain, which
58 usually includes estate fencing and supplementary feeding, increases the
59 prevalence and transmission of diseases such as bovine tuberculosis,
60 Aujeszky's disease, and type 2 porcine circovirus (Gortázar et al., 2006; Vicente
61 et al., 2004, 2005 and 2006).

62

63 Wild boar home range is very variable, and depends on season, food
64 availability, reproductive status, presence of refuge areas and risk avoidance
65 (Thurfjell et al., 2009). Spatial distribution may also be affected by forest
66 fragmentation (Virgós, 2002). Wild boars are mainly sedentary (Spitz et al.,
67 1984), but they may travel long distances sporadically (Andrezejewski and
68 Jezierski, 1978). Population and migratory movements are highly variable
69 depending on the location (Sarasa and Sarasa, 2013), which may have an
70 effect on the spread of diseases (Vieira-Pinto et al., 2011). Wild boars may
71 aggregate depending on refuge search, food availability, and social behaviour

within their matriarchal social structure, and this aggregation has a determining role in the epidemiology of several diseases (Vicente et al., 2005).

The objective of this study is to describe the spatial distribution and temporal evolution of the prevalence of antibodies against nine selected pathogens and *M. bovis* detection in the wild boar population in Sierra Nevada National Park (SNNP), taking into account wild boar density variation in space and time in an open unfenced environment.

Material and Methods

Study area

The SNNP is an 86,210 hectares protected area, belonging to 44 municipalities and surrounded by the 88,965 hectares of the Sierra Nevada Natural Park, accounting for a total of 175,175 hectares which form the Sierra Nevada Natural Space (SNNS). For the purpose of this study, only the 26,588 hectares of continuous forest within the SNNP, composed of large continuous pine tree (*Pinus* sp.) reforestations, dense scrubland areas and oak patches (*Quercus* sp.), were considered. The SNNS is an open unfenced area with a Spanish ibex (*Capra pyrenaica*) population of around 17,500 individuals, which move in the SNNP and the surrounding Natural Park. In the 44 municipalities of the SNNP, more than 76,000 domestic ruminants (38,927 sheep, 32,047 goats and 5,985 cattle) graze extensively in spring and summer (three to five months). Conversely, there is practically no domestic pig in the SNNS. Cattle herds without the officially tuberculosis-free status (as defined in the EU Directive 64/432/EEC) are present in 13 out of the 44 municipalities of the SNNP. There

is no supplementary feeding for wild boar, and hunting is permitted only for management purposes, which allowed sample collection for this study.

Sample collection

A total of 1,103 wild boars (395 males and 708 females) were sampled in hunting events carried out in seven hunting seasons (October to February 2002-2003, 2003-2004, 2004-2005, 2005-2006, 2006-2007, 2008-2009 and 2009-2010), in areas with known wild boar high density. Mean wild boar age, assessed according to Borgo et al., 2007, was over 24 months, ranging from 6 to 60 months and sex was biased toward females (males 35.81%, females 64.19%). Blood was collected by heart puncture, placed in tubes without anticoagulant and allowed to clot at room temperature until its arrival to the laboratory. Then it was centrifuged at 1,200 g for 15 minutes within 24 hours from its collection, and sera frozen at -18 °C until analysis. For *M. bovis* analyses, tonsils and submandibular and retropharyngeal-parotid lymph nodes were collected and frozen also at -18 °C. Pulmonary parenchyma was also collected when tuberculosis-like lesions were observed. Sample size allows a good (3 to 5%) or moderate (5 to 10%) accuracy for an expected prevalence lower and higher than 10%, respectively (Epidat 3.1, Xunta de Galicia, Spain).

Sample analysis

Table 1 shows the serological analyses performed to detect antibodies against nine of the selected pathogens included in this study. The diagnosis of *M. bovis* infection was based on gross pathology and bacteria isolation and identification, as well as molecular detection (PCR).

Population density assessment

Population density was estimated for each hunting event, from the information obtained in the hunting events, as previously reported by Tellería and Saéz-Royuela (1985). Briefly, the number of wild boars observed during a hunting event was related to the known surface of the hunted area, in order to obtain a minimum density value for each area, expressed as observed wild boars by square kilometre. This methodology is adequate and reliable for areas with a continuous structure or forests (Mauget et al., 1984), provided it is properly designed in space and time to ensure representativeness (Pucet et al., 1975).

Epidemiological indexes

For each pathogen, prevalence was determined both for all the studied areas altogether (total prevalence or TP) and taking into account only the areas where each pathogen was detected (prevalence in positive areas or PPA).

Each pathogen was considered to be actively circulating when PPA was over 35% and mean optical density (MOD) or mean antibody titer (MAT) was as indicated for ADV (MOD<0.299), PRRSV (MOD<0.249), porcine influenza virus (MAT>1:80), porcine parvovirus (MAT>1:1,280), *Erysipelothrix rhusiopathiae* (MAT>1:300), *Leptospira pomona* (MAT>1:1,600), and *Salmonella* sp. (MAT>1:160). For porcine circovirus, *Chlamydia/Chlamydiaceae* sp., and *M. bovis*, where MOD or MAT could not be calculated, PPA>35% was the only criteria. The presence of a pathogen was considered endemic when active circulation (as defined above) was present more than half of the seasons

studied (Thrusfield, 1990). Finally, the presence of a pathogen has been considered localized when there was territorial association in the active infections detected (Thrusfield, 1990; White and Harris, 1995).

Statistical analysis

Pathogen presence

The effects of game season and spatial evolution on the presence (categorical response variable, 1 infected and 0 for uninfected) of the analyzed pathogens were explored by generalised lineal models (GLM) with binomial errors and logit link function.

Similarly, the effects of sex, and age (categorical predictive variable, age classes 6-9 months, 10-12, 13-24 and > 24 months) and wild boar density (using the values obtained from the 141 hunting events) on the presence of the analyzed pathogens were explored in this case by mixed generalised lineal models (GLMM), also with binomial errors and logit link function. Sampling season was included in the model as random factor in the density analysis.

Number of pathogens

The number of pathogens detected in the same individual was defined as dependent variable, and the effects of sex, age and wild boar density (as defined for pathogen presence) explored with GLMM, in this case with Poisson error and logit link function. As for pathogen presence, sampling season was included in the model as random factor in the density analysis.

Coinfection

The relationship between positivity to more than one pathogen in an individual, which could indicate interaction (either positive or negative) between pathogens, was analyzed. Briefly, the ratio of wild boars positive to one pathogen (dependent variable) which were also positive to each one of the remaining pathogens (predictive variable) was calculated and classified in four categories: up to 24.9%, 25.0 to 49.9%, 50.0 to 74.9% and over 75.0%. GLM with binomial errors and logit link function were used to assess the signification of all the associations among pathogens.

All the statistical analyses were performed in SAS 9.2 with PROC GLIMMIX (SAS Institute, Cary, NC, USA). R Package V.2.15.1 was applied to test the statistical significance.

Results

From all the wild boars analyzed, 67% showed evidences of contact with at least one of the studied pathogens. Table 2 shows TP and PPA values. TP did not differ significantly according to sex ($F_{1,1019}=0.12$; $p=0.730$), age ($F_{3,1019}=0.40$; $p=0.755$) or their interaction ($F_{3,1019}=1.46$; $p=0.225$). However, PPA was significantly higher in females for Aujeszky's disease virus (ADV) ($\chi^2=35.34$; 6 d.f.; $p<0.001$), *Erysipelothrix rhusiopathiae* ($\chi^2=47.72$; 6 d.f.; $p<0.001$), *Leptospira pomona* ($\chi^2=76.52$; 6 d.f.; $p<0.001$), *Chlamydia/Chlamydiaceae* sp. ($\chi^2=19.76$; 3 d.f.; $p<0.001$) and *Salmonella* sp. (serovar C) ($\chi^2=119.92$; 5 d.f.; $p<0.001$), and in males for *Mycobacterium bovis* ($\chi^2=18.69$; 6 d.f. $p=0.002$) (Table 2). *M. bovis*-positive wild boars were detected

in 19 out of the 44 municipalities of the SNNP. In six out of these 19 municipalities cattle herds without the officially tuberculosis-free status were present.

Mean wild boar density in the study areas reached 16.35 individuals/Km² (range: 0.20 - 81.50; Figure 1). Figure 2 shows the wild boar density values obtained for the SNNP throughout the study years, which varied significantly among years ($\chi^2=18.16$; d.f.=6; p=0.006) (Kruskal-Wallis $\chi^2= 34.53$; d.f.=6; p<0.001; range: 5.4 to 21.7 wild boars/Km²). Wild boar density was significantly and positively correlated with prevalence of ADV ($\chi^2=10.10$; d.f.=1; p=0.001; n=1,100), *E. rhusiopathiae* ($\chi^2=4.09$; d.f.=1; p=0.043; n=1,100), and for all the pathogens altogether ($\chi^2=8.48$; d.f.=1; p=0.003; n=1,103) (Figure 3).

Table 3 summarizes the epidemiological results and the classification for each pathogen (as defined in Table 2).

Among the significant associations found amongst the pathogens analyzed, only those of porcine parvovirus with ADV, porcine circovirus, and *Erysipelothrix rhusiopathiae* showed a relatively high value with an acceptable sample size. No spatial relationship between pathogen prevalence was observed.

Discussion

This longitudinal study provides the first data on the health status of the relatively unmanaged and low density wild boar population of SNNP, an open unfenced area. In spite of the low density of the wild boar population and the

relatively low prevalence of several of the pathogens studied, as compared with previous publications on fenced areas in Spain (Ruiz-Fons et al., 2006), eight out of the ten pathogens analysed were present throughout the whole study period and are considered endemic in the wild boar population of the SNNP (Tables 2 and 3). Since there is not a relevant domestic pig population in the SNNP and its surroundings, this finding suggests that wild boar act as true host for these pathogens, maintaining active infection focuses and pathogen circulation. The natural aggregation and unrestricted movements in unfenced areas of wild boar populations (Andrezejewski and Jezierski, 1978; Sarasa and Sarasa, 2013) may favour the maintenance and spread of pathogens. However, the low prevalences found in the SNNP seems opposite to the general increasing pathogen prevalence trend reported in wild boars populations elsewhere in the Iberian Peninsula (Vicente et al., 2002; Acevedo et al., 2006; Ruiz-Fons et al., 2006 and 2008).

It is widely accepted that for most diseases the effect and prevalence of pathogens increases with host population density (Ewald, 1993). The relationship between local population density and pathogen prevalence (ADV, *E. rhusiopathiae*, and total number of pathogens) agrees with previous reports for ADV in high density wild boar populations in estates fenced for game purposes (Ruiz-Fons et al., 2008).

The heterogeneous distribution in space and time of the pathogens analyzed in the SNNP are probably determined by several factors which interact, such as local population density, species behaviour, pathogen specific transmission

route, population management and environmental aspects, including climatology (Vicente et al., 2002). Natural reservoirs maintain pathogens in geographically restricted areas (White and Harris 1995) due to ecosystem features (Collins et al., 1986; Fulford et al., 2002). In wild boar, this has also been reported for porcine parvovirus in Croatia (Roic et al., 2005) and for tuberculosis in Spain (Vicente et. al., 2006). The finding of *M. bovis* in wild boars in six municipalities with cattle herds without the officially tuberculosis-free status leaves a door open to the possibility that tuberculosis could circulate in the interface between domestic livestock and the wild boar population of SNNP. However, a more thorough study focused only in *M. bovis*, including cattle sampling as well as wild boar sampling and *M. bovis* strain identification and characterisation should be carried out to assess this possibility.

The absence of sex- and age-related differences in population prevalence agrees with previous reports for ADV, porcine reproductive and respiratory syndrome virus, porcine influenza virus, porcine circovirus, porcine parvovirus, *E. rhusiopathiae*, and *Salmonella* (Closa-Sebastià et al., 2011). Conversely, age-related differences in the seroprevalence porcine influenza virus, *Salmonella*, (Closa-Sebastià et al. 2011), porcine parvovirus (Roic et al., 2005; Ruiz-Fons et al., 2006), ADV and porcine circovirus (Vicente et al., 2005; Ruiz-Fons et al., 2006) have been previously reported.

The higher prevalence observed in the female wild boars for ADV, *E. rhusiopathiae*, *L. pomona*, *Salmonella* sp. and *Chlamydia/Chlamydiaceae* sp. is similar to those previously reported (Jridi et al., 1996; Lutz et al., 2003; Vicente

et al., 2005), and is probably related to higher intraspecific contacts and/or earlier breeding age in females as compared to males (Mauget and Pepin, 1985; Rosell et al., 2001). Conversely, the higher *M. bovis* prevalence observed in male wild boars could be related either to age, since there were more males than females younger than 24 months in matriarchal and mixed groups, and intraspecific transmission of tuberculosis has been reported to be high at early ages in wild boar from Spain (Vicente et al., 2006); or to the higher home range of males as compared to females (Massei et al., 1997), which would make males more likely to get in contact with *M. bovis*.

Regarding coinfection, porcine circovirus has been reported to increase susceptibility and lesion extension for bovine tuberculosis in wild boar, therefore discarding a mere exposure effect but suggesting a favouring pathological mechanism (Risco et al., 2013). Further research, including thorough post-mortem and analytical studies and even experimental consecutive infection should be carried out in order to clarify whether the relationships found in the present study are causal, consequential or related to exposure risk.

To summarize, this study shows non-intensively managed (i.e. free-ranging, not overcrowded, not artificially fed) wild boar populations are able to maintain the circulation of several pathogens in natural undisturbed open unfenced areas, with natural density variation both in time and space. Although badger (*Meles meles*) culling for tuberculosis management has been reported to contribute to disease dispersal due to increased movements and susceptible population (Jenkins et al., 2010; Riordan et al., 2011), this effect has not been observed in

wild boar (Mentaberre et al., 2014). Therefore, and since population density is statistically related with ADV, *E. rhusiopathiae*, and total pathogen prevalence, and since intensively managed high-density populations report higher prevalences than those found in the SNNP, keeping population density low seems an efficient management tool to control pathogen spread in wild boar populations. Maintaining a low wild boar population density should decrease contact possibility among populations and intraspecific transmission, but managers must be aware of the possibility of disease spread by high hunting pressure.

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Figure captions

Figure 1. Wild boar density in the municipalities from the SNNP. Low density below 2 wild boar/Km²; medium density between 2 and 4 wild boar/Km²; high density between 5 and 10 wild boar/Km²; and very high density over 10 wild boar/Km².

Figure 2. Wild boar density trend throughout the study years in the SNNP.

Figure 3. Statistically significant ($p < 0.05$) correlations found between wild boar density (wild boars/Km²) and pathogen prevalence.

Table 1

Table 1. Serological techniques used to detect antibodies against selected pathogens in wild boars from the SNNP.

Pathogen	Technique	Reactive origin
Aujeszky's disease virus (serovar 1)	ELISA	Ingenasa [®]
Porcine reproductive and respiratory syndrome virus	ELISA	Ingenasa [®]
Porcine influenza virus	Inhibition of haemagglutination	Infectious Diseases Department, University of Murcia
Porcine circovirus type 2	ELISA	Ingenasa [®]
Porcine parvovirus	Inhibition of haemagglutination	Infectious Diseases Department, University of Murcia
<i>Erysipelothrix rhusiopathiae</i>	ELISA	Ingenasa [®]
<i>Leptospira pomona</i>	Lysis microagglutination	Infectious Diseases Department, University of Murcia
<i>Chlamydia/Chlamydiaceae</i> sp.	Plate microagglutination	Vetoquinol [®]
<i>Salmonella</i> sp. (serovar C)	Plate agglutination	Microkit [®]

Table 2

Table 2. Prevalence in all the studied areas altogether (total prevalence or TP) and taking into account only the areas where each pathogen was detected (prevalence in positive areas or PPA).

	Total prevalence (TP)		Prevalence in positive areas (PPA)			
	N	TP (%) Mean $\pm$ SD	N	PPA (%) Mean $\pm$ SD	Male PPA Mean $\pm$ SD	Female PPA Mean $\pm$ SD
Aujeszky's disease virus	141	16.88 $\pm$ 7.96* (n=1,103)	41	40.68 $\pm$ 4.82 (n=410)	38.69 $\pm$ 9.40 (n=152)	44.15 $\pm$ 12.74 (n=258)
Porcine reproductive and respiratory síndrome virus	141	1.90 $\pm$ 2.85* (n=1,100)	8	22.38 $\pm$ 5.38 (n=147)		
Porcine influenza virus	141	13.87 $\pm$ 7.87* (n=1,102)	51	33.31 $\pm$ 7.07 (n=548)		
Porcine parvovirus	141	24.00 $\pm$ 5.01 (n=1,103)	84	35.94 $\pm$ 5.30 (n=738)		
Porcine circovirus	106	15.16 $\pm$ 9.44* (n=810)	32	46.66 $\pm$ 4.62 (n=305)		
<i>Erysipelothrix rhusiopathiae</i>	141	6.75 $\pm$ 2.48 (n=1,101)	39	25.39 $\pm$ 6.86 (n=378)	23.42 $\pm$ 16.19 (n=185)	29.86 $\pm$ 9.36 (n=193)
<i>Leptospira pomona</i>	141	3.23 $\pm$ 0.89 (n=1,102)	16	21.10 $\pm$ 8.26 (n=186)	18.18 $\pm$ 16.65 (n=69)	23.50 $\pm$ 12.45 (n=117)
<i>Salmonella</i> sp.	141	1.75 $\pm$ 1.39 (n=1,103)	15	25.75 $\pm$ 33.58 (n=134)	12.49 $\pm$ 11.03 (n=53)	25.49 $\pm$ 29.74 (n=81)
<i>Chlamydia/Chlamydiaceae</i> sp.	106	9.37 $\pm$ 8.86* (n=810)	24	31.99 $\pm$ 11.87 (n=256)	26.25 $\pm$ 19.07 (n=99)	35.15 $\pm$ 9.06 (n=157)
<i>Mycobacterium bovis</i>	141	5.73 $\pm$ 4.83* (n=1,102)	30	21.97 $\pm$ 12.17 (n=280)	28.76 $\pm$ 17.43 (n=94)	17.77 $\pm$ 11.63 (n=186)

SD = Standard deviation. Male and female values have been provided only when statistically significant differences were found. Asterisks indicate statistically significant differences among sampling years.

Table 3

Table 3. Status for each pathogen in the SNNP.

Pathogen	Areas with pathogen detection positive/sampled (%)	Areas with active infection positive/sampled (%)	Years with pathogen detection positive/sampled	Status
Aujeszky's disease virus	41/141 (29.08%)	23/141 (16.30%)	7/7	Endemic, active, localized
Porcine reproductive and respiratory syndrome virus	8/141 (5.67%)	5/141 (3.54%)	3/7	Sporadic, scarcely active, localized
Porcine influenza virus	51/141 (36.17%)	18/141 (12.76%)	6/7	Endemic, active, spread
Porcine parvovirus	84/141 (59.57%)	35/141 (24.82%)	7/7	Endemic, active, spread
<i>Erysipelotrix rhusiopathiae</i>	39/141 (29.08%)	8/141 (5.67%)	7/7	Endemic, scarcely active, spread
<i>Leptospira pomona</i>	16/141 (11.35%)	9/141 (6.38%)	7/7	Endemic, scarcely active, spread
<i>Salmonella</i> sp.	15/141 (10.64%)	4/141 (2.83%)	6/7	Endemic, scarcely active, spread
Porcine circovirus	32/106 (30.19%)	21/106 (19.81%)	4/5	Endemic, active, spread
<i>Mycobacterium bovis</i>	30/141 (21.28%)	11/141 (7.80%)	6/7	Endemic, active, spread
<i>Chlamydia/Chlamydiaceae</i> sp.	24/106 (22.64%)	15/106 (14.15%)	4/5	Endemic, active, spread

Figure 1
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Figure 2
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Figure 3
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