



Possible interaction between a rodenticide treatment and a pathogen in common vole (*Microtus arvalis*) during a population peak

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ABSTRACT

A common vole (*Microtus arvalis*) population peak in Northern Spain in 2007 was treated with large scale application of chlorophacinone, an anticoagulant rodenticide of the indandione family. Voles found dead and trapped alive were collected in treated and untreated areas. Residues of chlorophacinone were analyzed in liver of voles by HPLC–UV. Also, the presence of the pathogen *Francisella tularensis* was analyzed by PCR in samples of vole spleen. Chlorophacinone (82–3800 ng/g; wet weight liver) was only detected in voles found dead in treated areas (55.5%). The prevalence of *F. tularensis* in voles found dead in treated areas was also particularly high (66.7%). Moreover, chlorophacinone levels were lower in voles that were PCR-positive for *F. tularensis* (geometric mean [95% CI], 418 [143–1219] ng/g) than in those that were PCR-negative (1084 [581–2121] ng/g). Interactions between pathogens and rodenticides might be considered to reduce the doses used in baits or to avoid the use of the more toxic 2nd generation anticoagulant rodenticides.

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1. Introduction

The European common vole (*Microtus arvalis*) is widely distributed within Europe, ranging from the Atlantic coast of France to central Russia. The southern limit of its range in Europe lies on the Iberian Peninsula, where isolated populations exist (Mitchell-Jones et al., 1999). Before the early 1980s *M. arvalis* populations on the Iberian Peninsula were mainly restricted to northern and central mountainous locations (González-Esteban and Villate, 2002). Since then, the species has extended its range into lower areas of the Duero valley in NW Spain, spreading into the agricultural landscape of the Castilla y León region. Several population peaks have been reported in these agricultural areas in recent years (Delibes, 1989; González-Esteban et al., 1995).

Microtine populations are used to exhibit demographic cycles (Lambin et al., 2006). One of the main traits of these cycles is a marked and fast population crash after reaching peak numbers (Norrdahl, 1995). Among several possible causes for observed increases in mortality during population crashes are, increased predation, depleted food, and delayed density-dependent diseases (Soveri et al., 2000; Hanski et al., 2001; Turchin and Batzli, 2001; Korpimäki et al., 2004). Recent work has indicated that infectious agents could play a key role in regulating population cycles in microtines (Cavanagh et al., 2004; Smith et al., 2007; Burthe et al., 2008). Moreover, it is well documented

that voles are hosts for several pathogens, some of which are zoonotic (González-Esteban and Villate, 2002). *Francisella tularensis* has been isolated from common voles in Russia (Shlygina et al., 1987), and in the Austrian and Slovakian borderland, where tularemia is considered to be endemic (Výrosteková et al., 2002). More recently, it was also isolated in Spain (García del Blanco et al., 2004).

Between late summer 2006 and mid autumn 2007, populations of *M. arvalis* in central areas of Castilla y León region rapidly increased (Olea et al., 2009). In March 2007 the regional government initiated a control campaign which utilized rodenticides to reduce the potential for crop damage. By late July of that year, an extensive control campaign was in place which affected circa 500,000 ha. Rodenticides represent a significant risk for non-target species such as granivores, and their predators (Berny et al., 1997; Shore et al., 2003; Sage et al., 2008; Sarabia et al., 2008; Walker et al., 2008; Olea et al., 2009). Moreover, the storage of rodenticide baits by voles in cavities increases the persistence of the rodenticide in the environment, which increases the risk of secondary poisoning in predator species (Sage et al., 2007).

In terms of possible interactions with pathogens, rodenticides are known to affect the physiology and behavior of exposed individuals (Buckle and Smith, 1994), which may in turn influence population dynamics and species-assemblages (e.g., host–parasite). Thus, the possibility of interaction between rodenticide treatment, and variation in relevant disease parameters certainly deserves attention. Moreover, the health status of an individual may affect its sensitivity to certain toxicants, i.e., in this case, anticoagulant rodenticides. The aim of this paper is to investigate the effectiveness of chlorophacinone treatment for vole control, and consider its possible interaction with the presence of tularemia within the same common vole population.

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2. Material and methods

2.1. Study area

Samples were collected in 2007, between February 19th and April 12th, at five locations in the province of Palencia, and one in the province of Segovia (Fig. 1). All sample sites were within flat agricultural land, that was composed of a mosaic of crop types. The five study areas in Palencia were located in the centre of the Duero Basin, spatially coinciding with the apparent vole outbreak origin. High vole densities had been reported by farmers in this area since September 2006, and complaints had risen during the winter. This area was assessed to be the likely origin of what became an expanding plague, that by August 2007 extended across 1.5 million ha.

During February 2007, the indandione anticoagulant rodenticide chlorophacinone (CP) was experimentally applied in the area surrounding Castromocho (Fig. 1). Later (by March), rodenticide treatment had been extended to cover approximately 20,000 ha, which included our sampling locations at Baquerín and Lomas de Campos (Fig. 1). The treatment was applied in association with cereal grain which was widely spread across fields with sowing machines, at the maximal authorized dose of 20 kg of grain/ha (with 0.0075% CP). We selected three similar locations in Boada, Tamara and San Pedro de Gaillos, as control areas, where no rodenticide treatment had been applied during the study period (Fig. 1). All sampling locations were considered to have been affected by the vole plague.

2.2. Estimation of vole density, and vole sampling

We trapped voles in alfalfa fields during February and March 2007 at two experimental and two control sites in Castromocho (Fig. 1). We selected alfalfa fields to obtain an estimate of maximal densities because this type of crop is known to be a preferred habitat for voles. By using a consistent habitat type, we looked to reduce the variability

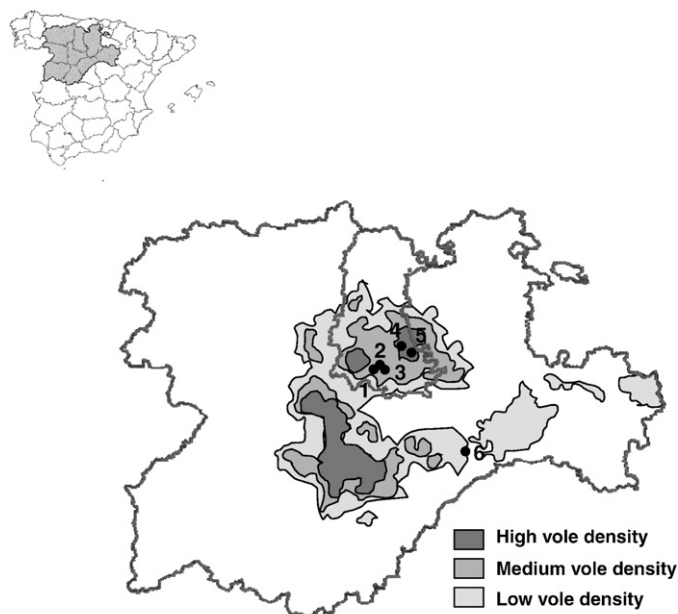


Fig. 1. Vole sampling site locations in Palencia (1–5) and Segovia (6) provinces. (1). Boada (a control area, untreated), 3 live voles trapped. (2). Castromocho (a control area and subsequently a rodenticide treated area), 10 live voles trapped, 11 found dead post-treatment (1 live vole trapped and 1 found dead before treatment). (3). Baquerín (treated area), 1 live vole trapped and 16 found dead. (4). Lomas (treated area), 1 vole found dead. (5). Tamara (a control area), 5 live voles trapped. (6). San Pedro de Gaillos (a control area), 2 live voles trapped and 3 found dead. Density data for summer 2007 is taken from the Instituto Tecnológico Agrario de la Junta de Castilla y León, JCCM, Spain, http://es.wikipedia.org/wiki/Plaga_de_topillos_en_Castilla_y_León_de_2007.

that may have existed between different microhabitats/types of crop. In each field we set three 8-trap lines spaced 15 m apart across a regular grid (24 traps in total). One line was installed on the field verge (normally adjacent to a ditch or herbaceous hedgerow). Trapping was carried out on the 19th of February (pre-treatment), 24th of February (3 days after treatment was applied on the 21st of February), and 4th of March (14 days post-treatment). Overall trapping effort was 288 traps/night. All trapped voles were individually implanted with transponders (Trovan ID 100) which allowed them to be subsequently identified. Population density was estimated using capture–implant–recapture data, following Petersen's method with Chapman fitting (Tellería, 1986). All data were pooled to increase accuracy, and the effective grid catching area was 2500 m². Similar trapping designs were then used at all other sampling sites between February and April 2007.

During trapping, 21 voles out of all those trapped were randomly selected from rodenticide treated and control locations, and sacrificed by cervical dislocation at the time of collection. These were then stored frozen in individual zip-lock bags until post-mortem investigations were undertaken. Additionally, while undertaking trapping work and/or during other field work regularly undertaken in the area, any fresh and entire dead voles encountered were also collected. Thirty one such voles were collected in total by this method, from both treated and untreated areas, and processed as described above. For each vole sample, we considered the following variables: date of collection, location, rodenticide (CP) exposure (control or treated site), and method of collection (found dead or live-trapped). A necropsy was carried out and the spleen and/or liver were collected into individual tubes for further analysis.

2.3. Analysis of chlorophacinone residues

Chlorophacinone was analyzed by high performance liquid chromatography (HPLC) (Fauconnet et al., 1997) with some modifications (Sarabia et al., 2008). Liver (1 g) was homogenized (in a mortar and pestle) with 10 g of anhydrous sodium sulfate. The homogenate was then extracted by agitation (on a reciprocating shaker; SH30L, FINEPCR, Seoul, Korea) in a Teflon capped glass-tube with 20 ml of dichloromethane for 10 min, followed by 5 min of sonication (Ultrasons-H, Selecta, Abrera, Spain). The supernatant was filtered and the homogenate extracted again (in the same way) with 5 ml of dichloromethane twice. The combined extract was dried in a rotary evaporator (Büchi, Flawil, Switzerland) and the residue re-dissolved with 2.5 ml of a mixture of methanol and 65 mM phosphate buffer, pH 7.6 (20:80, v/v). An Upti-Clean solid phase extraction column containing 100 mg of octadecyl silica (Interchrom, Montluçon, France) was then conditioned with 2 ml of methanol, then 2 ml of the mixture of methanol:phosphate buffer. The re-dissolved extraction residue was then passed through the column and the eluted phase was discarded. The column was then eluted with 4 ml of methanol, which was collected and then dried in the rotary evaporator and re-dissolved in 0.5 ml of methanol. This was then filtered through a 0.45 µm nylon membrane (Acrodisc 4 mm syringe filter, Pall, New York, USA) and 100 µl were placed in a vial for HPLC analysis. The recovery obtained using this method was 61% for liver samples spiked with CP standard (Dr. Ehrenstorfer, Augsburg, Germany). The HPLC system consisted of an Agilent 1100 Series quaternary pump, autosampler, column heater and diode array detector (Agilent Technologies, Waldbronn, Germany). The chromatographic conditions and quantification were controlled using Chemstation software (ver. A.10.02, Agilent Technologies). The HPLC column was a Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm, 5 µm particle size; Agilent Technologies, Santa Clara, USA). The flow rate used was 1 ml min⁻¹ and the mobile phase was a mixture of 65 mM phosphate buffer and acetonitrile, pH 7.6 (55:45, v/v). The injection volume was 10 µl, and the diode array detector monitored at 281 nm. The retention time for CP was 3.1 min (and the total run time per sample was 7 min). Identification of CP

was made using the characteristic retention time, characteristic absorbance and the overall UV spectra recorded between 240 and 380 nm. Chlorophacinone shows a characteristic UV spectrum with three peaks of absorbance at 286, 314 and 326 nm. The calibration was performed with liver spiked with 0.125–10 µg/g of CP and the linear correlation coefficient was 0.998 (using peak area). Coefficient of variation of calibration slopes was 10.3%. Limit of quantification of CP was established at 80 ng/g of wet weight of liver. Liver CP levels >200 ng/g wet weight were considered indicative of anticoagulant poisoning (Berny et al., 1997).

2.4. Detection of *Francisella tularensis*

A previously described (Long et al., 1993) polymerase chain reaction (PCR) technique was used to detect *Francisella tularensis*, with some modifications as described below. This PCR technique is as sensitive as culture in detecting *F. tularensis* under ideal conditions, so PCR can be expected to be considerably more reliable and specific in clinical situations (Long et al., 1993). Approximately 25 mg of spleen were collected aseptically and digested with a standard protocol using proteinase K and 4 M ammonium acetate, followed by precipitation of DNA with ethanol. Dilutions of DNA were then prepared with Tris–EDTA buffer, to a final concentration between 50 and 100 ng/µl (as measured with a spectrophotometer; UV-1603, Shimadzu, Japan). PCR reaction was performed in 25 µl reaction volumes with 2.5 µl of DNA extraction, 0.2 µM of primer concentration, 2.5 mM MgCl₂, 200 µM from a mix of dNTPs, and 1.25 U/µl of Taq polymerase (Biotools, BandM Labs, Madrid, Spain) PCR reactions were performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster city, USA) under the following conditions: one cycle of denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 57 °C, extension for 1 min at 72 °C, and a final extension for 5 min at 72 °C. Finally, 10 µl of the amplification reaction solution was resolved by electrophoresis in 2% agarose gel. DNA amplified bands were visualized by ultraviolet transillumination (Gel Doc 2000, Bio-Rad, USA) after staining with ethidium bromide. Positive samples showed an expected band of 250 bp.

2.5. Statistical analyses

Aside from the ecological data recorded during field collection of individuals, we created a database including the following complementary information for each animal analyzed: CP concentration (ng/g wet weight); *F. tularensis* PCR result; macroscopic lesions classified as splenomegaly, hepatomegaly, hemorrhages (in the cervical zone and/or in the thoracic musculature) and hemothorax. All trapped animals had hemorrhages in the cervical–thoracic zones because of the method of sacrifice. Therefore, to study the relationship between hemorrhages and CP residues or tularemia, statistical analyses were only performed on those animals found dead.

Associations between the presence of CP residues, the prevalence of PCR-positives for *F. tularensis*, the method of collection of voles, the treatment regime (treated or not with rodenticide), and the presence of lesions, were studied on different tables of contingency using Chi-square or Fisher exact tests. In order to evaluate the effect of *F. tularensis* in CP exposed voles, the concentration of CP was compared between vole groups that were PCR-positive and negative with a Mann–Whitney *t*-test. Statistics were performed with the SPSS 15.0 program; the level of significance used was $p < 0.05$.

3. Results

Estimated mean vole density at the centre of the main study area (Castromocho, Fig. 1) during the vole population peak, and immediately before the rodenticide treatments were applied, was 972.8 ± 341.6

voles/ha (mean \pm SD), which may be considered as an extremely high density typical of peaking populations.

Chlorophacinone was only detected in voles found dead in the treated areas (55.5% of this group; Fig. 2). Liver chlorophacinone concentrations ranged from 82 to 3800 ng/g wet weight (w.w.). The data were log-normally distributed, and the geometric mean (with 95% CI) was 650 (349–1212) ng/g w.w. In most cases (73%), the levels recorded were above those associated with CP poisoning (≥ 200 ng/g w.w.) (Fig. 3). None of the live-trapped voles in treated areas, or those sampled in control areas, had detectable chlorophacinone levels.

Tularemia PCR-positive voles were found in both control and treated areas, but there were a significantly higher percentage of PCR-positive voles (found dead) in treated areas (66.7%) ($\chi^2_3 = 11.2$, $p = 0.011$; Fig. 2). Among voles that had CP residues, the liver CP concentration was also lower in PCR-positives (geometric mean [95% CI], 418 [143–1219] ng/g w.w.) than in PCR-negatives (1084 [581–2121] ng/g w.w.) ($U = 11$, $p = 0.049$) (Fig. 4). However, the percentage of PCR-positive voles found dead with (53.3%) and without (83.3%) CP residues was not significantly different in treated areas ($p = 0.21$).

The most common lesions observed were hemorrhages, and splenomegaly or hepatomegaly was also noted. Hemorrhages were mainly in the thoracic zone, followed by the cervical zone, thoracic musculature, and hemothorax. Hemorrhages were present in voles with (67%) and without CP residues (75%) ($p = 0.7$). Splenomegaly and hepatomegaly were more frequent in tularemia PCR-positive voles (33.3% and 13% respectively) than in PCR-negative voles (12% and 0%), but these associations were not statistically significant ($p = 0.23$ and $p = 0.09$).

4. Discussion

The majority of voles (93%) found dead in treated areas during the observed population peak studied here, had CP residues in liver and/or were PCR-positive for *F. tularensis*. Thus, vole mortality in CP-treated areas was clearly caused by exposure to CP, but also, in all probability, by infectious agents including *F. tularensis*. The link between the presence of CP in dead vole livers, and mortality of voles is as expected, very clear, since no CP residues were detected in voles trapped alive, and the liver residue levels recorded in dead voles exceeded (in most cases) the levels associated with CP poisoning (Berny et al., 1997). However, the prevalence of tularemia was also higher in animals found dead, than in those live-trapped, which may indicate that *F. tularensis* was playing a role in the observed mortality. As the limit of quantification for CP was 80 ng/g in liver and animals poisoned by rodenticides usually have

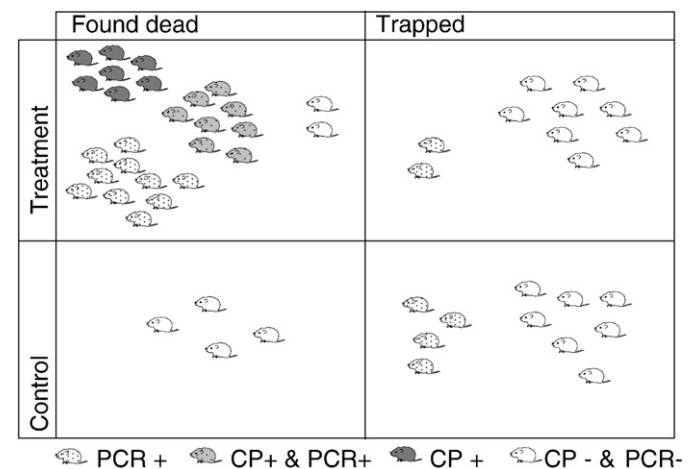


Fig. 2. Distribution of voles analyzed, according to collection method (found dead or trapped) and area (whether treated with chlorophacinone or not – control areas). PCR+ = PCR-positive for *F. tularensis*; CP+ and PCR+ = positive for chlorophacinone residues and PCR-positive for *F. tularensis*; CP+ = positive for chlorophacinone residues; CP- and PCR- = negative for chlorophacinone residues and PCR-negative for *F. tularensis*.

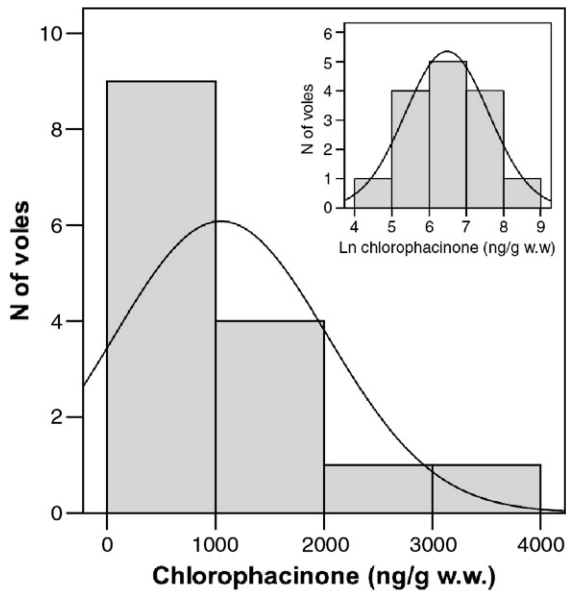


Fig. 3. Distribution of chlorophacinone residues in samples found to be positive, expressed as ng/g wet weight in liver.

anticoagulant levels >200 ng/g, we cannot conclude that some of the voles trapped alive in the treated areas had not been exposed to CP. Moreover, some of the voles found dead in the treated areas with undetectable CP residues in liver may have died due to CP poisoning, because in 8 voles hemorrhages were observed.

Likewise, an interaction between the toxic effects of CP and *F. tularensis* infection may have occurred, since CP concentrations were actually lower in PCR-positive voles. CP concentrations in some voles found dead were actually relatively low, which suggests that in voles at least, this toxicant was effective even at quite low doses. If this could be confirmed experimentally with captive voles, such results may permit a reduction in the CP dose used in bait, and thus potentially mitigate unwanted and important effects upon non-target species (Sage et al., 2008; Olea et al., 2009). Moreover, since CP was found to be effective in killing voles, this may indicate that the large scale use of more persistent 2nd generation rodenticides (which carry a higher risk of secondary poisoning for scavengers) may be unneces-

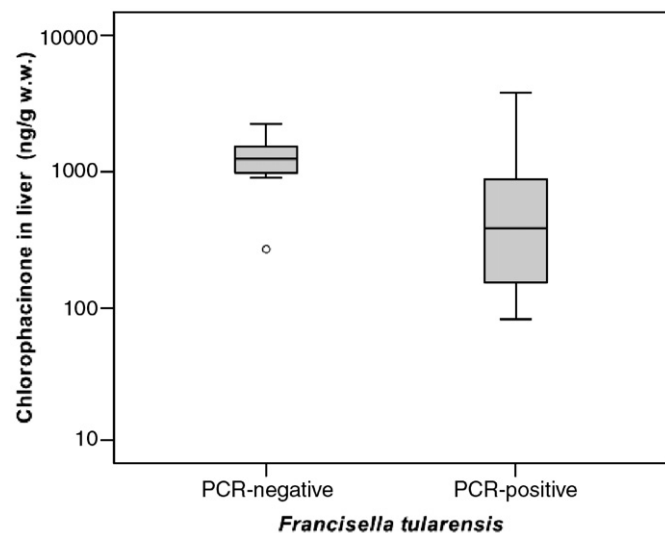


Fig. 4. Differences in chlorophacinone residue levels (expressed as ng/g wet weight in liver) between *Francisella tularensis* PCR-negative ($n=7$) and PCR-positive ($n=8$) voles. Box-plots give median, quartile, minimum–maximum, and an outlier value.

sary (Berny et al., 1997; Walker et al., 2008; Sage et al., 2008). Some voles with CP residues in liver did not show signs of hemorrhage (33%), as was the case with pigeons (23.5%) found dead within the same study area (Sarabia et al., 2008). This may be because indandione rodenticides can cause death via neurological, heart or lung damage, which may incur death before animals develop hemorrhages that are detectable at necropsy (Reigart and Roberts, 1999). However, these voles may have died by other causes before vitamin K was depleted and hemorrhages were developed.

In addition to the role that *F. tularensis* may be playing here, in terms of direct vole mortality, or by causing increased sensitivity to rodenticides, it is also important to note that rodenticide treatment itself may cause bacteria to spread (within and between fields). Tularemia prevalence was higher in treated areas amongst voles found dead. These treated areas were selected by the regional authorities for rodenticide application, because of the impact that voles were having on agriculture. As such, vole density was probably higher in these zones (than in control areas), and this could in part explain why disease prevalences were also higher. Alternatively, this may also suggest that the rodenticide treatment itself could have acted to increase tularemia spread and therefore prevalence (and subsequent vole mortality). Tularemia transmission between voles could be enhanced by cannibalism, since voles are known to eat corpses of their kin (Olsufjev et al., 1984), or, it may be spread by inhalation of bacteria that are likely to be more abundant in the air when infected vole corpses are abundant. The possibility that rodenticide use could itself increase susceptibility to pathogens and indirectly enhance their spread by creating conditions where large numbers of vole corpses (with tularemia) exist over a restricted spatial area, deserves careful additional research. Whilst rodenticide use may be required, at the same time diseases need to be controlled, because *F. tularensis* may survive a long time within dead animals (Acha and Szyfres, 2003). Moreover, the interaction between rodenticides and pathogens may be more complex and diverse depending on the transmission of the pathogen. A study in a repeatedly treated area with rodenticides to kill water voles (*Arvicola terrestris*) has shown the possibility that secondary poisoning of foxes affected their population density and consequently decreased their infection by *Echinococcus multilocularis*, for which vole is the intermediate host and fox is the definitive host (Raoul et al., 2003). The elevated prevalence of tularemia found in voles here is worthy of further study, and work should perhaps focus on improving our knowledge regarding the spatio-temporal dynamics of common vole populations, and consider the effects of disease as natural limiting factors in population cycle events (such as population peaks).

Another aspect to take into account is the risk of zoonotic transmission to humans because an outbreak of human tularemia has occurred in the same region in 2007 (Martín et al., 2007; Allue et al., 2008). It started on May in the Palencia province and peaked on mid July, then decreasing up to December with a total of 507 patients infected. Half of the cases were people who presumably had contact with the environment and with wild rodents (Allue et al., 2008). The risk of transmission of tularemia from wild rodents to humans is well documented (Ellis et al., 2002; Christova and Gladnishka, 2005; Petersen and Schriefer, 2005; Eliasson et al., 2006). In an area in Bulgaria where human tularemia is endemic, 21.9% of trapped rodents were also PCR-positive for *F. tularensis* (Christova et al., 2004). The first outbreak of human tularemia in Spain was described in 1997, associated with contact with hares (Eiros Bouza and Rodríguez Torres, 1998), in contrast with 2007, in which the transmission was mainly due to inhalation (Allue et al., 2008). The large scale use of rodenticides during 2007 increased the number of dead voles in the environment during harvesting (many of which were infected with tularemia). Although voles may die in their burrows, corpses were also frequently found on the surface in the study area. This is likely to have increased at short term the risk of transmission of tularemia to humans via inhalation of dust generated by harvesting machinery

(Dahlstrand et al., 1971; Eliasson et al., 2006). Further epidemiological studies are therefore required, to establish possible spatio-temporal relationships between human tularemia cases, and large scale rodenticide treatment. On the other hand, CP treatment, as well as natural causes of mortality, would ultimately reduce vole density and therefore the risk of tularemia infection among them and to humans.

The large scale application of rodenticides for vole control is always controversial due to the risk of poisoning of non-target species (Beryny et al., 1997; Shore et al., 2003; Fournier-Chambrillon et al., 2004; Olea et al., 2009). Treatments with CP seemed effective in killing voles, but we found a natural mortality rate almost as important as that caused by CP. This may explain why vole populations rapidly declined in areas treated with rodenticide, but also in treatment-free areas (Olea et al., 2009). Future vole control campaigns in Spain should carefully balance the benefits and risks of rodenticides to control tularemia spreading, the sanitary risk of contact with rodents by rural population, and the negative environmental effects of rodenticide baits on the surface such as the poisoning of non-target species of game and wildlife (Sarabia et al., 2008; Olea et al., 2009).

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