Accepted Manuscript

Title: Drivers of *Bartonella* infection in micromammals and their fleas in a Mediterranean peri-urban area

Authors: Aitor Cevidanes, Laura Altet, Andrea D. Chirife, Tatiana Proboste, Javier Millán

PII: S0378-1135(16)30611-3
DOI: http://dx.doi.org/doi:10.1016/j.vetmic.2017.03.023
Reference: VETMIC 7584

To appear in: *VETMIC*

Received date: 3-11-2016
Revised date: 10-3-2017
Accepted date: 13-3-2017

Please cite this article as: Cevidanes, Aitor, Altet, Laura, Chirife, Andrea D., Proboste, Tatiana, Millán, Javier, Drivers of Bartonella infection in micromammals and their fleas in a Mediterranean peri-urban area.Veterinary Microbiology http://dx.doi.org/10.1016/j.vetmic.2017.03.023

Drivers of *Bartonella* infection in micromammals and their fleas in a Mediterranean peri-urban area

Aitor Cevidanes[a]*, Laura Altet[b], Andrea D Chirife[c], Tatiana Proboste[d], Javier Millán[e]*

[a] PhD Program in Conservation Medicine, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, República 252, Santiago, Chile

[b] Vetgenomics, Edificio Eureka, Research Facilities, Universitat Autònoma de Barcelona, Bellaterra, 08193, Spain

[c] El Mirador del Rosario P5, Peñaflor, Chile

[d] School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia.

[e] Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, República 252, Santiago, Chile

*Corresponding authors: Aitor Cevidanes (aitorcevi@gmail.com) and Javier Millán (syngamustrachea@hotmail.com)

Highlights

- *Bartonella* (12 haplotypes) were identified in 49% of periurban micromammals and in 58% of flea pools
- *Bartonella* prevalence varied depending on flea infestation level, host relative abundance, season and sex
- Prevalence in flea pools was only explained by *Bartonella* occurrence in the pool host.
- In general, prevalence did not differ between natural and residential areas.
- 66% of fetuses from all the analyzed litters were infected, indicating that vertical transmission could be important

Abstract

People living at the human/wildlife interface are at risk of becoming infected with *Bartonella* for which micromammals act as reservoir. We aimed to determine the factors related to the prevalence of *Bartonella* and its haplotype diversity in micromammals and in their fleas in a Mediterranean peri-urban environment. We analyzed 511 micromammals, chiefly 407 wood mice (*Apodemus sylvaticus*), captured into Barcelona metropolitan area (Spain) in spring and autumn from 2011 to 2013 in two natural and two adjacent residential areas, their fleas (grouped in 218 monospecific pools) and 29 fetuses from six *Bartonella*-positive female wood mice. Amplification of a fragment of ITS was carried out by real time PCR. Prevalence was 49% (57% in the dominant species, the wood mouse), and 12 haplotypes were detected. In general, prevalence was higher in those hosts more heavily infested by fleas, coincident with higher rates of capture, in autumn than in spring, and in adults than in juveniles. Prevalence did not differ between natural and residential areas except for one prevalent haplotype, which was more frequent in natural areas. Prevalence in flea pools (58%) was only explained by *Bartonella* occurrence in the pool host. In 56.4% of the flea pools with identified *Bartonella* haplotypes, we found the same haplotype in the host and in its flea pool. Prevalence in wood mouse fetuses was 66%, with at least one infected fetus in all litters, and two litters with all the fetuses infected. indicating that vertical transmission might be important in *Bartonella* epidemiology in the wood mouse. There is a hazard of *Bartonella* infection for people living in residential areas and those visiting peri-urban natural areas in Barcelona.

Keywords: Algerian mouse, Muridae, shrew, vector-borne

Introduction

Bartonella species are Gram-negative bacteria belonging to the class Alphaproteobacteria, which parasitize mammalian erythrocytes and endothelial cells (Birtles, 2005). More than 30 *Bartonella* species are known, infecting a wide variety of hosts including humans. Rodents are natural reservoirs of many *Bartonella* species (Gutiérrez et al., 2015), presenting subclinical and persistent bacteremia (Schülein et al., 2001). At least six *Bartonella* species infecting rodents have been reported to be related with human infections [see Buffet et al. (2013) for review of rodent-adapted *Bartonella* species, their identified rodent hosts, vectors and their potential as human pathogens]. This number may increase, because some animal-associated species that previously were not considered as zoonotic are currently being associated with disease in humans (Vayssier-Taussat et al., 2016). *Bartonella* is commonly considered a vector-borne pathogen, with sand flies, lice, fleas and ticks acting as vectors of *Bartonella* (Breitschwerdt and Kordick, 2000). The role of fleas in rodent-associated *Bartonella* is important in the ecology of these bacteria, not only because fleas are known to be efficient vectors, but also because these arthropods act as additional reservoirs (Gutiérrez et al., 2015).

Epidemiological studies on *Bartonella* carried out in micromammals showed high prevalence, with marked variations among seasons and/or rodent sex and age, among other factors (Jardine et al., 2006; Kosoy et al., 2004; Telfer et al., 2007b; Welc-Faleciak et al., 2010). However, although the rodent habitat could represent an important factor related to the efficiency of the *Bartonella* transmission cycle (Gutiérrez et al., 2015), the effect that urbanization may have on *Bartonella* prevalence in wildlife has never been studied. It is well known that urbanization of natural areas can alter abiotic factors of the environment in which animals thrive. For example, urbanization can attenuate the temperature range, known as the “urban warming

effect" (Grimmond, 2007), and can provide artificial sources of humidity (Shochat et al., 2006). This is why several vector-borne diseases might respond to changes associated with urbanization. For example, urbanized areas can provide suitable environments for vector reproduction (Bradley and Altizer, 2007). In addition to the effect of microclimatic conditions on flea development (Krasnov et al., 2001), urbanization could also alter the types, abundances and distribution of resources available to wildlife, especially food (Becker et al., 2015), promoting higher aggregation and host density. Such changes may alter *Bartonella* prevalence due to increased host-to-host contact rates. Differences in prevalence of some rodent-borne pathogens related to anthropogenic landscape changes have been previously observed. For example, Langlois *et al*. (Langlois et al., 2001) found higher Sin Nombre virus seroprevalence among deer mice trapped in buildings than among those trapped in the wild. These authors suggested that the conditions that probably attract deer mice into buildings, such as concentrated food supply and moderate microclimate, may increase the transmission of the virus by increasing mouse population density and out-of-host survival of the virus, respectively (Langlois et al., 2001). Similarly, the predicted abundance of *Yersinia pestis* seropositive rodents was higher in agricultural sites relative to conserved sites of Tanzania, due to differences in host and flea community composition (McCauley et al., 2015). However, in that same study no significant differences in rodent abundance, flea prevalence or intensity between two types of habitats was demonstrated (McCauley et al., 2015).

The Barcelona (Spain) metropolitan area (BMA) has a population of about 5.5 million inhabitants (Nations, 2011) and is the most populous metropolitan area on the Mediterranean coast. Within this area, there are a number of natural areas, some of which are completely surrounded by built-up land, where outdoor activities are frequent. In addition, there are an

increasing number of residential areas on the edges of these natural areas. Hikers, nature lovers and local residents, along with their dogs and cats, can thus easily come into contact with wild animals and the infections they host. Previous studies have shown that *Bartonella* is highly prevalent and shows high genetic diversity in wild micromammals captured in one of these natural areas (Gil et al., 2010). Recently, we compared the ectoparasitefauna retrieved from more than 600 micromammals captured in natural and residential areas into BMA. Infestation by fleas, the most important vectors for *Bartonella* (Gutiérrez et al., 2015), was prevalent among the analyzed individuals, but we found little support for an effect of urbanization on prevalence of infestation by arthropods (Cevidanes et al., 2016). However, we found that one flea (*Leptopsylla taschenbergi*) showed marked differences in prevalence between seasons in natural areas, but prevalence was constant in residential areas, suggesting that narrower variations in environmental factors may had enhanced the survival probabilities in these habitats (Cevidanes et al., 2016). However, no such differences were found for other species of flea, which were more influenced of other factors such as season and host sex (Cevidanes et al., 2016). In consequence, in the present study we aimed to get insight on the factors related to the prevalence of *Bartonella* in micromammals and their fleas in a Mediterranean peri-urban environment.

Material and Methods

Study area, micromammal capture and sampling

The study was conducted in four areas (two natural parks and two residential areas located within the boundaries of these parks) in peri-urban Barcelona (northeast Spain). The natural parks studied were Collserola (CoNP; 41°26′ N, 2°08′ E) and Sant Llorenç del Munt (SLNP; 41°38′ N, 2°01′ E). CoNP is completely surrounded by the metropolitan area and receives about

2 million visitors annually. SLNP is located to the north of the metropolitan area and annually receives about 250,000 visits. In CoNP, the residential area (La Floresta, belonging to San Cugat del Vallés municipality) is only 600 m away from the selected natural study area, which is the one with the highest level of protection in the Park. In SLNP, an area less impacted by urbanization pressure, the residential area (St. Feliú del Racó, Castellar del Vallés municipality) is about 10 km away from the corresponding natural study area. These residential areas are characterized by the presence of detached houses in a density of about 100 houses per square kilometer, scattered within habitats that are not completely built-up and are not dissimilar to natural areas (Figure 1). More detailed information about the study area can be found in Millán *et al.* (2014).

Animals were captured with Sherman traps (H.B. Sherman Traps, Inc., Tallahassee, Florida) in two seasons (before and after the dry season, i.e., spring and autumn) from 2011 to 2013. Seventy traps were set for five consecutive days in each of the trapping sessions. Traps were baited with a breadcrumbs spread with fish oil. A total of 407 wood mice (*Apodemus sylvaticus*), 44 white-toothed shrews (*Crocidura russula*), 36 Algerian mice (*Mus spretus*), 16 black rats (*Rattus rattus*), three brown rats (*R. norvegicus)*, two house mice (*M. musculus*), two *Rattus* sp. and one *Mus* sp. were included in this study. Detailed information on capture success for species and area can be found in (Cevidanes et al., 2016). Animals were transferred without handling to a plastic bag and weighed using a Pesola scale to the nearest 0.5 g and anaesthetized with a combination of ketamine (Domtor©, Esteve, Barcelona, Spain) and medetomidine (Imalgene©, Merial, Barcelona, Spain) (Chirife and Millán, 2014). Rodents were then euthanized by retro-orbital bleeding and necropsied in detail. A spleen sample was obtained and kept at -20 °C until analysis. To study the relative importance of vertical transmission, fetuses of *Bartonella*-positive

pregnant wood mouse females (n=29 fetuses belonging to 6 females; mean litter size= 4.83) were collected. During necropsy, complete uterus was retrieved carefully and placed into a separated vial. Later, fetuses were individually retrieved from the uterus into a dissection hood using sterilized instrumental and placed into separate vials. Different sets of dissection tools were used for each fetus, which were cleaned with 95% ethanol and fire-sterilized after every sample. Before euthanasia, the fur of each animal was systematically examined and all fleas seen were removed and preserved in 96% ethanol.

This study complied with the regulations of animal experimentation and welfare issued by the European Union (Directive 86/609/CE). Specifically, capture and handling of animals was approved by authorities in bioethics under permit CEEAH 1871 (Univeristat Autònoma de Barcelona, Spain), and by annual capture authorizations from the Catalonia regional government (permits SF/111/2011, SF/153/2012, and SF/064/2013).

Laboratory analysis

Fleas were identified based on their morphological characteristics following the keys and descriptions of Beaucournu and Launay (1990). Fleas were directly identified in ethanol to avoid DNA degradation. Once identified, fleas were grouped in 218 monospecific pools belonging to the following species: *Ctenophtalmus andorrensis catalanensis* (144 pools), *Leptopsylla taschenbergi amitina* (68 pools), *Typhloceras poppei poppei* (three pools), *Nosopsyllus fasciatus* (one pool) and *Hystrichopsylla talpae* (one pool). Detailed information about the prevalence and intensity of the retrieved fleas per species can be found in Cevidanes et al. (2016). DNA was isolated from flea pools, host samples and fetuses from six pregnant *Bartonella*-positive wood mouse females using the High Pure PCR template preparation kit (Roche, Mannheim, Germany)

according to the manufacturer's instructions with some modifications from Solano-Gallego et al. (2012). A blood or liver sample from a clinically healthy non-infected dog was used as a control for DNA contamination in every DNA extraction performed for host samples and fetuses (blood) and fleas (liver) respectively.

Amplification of a fragment of 16S-23S ribosomal RNA intergenic spacer (ITS) was used for detection of DNA from Bartonellae in these extracts; amplification products were sequenced as a means of identifying which *Bartonella* species were present. The advantage of the ITS region (the intergenic spacer between 16SrRNA and 23SrRNA genes) is that it is extremely diverse between *Bartonella* species, allowing characterization to the species or even strain level, even when short amplicons are sequenced (Birtles et al. 2000). Primer pairs used were as follows: Forward 5'- AGATGATGATCCCAAGCCTTCTG-3' [modified from Maggi et al. (2005)] and Reverse 5'-CCTCCGACCTCACGCTTATCA-3' [modified from Gil et al. (2010)]. Real time PCR was carried out in a final volume of 20 µl using SyberSelect MasterMix (Thermofisher), 0.3 µM of each primer and 4 µl of diluted DNA (1/2 for blood and 1/10 for spleen). The thermal cycling profile was 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. Real time PCR specificity assessment was performed by adding a dissociation curve analysis at the end of the run. Amplification of 18S RNA was used as an internal reference for genomic DNA amplification to ensure (i) the proper PCR amplification of each sample and that (ii) negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation or PCR inhibition. The eukaryotic 18S RNA Pre-Developed TaqMan Assay (Thermofisher) was used for host samples and fetuses and an in-house design for fleas (Forward primer 5'- CGAAAGCATTTGCCAAATGTG-3' and reverse primer 5'- TCATCGGAGGAACTTCGGC-3'). As rodents are believed to be refractory to *B.*

henselae infection, DNA extracted from a slide coated with *B. henselae*-infected HEp2 cells (MegaScreen® *Bartonella henselae*, Megacor) was used as a positive PCR control. When a plate included any sample positive for *B. henselae*, the PCR was repeated without positive control. Water was used as a negative PCR control. The amplification products were sequenced to identify which *Bartonella* haplotype was present. The sequencing was carried out with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Thermofisher) previous enzymatic purification of the PCR product using Alkaline Phosphatase and Exonuclase I. Sequencing was performed in a conventional Thermocycler with the thermal cycling profile recommended in the sequencing kit (96ºC 1 min, followed by 25 cycles of 96ºC 10 sec, 50ºC 5 sec and 60ºC 4 min) using the same primers or an internal primer as necessary [5'-ATGATCCCAAGCCTTCTGGC-3'; modified from Maggi et al. (2005)]. Sequences obtained were compared with the GenBank database (www.ncbi.nlm.nih.gov/BLAST). The resulting novel *Bartonella* DNA sequences for the ITS were then submitted to the GenBank or to the EMBL-EBI database (http://www.ebi.ac.uk) for the assignment of an accession number (from HE974357 to HE974360 and from JX276642 to JX246644).

Statistical analysis

Confidence intervals for prevalence and standard error for mean intensity were calculated using the "EpiR" package of the R software. Factors associated with *Bartonella* sp. occurrence probability (a dichotomous dependent variable) in wood mouse and flea pools were analyzed using a Generalized Linear Mixed Model with binomial error and logit-link function. In the model for the wood mouse, the most frequently captured species, fixed effects included were habitat type (natural/residential), season (spring/autumn), sex (male/female), age (adult/juvenile), host abundance (low/medium/high) and flea abundance (no infestation / low infestation / high

infestation). Host abundance was calculated using a "captures/100 traps/night" index. Low host abundance was considered between 0.1 and 5 animals/traps/night; medium between 5 and 10; and high more than 10. One of these categories was assigned to each individual depending on the host abundance of the sampling period and area in which it was captured. Flea abundance was transformed into a categorical variable according to the flea burden of each host individual: no infestation= no fleas; low infestation= between one and five fleas; and high infestation= between five and ten fleas. In the model for flea pools, fixed effects were habitat type (natural/residential), season (spring/autumn), species from which the pool was retrieved and *Bartonella* occurrence in the host (positive/negative). The random effects for all models were sampling year (2011/2012/2013) and study area (CoNP/SLNP). Models were analyzed using the "lme4" package of the R software. The best model was selected using the "dredge" function of the "MuMIn" package of R, based on the Akaike Information Criterion corrected for sample size (AICc). The best model was compared to the model with an intercept and random effects only, using the likelihood ratio test to evaluate the overall fit. Only individuals with information on all factors were included in the models (n=405). Nonparametric bivariate analysis (Pearson`s chi-square test and Fisher´s exact test) were carried out for those host species with small sample size and to determine the probability of occurrence of the three most prevalent haplotypes.

Results

Bartonella *in micromammals*

Bartonella DNA was detected in 249 individuals (48.7%; 95% Confidence Intervals = 44.3%-53.0%) (Table 1). The prevalence of infections was higher in wood mice (57.0%; 95% CI = 52.1% – 62.8%) than in white-tooth shrews (22.7%; 95% CI = 11.7% - 37.2%) or Algerian mice

(19.4%; 95% CI = 8.8% - 35.7%) (χ^2= 27.24, p<0.001). *Bartonella* DNA was not detected in any rat or house mouse.

Results of the best model for *Bartonella* occurrence probability in wood mice indicated that prevalence was higher in autumn than in spring, in adult than in juvenile mice, in "high abundance" than in "low abundance" capture events, and in the "high infestation" group (i.e., more than five fleas) than in the "no infestation" or "low infestation" groups (Table 2; Figure 2). It is noteworthy to mention that no correlation was found between flea infestation intensity and host abundance.

Regarding the nonparametric bivariate analysis for *Bartonella* prevalence in the Algerian mouse and shrew, we only detected differences in the Algerian mouse between seasons; prevalence was greater in autumn (35.3%) than in spring (5.3%) (Fisher's p =0.03).

The prevalence of *Bartonella* in wood mouse fetuses was 68.9% (20/29), with at least one infected fetus found in all six litters, and two litters with all the fetuses infected. Unfortunately, we were not able to obtain readable sequences from fetuses.

Bartonella *in fleas*

Bartonella DNA was detected in 125 flea pools (57.6%, 95% CI = 50.9% –64.1%; Table 1). Neither the *N. fasciatus* pool nor the *T. poppei p.* or *H. talpae* pools were positive for *Bartonella* DNA.

The only significant variable included in the best model for *Bartonella* occurrence probability in flea pools was the *Bartonella* occurrence in the host from which the fleas were retrieved (Table 2).

Bartonella *genetic diversity*

Twelve different haplotypes (HT) were detected. Amplicon length and homologies are summarized in Annex 1. HT1 was identical to a sequence reported from a wood mouse in the UK (*Bartonella* sp. wbs68, accession number AJ269796); HT2 and HT7 were similar to *B. taylorii* WM9, but with one less or two more copies, respectively, of a tandem repeat sequence (TTTTTTAGATAATGT); HT3 was identical to a strain of *B. birtlesii* (syn. *Bartonella* sp. N40, a. n. AJ269791); HT4 was identical to a strain described as *B. taylorii* WM9 (a. n. AJ269788); HT5 was identical to a sequence obtained from a *Bartonella* strain infecting a wood mouse in the UK (*Bartonella* sp. wbs11, a.n. AJ269792), which is closely related to *B. doshiae*. HT6 was identical to a sequence obtained from a *Bartonella* strain infecting an Algerian mouse from southern Spain (Uncultured *Bartonella* sp. clone MS_Seville-07021427, a. n. EU218552), which is closely related to *B. elizabethae*.; HT8 was similar to *B. birtlesii* (syn. *Bartonella* sp. N40) but with an additional copy of the tandem repeat described above; HT9 showed less than 86% similarity of isolates to any known *Bartonella* species, suggesting that this genetic group may be a new species; HT10 was identical to *B. henselae* (a. n. L35101), but this does not mean that these amplifications corresponded to *B. henselae*; HT11 had 99% similarity with *B. grahamii* (CP001562.1). Finally, HT12 showed 95% similarity with a *Bartonella* isolated from the blood of an American red squirrel (*Bartonella* sp. AR 15-3, a. n. FN645485.1), mostly related to *B. rochalimae*, though with only 91% identity (a. n FN645466.1), and thus might also represent a new species. Haplotypes detected in each host and flea species are summarized in Table 1.

Regarding the factors related to the prevalence of the dominant haplotypes (HT1, HT3, HT5) in wood mouse, the prevalence of HT5 was higher in natural (13.4%) than in residential areas (5.32%) (χ^2= 6.3, $p<0.05$). On the other hand, HT3 was more prevalent in autumn (26.5%)

than in spring (16.42) (χ^2= 8.4, p<0.005). We found that 56.4% of the flea pools with identified *Bartonella* haplotypes coincided with those haplotypes amplified in the host from it was retrieved (Annex 2). Concerning to the most prevalent haplotypes (HT1, HT3 and HT5), the concordance was around 50%: HT1 was amplified in 52.9% of the positive flea pools retrieved from hosts infected with HT1, and HT3 and HT5, in 50% of the pools from host with HT3 and HT5, respectively (Annex 2).

Discussion

This study, that included a three-year continued capture effort during two seasons in the peri-urban zone of a large metropolitan area, provides new information about the factors related to the prevalence of *Bartonella* in both micromammals and their fleas. Although other studies had already studied the prevalence of *Bartonella* in micromammals in Mediterranean areas (Bitam et al., 2008; Gil et al., 2010; Márquez et al., 2008), and elsewhere (Kosoy et al., 2004; Silaghi et al., 2016; Welc-Faleciak et al., 2010) our study helps to understand the biotic and abiotic factors related to the occurrence of these bacteria in hosts and vectors.

The wood mouse was both the species most frequently captured and the species showing the highest prevalence of infection during the survey. This is consistent with previous observations indicating that increasing prevalence is directly related to increasing dominance of a species in a micromammal community (Kosoy et al., 2004, 1997). In fact, we observed that host abundance was also positively correlated with *Bartonella* occurrence probability. Density-dependent *Bartonella* prevalence was previously observed in some *Bartonella*-rodent associations (e.g. Telfer et al., 2007b). Higher host density may increase flea transmission and contact rate between hosts. We observed that prevalence was also directly related to the intensity

of flea infestation, thus flea abundance may influence directly the chances of *Bartonella* infection in the host, due to the fundamental role of flea parasitism in the transmission of this bacterium (Gutiérrez et al., 2015).

Taking into account that according to Cevidanes et al. (2016) flea prevalence was similar between natural and residential areas, this explains the similar prevalence of *Bartonella* between these two habitats. Only one *Leptopsylla taschenbergi* was apparently affected by the type of habitat (Cevidanes et al., 2016), but this did not translated in differences in *Bartonella* prevalence. The only significant difference related to habitat type was found for one of the *Bartonella* haplotypes (HT5), which was found to be more prevalent in natural areas. The reasons for this finding are unknown. Taking into account that no other haplotype showed this pattern, it seems to be an exception, and that other factors have more influence in the probability of a rodent becoming infected. For example, *Bartonella* is suspected (but not confirmed) to be transmitted by gamasid mites (Kim et al., 2005; Reeves et al., 2007, 2006), which were very prevalent in the studied population [55% of wood mouse and 65% of Algerian mouse hosted gamasids (Cevidanes et al., 2016)]. In addition, we found support during the present study the vertical transmission of this bacterium in wood mouse, which is, as far as we know, the first description in this species. The isolation of *Bartonella* from offspring of naturally infected rodents was first observed by Kosoy et al. (1998), which reported 58% infected embryos from five litters out of seven infected mothers belonging to two species, *Sigmodon hispidus* and *Peromyscus leucopus*. Our rate of infection (66%) is in the range of Kosoy et al. (1998) observations, but we found all the litters to be infected. It is worthwhile to comment that, in the study by Kosoy et al. (1998), *Bartonella* was cultured from fetuses and newborns. Contrary to Kosoy et al. (1998) and our own studies, very infrequent or no vertical transmission at all was

detected from infected females to their offspring in other rodent species (Bown et al., 2004; Morick et al., 2013). Such as high rates of vertical transmission observed in wood mouse should significantly contribute to *Bartonella* transmission in the studied community.

We did find support for a marked seasonality in the prevalence of infection with *Bartonella*, with higher prevalences detected in autumn. It has been previously observed that the peak of *Bartonella* infection in small mammals in the Northern Hemisphere takes place from late summer to early autumn (Jardine et al., 2006; Paziewska et al., 2012; Telfer et al., 2007a), coinciding with the peak of flea infestation (Jardine et al., 2006; Krasnov et al., 2005). Fleas were indeed more prevalent in autumn than in spring in our sample (Cevidanes et al., 2016), supporting the hypothesis that *Bartonella* transmission in the studied micromammals is mostly flea-borne, which is further sustained by the fact that *Bartonella* prevalence was higher in hosts more severely infested by fleas (see below). An alternative or concomitant hypothesis to explain the season-related differences may be related to the annual recruitment because the proportion of pregnant or lactating wood mice females in our sample was significantly higher in autumn (42.8%) compared to spring (16.6%, χ^2= 17.03, p<0.001; data not shown), and we have discussed before the high rate of infection in fetuses from positive females. This suggests that vertical transmission may have more influence than expected in prevalence differences between seasons. Higher prevalence during autumn may also be related to other factors such as an increase in the susceptible proportion of the population, increased male mobility and/or immunosuppression during the reproductive period, among others (Gutiérrez et al., 2015).

We also detected age-related differences in *Bartonella* prevalence, being markedly higher in adult than in juvenile wood mice. Previous studies detected higher prevalence in juvenile small mammals (Jardine et al., 2006; Kosoy et al., 2004; Telfer et al., 2007a), which is in

opposition to our results. Juvenile-biased infections have been associated with mobile behavior and an immature immune system, among other factors (Welc-Faleciak et al., 2010). However, *Bartonella* in rodents presents with prolonged bacteremia (Schülein et al., 2001), and adult individuals have more time to become infected than juveniles, which may explain the higher prevalence found in adults during our survey. Other authors considered that no specific rule about age bias in *Bartonella* prevalence can be drawn (Gutiérrez et al., 2015).

No factor other than *Bartonella* occurrence in the host appeared to be related to the prevalence of *Bartonella* in fleas. In addition, we observed the same haplotype in the flea and its host in a considerable proportion. Fleas from positive hosts may have had more chances to become infected. However, this result may be deceptive, because we may be detecting *Bartonella* DNA from the host blood and not live bacteria.

Conclusions

Though it is well-known that micromammals in Mediterranean environments serve as reservoirs for a number of *Bartonella* spp., no information was available about the factors related to variations in their occurrence. In the present study we found that prevalence varied markedly among seasons, host age groups, host abundance and flea infestation levels, and that offspring of infected female wood mouse was frequently infected, indicating that *Bartonella* infection in micromammals is driven by a variety of biotic and abiotic factors. No differences in *Bartonella* prevalence were observed between natural and adjacent residential areas. Taking into account a likely increase in the risk of human-micromammal contact in residential areas, this means that that there is a hazard of infection with wild rodent-borne *Bartonella* for people living in those residential areas. The number of people moving to residential areas or visiting natural parks

around Barcelona is constantly increasing, as is their potential exposure to rodent-borne pathogens.

Acknowledgements.

This study was funded by project CGL2010-17931. We wish to thank S. Lavín (SEFaS, UAB), L. Cabañeros, F.Llimona, and S. Cahill (Collserola Natural Park) and J. Torrent and A.Miño (St. Llorenç Natural Park) for facilitating the work in the field; L. Lobato, B. López, C. Linares, and W. Flores for field assistance; M. Gómez for advice on flea identification; L. Serrano and E. Pérez for laboratory assistance and E. Serrano for his advice on the data analysis.

References

Beaucournu, J.C., Launay, H., 1990. Faune de France 76: les Puces de France et du Bassin méditerranéen occidental. Fédération française des Sociétés de Sciences Naturelles, Paris.

Becker, D.J., Streicker, D.G., Altizer, S., 2015. Linking anthropogenic resources to wildlife-pathogen dynamics: a review and meta-analysis. Ecol. Lett. 18, 483–95.

Birtles, R. J., Hazel, S., Bown, K., Raoult, D., Begon, M., & Bennett, M. 2000. Subtyping of uncultured bartonellae using sequence comparison of 16 S/23 S rRNA intergenic spacer regions amplified directly from infected blood. Mol Cell Probes, 14(2), 79-87.

Birtles, R.J., 2005. Bartonellae as elegant hemotropic parasites. Ann. N. Y. Acad. Sci. 1063, 270–9.

Bitam, I., Rolain, J., Kernif, T., Baziz, B., Parola, P., Raoult, D., 2008. *Bartonella* species detected in rodents and hedgehogs from Algeria. Clin. Microbiol. Infect. Dis. 15, 102–103.

Bown, K.J., Bennett, M., Begon, M., 2004. Flea-borne *Bartonella grahamii* and *Bartonella taylorii* in Bank Voles. Emerg. Infect. Dis. 10, 6–9.

Bradley, C.A., Altizer, S., 2007. Urbanization and the ecology of wildlife diseases. Trends Ecol. Evol. 22, 95–102.

Breitschwerdt, E.B., Kordick, D.L., 2000. *Bartonella* Infection in Animals: Carriership, Reservoir Potential, Pathogenicity, and Zoonotic Potential for Human Infection. Clin. Infect. Dis. 13, 428–438.

Buffet, J., Kosoy, M., Vayssier-Taussat, M., 2013. Natural history of Bartonella- infecting rodents in light of new knowledge on genomics, diversity and evolution. Future Microbiol. 8, 1117–1128.

Cevidanes, A., Proboste, T., Chirife, A.D., Millán, J., 2016. Differences in the ectoparasite fauna between micromammals captured in natural and adjacent residential areas are better explained by sex and season than by type of habitat. Parasitol. Res. 115, 2203–2211.

Chirife, A.D., Millán, J., 2014. Field immobilization of Wood Mice (*Apodemus sylvaticus*) with Medetomidine and Ketamine and Antagonism with Atipamezole. J. Wildl. Dis. 50, 961–963.

Gil, H., García-Esteban, C., Barandika, J.F., Peig, J., Toledo, A., Escudero, R., Jado, I., Rodríguez-Vargas, M., García-Amil, C., Lobo, B., Roales, P., Rodríguez-Moreno, I., Olmeda, A S., García-Pérez, a L., Anda, P., 2010. Variability of *Bartonella* genotypes among small mammals in Spain. Appl. Environ. Microbiol. 76, 8062–70.

Grimmond, S., 2007. Urbanization and global environmental change: local effects of urban warming. Geogr. J. 173, 83–88.

Gutiérrez, R., Krasnov, B., Morick, D., Gottlieb, Y., Khokhlova, I., Harrus, S., 2015. Bartonella infection in rodents and their flea ectoparasites: an overview. Vector Borne Zoonotic Dis. 15, 27–39.

Jardine, A.C., Waldner, C., Wobeser, G., Leighton, F.A., 2006. Demographic features of *Bartonella* infections in Richardson´s ground squirrels (*Spermophilus richardsonii*). J. Wildl. Dis. 42, 739–749.

Kim, C., Kim, J.-Y., Yi, Y.-H., Lee, M., Cho, M., Shah, D.H., Klein, T.A., Kim, H., Song, J., Chong, S., Guinn, M.L.O., Lee, J.S., Lee, I., Park, J., Chae, J., 2005. Detection of *Bartonella* species from ticks, mites and small mammals in Korea. J. Vet. Sci. 6, 327–334.

Kosoy, M., Mandel, E., Green, D., Marston, E., Childs, J., 2004. Prospective Studies of *Bartonella* of Rodents. Part I. Demographic and Temporal Patterns in Population Dynamics. Vector Borne Zoonotic Dis. 4, 285–295.

Kosoy, M., Regnery, R.L., Tzianabos, T., Marston, E.L., Jones, D.C., Green, D., Maupin, G., Olson, J.G., Childs, J.E., 1997. Distribution, diversity, and host specificity of *Bartonella* in rodents from the Southeastern United States. Am. J. Trop. Med. Hyg. 57, 578–588.

Kosoy, M.Y., Regnery, R.L., Kosaya, O.I., Jones, D.C., Marston, E.L., Childs, J.E., 1998. Isolation of *Bartonella* spp. from embryos and neonates of naturally infected rodents. J. Wildl. Dis. 34, 305–309.

Krasnov, B., Morand, S., Hawlena, H., Khokhlova, I., Shenbrot, G., 2005. Sex-biased parasitism, seasonality and sexual size dimorphism in desert rodents. Oecologia 146, 209–17.

Krasnov, B.R., Khokhlova, I.S., Fielden, L.J., Burdelova, N. V, 2001. Effect of Air Temperature and Humidity on the Survival of Pre-Imaginal Stages of Two Flea Species (Siphonaptera: Pulicidae). J. Med. Entomol. 38, 629–637.

Langlois, J.P., Fahrig, L., Merriam, G., Artsob, H., 2001. Landscape structure influences continental distribution of hantavirus in deer mice. Landsc. Ecol. 16, 255–266.

Maggi, R.G., Harms, C.A., Hohn, A.A., Pabst, D.A., Mclellan, W.A., Walton, W.J., Rotstein, D.S., Breitschwerdt, E.B., 2005. *Bartonella henselae* in Porpoise Blood. Emerg. Infect. Dis. 11, 1894–1898.

Márquez, F.J., Rodríguez-Liébana, J.J., Pachón-Ibáñez, M.E., Docobo-Pérez, F., Hidalgo-Fontiveros, A., Bernabeu-Wittel, M., Muniain, M.A., Pachón, J., 2008. Molecular screening of *Bartonella* species in rodents from south western Spain. Vector Borne Zoonotic Dis. 8, 695–700.

McCauley, D.J., Salkeld, D.J., Young, H.S., Makundi, R., Dirzo, R., Eckerlin, R.P., Lambin, E.F., Gaffikin, L., Barry, M., Helgen, K.M., 2015. Effects of land use on plague (*Yersinia pestis*) activity in rodents in Tanzania. Am. J. Trop. Med. Hyg. 92, 776–83.

Millán, J., Chirife, A.D., Proboste, T., Velarde, R., 2014. Factors associated with the prevalence and pathology of *Calodium hepaticum* and *C. splenaecum* in periurban micromammals. Parasitol. Res. 113, 3001–3006.

Morick, D., Krasnov, B.R., Khokhlova, I.S., Gottlieb, Y., Harrus, S., 2013. Transmission dynamics of *Bartonella* sp. strain OE 1-1 in Sundevall's Jirds (*Meriones crassus*). Appl. Environ. Microbiol. 79, 1258–1264.

Nations, U., 2011. Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat, World Population Prospects: The 2010 Revision and World Urbanization Prospects: The 2011 Revision.

Paziewska, A, Harris, P.D., Zwolińska, L., Bajer, a, Siński, E., 2012. Differences in the ecology of *Bartonella* infections of *Apodemus flavicollis* and *Myodes glareolus* in a boreal forest. Parasitology 139, 881–93.

Reeves, W.K., Dowling, A.P.G., Dasch, G.A., 2006. Rickettsial agents from parasitic Dermanyssoidea (Acari: Mesostigmata). Exp. Appl. Acarol. 38, 181–188.

Reeves, W.K., Loftis, A.D., Szumlas, D.E., Abbassy, M.M., Helmy, I.M., Hanafi, H.A., Dasch, G.A., 2007. Rickettsial pathogens in the tropical rat mite *Ornithonyssus bacoti* (Acari: Macronyssidae) from Egyptian rats (*Rattus* spp.). Exp. Appl. Acarol. 41, 101–107.

Schülein, B.R., Seubert, A., Gille, C., Lanz, C., Hansmann, Y., Piémont, Y., Dehio, C., 2001. Invasion and Persistent Intracellular Colonization of Erythrocytes: A Unique Parasitic Strategy of the Emerging Pathogen *Bartonella*. J. Exp. Med. 193, 1077–1086.

Shochat, E., Warren, P.S., Faeth, S.H., McIntyre, N.E., Hope, D., 2006. From patterns to emerging processes in mechanistic urban ecology. Trends Ecol. Evol. 21, 186–91.

Silaghi, C., Pfeffer, M., Kiefer, D., Kiefer, M., Obiegala, A., 2016. Bartonella, Rodents, Fleas and Ticks: a Molecular Field Study on Host-Vector-Pathogen Associations in Saxony, Eastern Germany. Microb. Ecol. 1–10. doi:10.1007/s00248-016-0787-8

Solano-Gallego, L., Rossi, L., Scroccaro, A.M., Montarsi, F., Caldin, M., Furlanello, T., Trotta, M., 2012. Detection of *Leishmania infantum* DNA mainly in *Rhipicephalus sanguineus* male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasit. Vectors 5, 98.

Telfer, S., Begon, M., Bennett, M., Bown, K.J., Burthe, S., Lambin, X., Telford, G., Birtles, R., 2007a. Contrasting dynamics of *Bartonella* spp. in cyclic field vole populations : the impact of vector and host dynamics. Parasitology 134, 413–425.

Telfer, S., Clough, H.E., Birtles, R.J., Bennett, M., Carslake, D., Helyar, S., Begon, M., 2007b. Ecological differences and coexistence in a guild of microparasites: *Bartonella* in wild rodents. Ecology 88, 1841–1849.

Vayssier-Taussat, M., Moutailler, S., Féménia, F., Raymond, P., Croce, O., Scola, B. La, Fournier, P., Raoult, D., 2016. Identification of Novel Zoonotic Activity of *Bartonella* spp ., France. Emerg. Infect. Dis. 22, 457–462.

Welc-Faleciak, R., Bajer, A., Behnke, J.M., Siński, E., 2010. The ecology of *Bartonella* spp. infections in two rodent communities in the Mazury Lake District region of Poland. Parasitology 137, 1069–77

Legends to Figures:

Figure 1. Map of the study areas. Dark gray: urbanized areas; light gray: non-urbanized areas; Striped: Natural Parks; (1) Sant Llorenç del Munt Natural Park SLNP; (2) Collserola Natural Park (CoNP); Up-left photo: SLNP natural area; Up-right photo: SLNP residential area; Down-left photo: CoNP natural areas, Down-right photo: CoNP residential area.

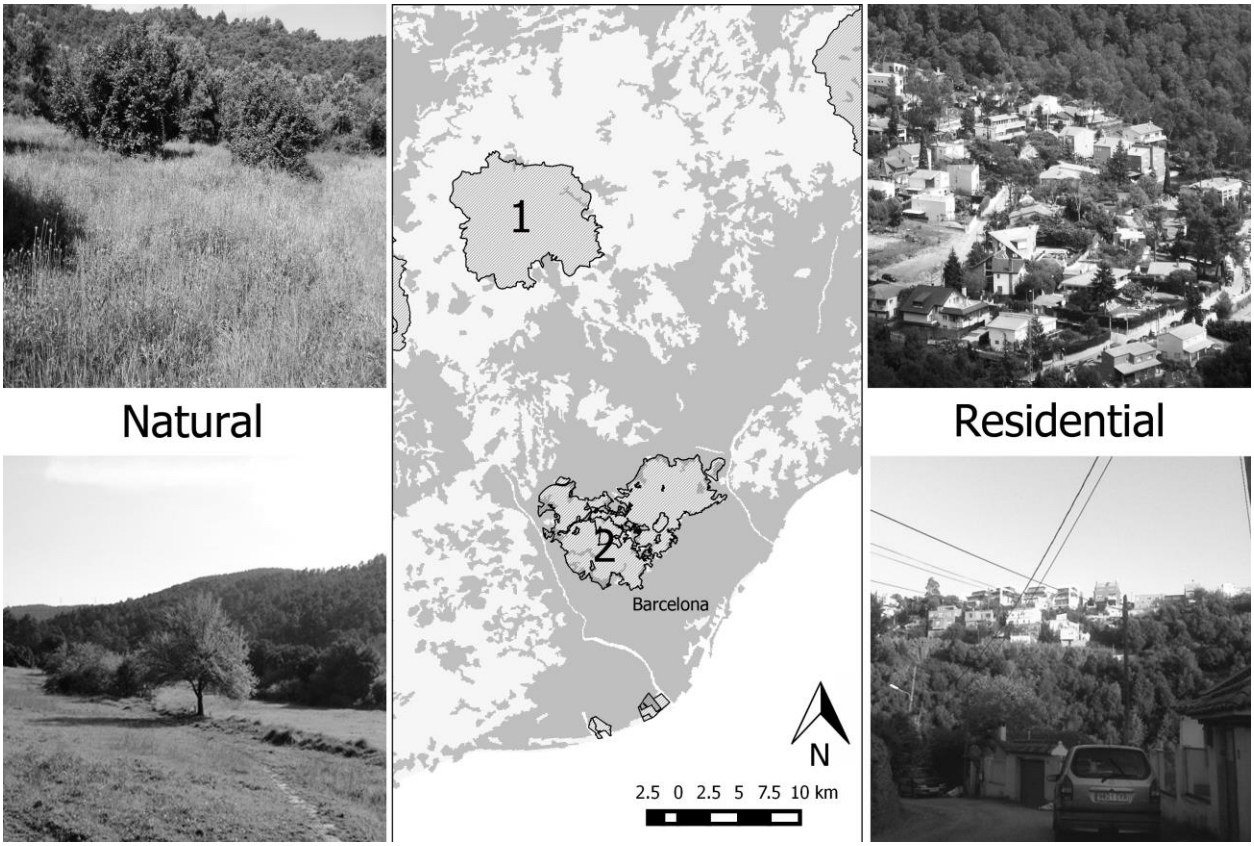

Figure 2. Variations in *Bartonella* prevalence in wood mice (*Apodemus sylvaticus*) depending on the rate of captures during the capture event and the level of flea infestation. Host abundance was calculated using a "captures/100 traps/night" index. Low host abundance was considered between 0.1 and 5 animals/100 trap/night; medium between 5 and 10; and high more than 10. One of these categories was assigned to each individual depending on the host abundance of the sampling period and area in which it was captured. Flea abundance was transformed in a categorical variable depending on the flea burden of each host individual: no infestation= no fleas; low infestation= between one and five fleas; and high infestation= between five and ten fleas.

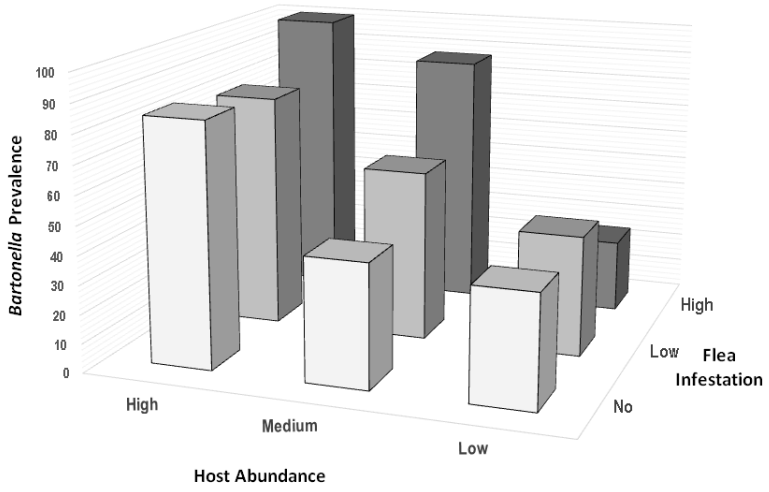

Table 1. Genetic variants of *Bartonella* detected in small mammals and their fleas in periurban Barcelona. Two *Mus musculus*, 16 *Rattus rattus* and three *R. norvegicus, t*wo *Rattus* sp. and one *Mus* sp. hosted no *Bartonella*.

Haplotype	**Wood mouse (*Apodemus sylvaticus*)**			**White-toothed shrew (*Crocidura russula*)**		**Algerian mouse (*Mus spretus*)**		
		Flea pools			**Flea pools**		**Flea pools**	
	Tissue	***C.a.c***	***L.t.a***	**Tissue**	***C.a.c***	**Tissue**	***C.a.c***	***L.t.a***
	n=407	n=129	n=65	n=44	n=10	n=36	n=6	n=4
HT1	40 (17.2%)	18 (20.9%)	1 (1.5%)	1 (10%)	0	0	2 (100%)	0
HT2	16 (6.8%)	8 (9.3%)	4 (14.2%)	0	0	5 (71.4%)	0	1 (100%)
HT3	48 (20.2%)	10 (11.6%)	4 (14.2%)	1 (10%)	0	0	0	0
HT4	21 (9.0%)	9 (10.4%)	2 (7.1%)	1 (10%)	2 (33.3%)	1 (14.2%)	0	0
HT5	41 (17.6%)	22 (25.5%)	4 (14.2%)	1 (10%)	0	0	0	0
HT6	5 (2.1%)	1 (1.1%)	7 (25%)	0	0	0	0	0
HT7	12 (5.1%)	7 (8.1%)	0	0	0	0	0	0
HT8	5 (2.1%)	1 (1.1%)	1 (3.5%)	0	0	0	0	0
HT9	2 (0.8%)	0	0	5 (50%)	2 (33.3%)	0	0	0

HT10	3 (1.2%)	0	0	1 (10%)	0	0	0	0
HT11	3 (1.2%)	1 (1.1%)	0	0	1 (16.6%)	0	0	0
HT12	0	0	1 (3.5%)	0	0	1 (14.2%)	0	0
Unidentified HT	36 (15.5%)	9 (10.4%)	4 (14.2%)	0	1 (16.6%)	0	0	0
Overall [95% C.I.]	232 (57.0%) [52.1%-62.8%]	86 (66.6%) [58.1%-74.5%]	28 (43.0%) [31.2%-55.4%]	10 (22.7%) [11.7%-37.2%]	6 (60.0%) [28.2%-85.0%]	7 (19.4%) [8.8%-35.7%]	2 (33.3%) [6.2%-72.8%]	1 (25%) [1.2%-75.1%]

[1] Number of positives for each haplotype and percentage of the variants detected in each host and flea pool species (in parenthesis); C.a.c, *Ctenophtalmus andorrensis catalanensis*; L.t.a, *Leptopsylla taschenbergi amitina*.

Table 2. Summary of GLMM best model results for *Bartonella* and wood mouse and flea pool association. Year and area of sampling were introduced as random effects. Reference levels for the fixed effects were, adult (for age), autumn (for season), low (for host abundance), none (for flea abundance) and negative (for *Bartonella* occurrence in hosts). AICw – Akaike Information Criterion weight of the best model from a set of models derived from a full model (all fixed effects and year and area of sampling as random effects; see main text for explanations).

sociation	**Nº Obs**	**Fixed effect**	**Estimate ±SE**	**z-value**	**AICw**	**χ^2**
rtonella – Wood mouse	405	**Season (Spring)**	-1.30 ±0.29	-4.47***	0.187	83.24 ***
		Age (Juvenil)	-0.76 ± 0.36	-2.14*		
		Host abundance (Medium)	1.63 ± 0.41	3.853***		
		Host abundance (High)	1.86±0.46	4.04***		
		Flea abundance (High)	1.405 ± 0.59	2.36*		
		Flea abundance (Low)	0.09 ± 0.26	0.33NS		
rtonella – Flea pools	218	***Bartonella* occurrence in host**	1.94 ±0.32	5.95***	0.18	39.48***

Significance levels: '***', <0.0001; '**', <0.01; '*', <0.05; '^', <0.1 ; 'NS', non-significant. $\chi 2$, likelihood ratio when comparing the best model with the model of intercept and random effects only.

Annex I.- Amplified *Bartonella* haplotypes amplicon lenght and homologies with sequences available in GenBank

Haplotype	Amplicon length (without primers)	% coverage	% identity	nt	with the sequence:
HT1	158	100	100	158/158	AJ269796.1/KU886518.1
HT2	158	100	91	158/173	AJ269788.1
HT3	173	100	100	173/173	AJ269791.1/AJ269787.1
HT4	173	100	100	173/173	AJ269788.1
HT5	150	100	100	150/150	AJ269792.1
HT6	209	100	100	209/209	EU218552.1/JF766264.1
HT7	203	100	85	173/203	AJ269788.1
HT8	188	100	92	173/188	AJ269791.1/AJ269787.1
HT9	228	100	86	197/229	DQ166944.1*
HT10	142	100	100	142/142	KX062703.1/FJ010195.1/AY763103.1/BX897699.1/L35101.1
HT11	238	100	99	236/238	CP001562.1/AJ269790.1/AJ269785.1/AF442953.1
HT12	123	100	95	117/123	FN645485.1

*sequence that show the highest identity

Annex II.- Proportion of each identified haplotype in flea pools depending on the haplotype (HT) found in its host.

	Flea pools											
Host samples	HT1	HT2	HT3	HT4	HT5	HT6	HT7	HT8	HT9	HT11	HT12	Overall flea pools with identified HT by host HT
HT1	**52.9% (9)**	11.8% (2)	17.6% (3)	5.9% (1)	11.8% (2)	0	0	0	0	0	0	17
HT2	9.1% (1)	**63.6% (7)**	0	9.1% (1)	0	18.2% (2)	0	0	0	0	0	11
HT3	11.1% (2)	0	**50.0% (9)**	5.6% (1)	27.7 % (5)	5.6% (1)	0	0	0	0	0	18
HT4	0	0	0	**50.0% (2)**	25.0%(1)	0	0	0	25.0%(1)	0	0	4
HT5	8.3% (1)	0	0	16.7% (2)	**50.0% (6)**	8.3% (1)	16.7% (2)	0	0	0	0	12
HT6	0	**50.0% (3)**	0	0	0	33.3% (2)	0	0	0	0	16.7% (1)	6
HT7	0	0	0	0	0	0	**100% (4)**	0	0	0	0	4
HT8	0	0	0	0	0	0	0	**100% (2)**	0	0	0	2
HT9	0	0	0	0	0	0	0	0	100%(2)	0	0	2
HT10	100% (1)	0	0	0	0	0	0	0	0	0	0	2
HT11	0	0	0	0	0	0	0	0	**0**	**100% (1)**	0	1
Overall proportion of HT in flea pools *	17.9% (14)	15.4% (12)	15.4% (12)	8.9% (7)	17.9% (14)	7.7% (6)	7.7% (6)	2.6% (2)	3.8% (3)	1.3% (1)	1.3% (1)	78

*Overall proportion of haplotypes (HT) in flea pools respect to the total flea pools with identified haplotypes.