International Journal for Parasitology 49 (2019) 779–787

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Changes in host densities and co-feeding pattern efficiently predict tick-borne encephalitis hazard in an endemic focus in northern Italy

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ARTICLE INFO

Article history:
Received 21 February 2019
Received in revised form 3 May 2019
Accepted 8 May 2019
Available online 23 July 2019

Keywords:
Tick-borne encephalitis
Ixodes ricinus
Apodemus flavicollis
Capreolus capreolus
Rodent density
Autumnal cooling
TBE hazard

ABSTRACT

Tick-borne encephalitis is an important zoonosis in many parts of north-western, central and eastern Europe, Russia and the Far East, with considerable altitudinal and latitudinal shifts described during recent decades. The reported routes of transmission for TBE virus include the saliva-activated non-viraemic transmission between co-feeding ticks taking place on rodent hosts. During the period 2001–2014, a population of the yellow-necked mouse (*Apodemus flavicollis*), which is considered among the most efficient TBE competent host, especially in central and western Europe, was intensively live-trapped in a known TBE focus in the Province of Trento, Italy. Individual live-trapped mice were checked for the number and position of feeding ticks and serologically screened for TBEv antibodies. A combined effect of climatic conditions and density of both roe deer and mice on the number of co-feeding tick groups was observed. Specifically, the occurrence of co-feeding ticks on mice during the questing season was affected by autumnal cooling in the previous season. On the other hand, co-feeding occurrence was also positively associated with roe deer abundance, while mouse density showed a hump-shaped pattern. Individual features of *A. flavicollis* such as weight and sex also affected co-feeding occurrence with the heaviest (breeding adult) males carrying more co-feeding ticks. We also found that the overall number of co-feeding ticks on mice positively affected TBEv antibody detection in this species the following year. In conclusion, a specific combination of climatic conditions in conjunction with certain rodent and roe deer densities are the principal determinants of the number of co-feeding ticks on *A. flavicollis* and, consequently, TBEv circulation. These variables can be used to provide an early warning signal for a TBE hazard, thus representing a useful tool for Public Health authorities to prepare action for prevention and control within TBEv circulation areas.

1. Introduction

Tick-borne encephalitis (TBE) is a zoonotic infection of the central nervous system caused by the TBE virus (TBEv), which is transmitted to humans principally by ticks belonging to the *Ixodes ricinus* complex. Despite the availability of a safe vaccine (De Micheli et al., 2009; Loew-Baselli et al., 2011), TBE has been a growing public health problem in Europe and several parts of Asia in recent decades, with both an increasing number of human cases in endemic regions and the emergence of new TBE foci in geographic areas and countries not previously affected (Kunze and ISW-TBE 2019, https://iswtbe.com).

TBEv (Family *Flaviviridae*) currently includes three different subtypes: the western European subtype, transmitted mainly by the sheep tick *I. ricinus*, and the Siberian and Far Eastern subtypes, both of which are transmitted mainly by the taiga tick *Ixodes persulcatus* (Bogovic and Strle, 2015; Varlacher et al., 2015). In nature, TBEv is maintained in a cycle involving hard ticks (Ixodidae), which act as the main vector, and micromammals, especially those belonging to the genera *Apodemus* and *Myodes*, from which the ticks obtain their blood meals (Varlacher et al., 2015). The circulation of this virus in Europe is spatially and temporally limited to natural foci (endemic areas) which can vary from a few square metres to several square kilometres. These foci are usually unstable, both in term of impact and location. For example, human TBE cases usually fluctuate widely even within endemic foci (Zeman, 2017), and latitudinal and altitudinal shifts have been

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https://doi.org/10.1016/j.ijpara.2019.05.006

reported in several European Union (EU) countries during the last decade (Daniel et al., 2003; Rezza et al., 2015). Among European countries, Italy is considered ‘low incidence’ for TBE (0.38/100,000 inhabitants; Rezza et al., 2015), which has been only recently included in the list of notifiable diseases by the Italian Ministry of Health. The occurrence of human TBE cases appears to be geographically clustered in the forested areas in the mountainous north-east of the country, where the number of TBE cases has increased three- to four-fold in the past 15 years (Rezza et al., 2015).

The transmission of TBEv among ticks, and between ticks and competent vertebrate hosts, may be vertical (from an infected tick female to her eggs) (Slovák et al., 2014), horizontal viraemic (from an infectious vertebrate competent host to an uninfected tick) (Nuttall and Labuda, 2003) or horizontal non-viraemic (also called ‘co-feeding’, i.e. infected and uninfected ticks simultaneously feed on susceptible or immune vertebrate hosts, resulting in all ticks in the co-feeding group potentially becoming infected) (Labuda et al.. 1993a; Labuda et al., 1996, 1997). Both vertical and viraemic transmissions are considered ineffective (Danielová et al., 2002) or less important for the maintenance of TBEv in nature, while co-feeding transmission is recognised as the most efficient way to sustain the presence of TBEv in an endemic area for several reasons. First of all, ticks are aggregated in the environment and tend to more intensively parasitize a small proportion of the host population (20/80 rule) (Shaw and Dobson, 1995). Secondly, on the hosts, ticks show an overdispersed (variance/mean > 1) distribution, feeding continuously for several days in close proximity (within 0.5 cm). This last feature accomplishes virus transmission through the local skin cells at the feeding site, avoiding any involvement of the host immune system (Labuda et al., 1996, 1997; Nuttall, 1998; Nuttall and Labuda, 2003; Nonaka et al., 2010).

An essential prerequisite for co-feeding is the simultaneous presence of infected (usually nymphs) and uninfected (usually larvae) tick stages in the environment. Several studies have assessed the importance of specific climatic requirements that favour the seasonal synchronicity of larval and nymphal tick activity (Randolph et al., 1999; Randolph and Rogers, 2000). In particular, Randolph et al. (2000), showed that a high rate of autumnal cooling (i.e. a rapid decrease in late summer temperatures) favours the synchronous emergence of larvae with nymphs in the spring of the following year. This is achieved by the induction of a behavioural diapause where ticks overwinter as unfed stages. Nonetheless, this temperature regime and the associated synchronous feeding by larvae and nymphs are not always sufficient to maintain the enzootic cycle of TBEv (Randolph et al., 2000). Other important conditions need to be met such as habitat suitability for ticks, availability of both tick maintenance hosts (such as large vertebrates which provide blood meals for reproductive adult ticks), and transmission-competent hosts (such as rodents) which favour viraemic or non-viraemic transmission (Rizzoli et al., 2009).

Although many studies have focussed on identifying the biotic and abiotic factors causing TBE emergence and foci occurrence (Sumilo et al., 2007; Moshkin et al., 2009; Rizzoli et al., 2009; Tonteri et al., 2015), few have attempted to identify the ecological mechanisms which affect TBEv circulation within endemic foci. However, an understanding of these mechanisms is essential for identifying early indicators of changes in a TBE hazard (i.e. occurrence of the pathogen in the enzootic cycle; Randolph et al., 2010).

Here, we carried out a long-term longitudinal study in a well-known natural TBE focus (Hudson et al., 2001; Rizzoli et al., 2009), monitoring the population dynamics of a TBE-competent host, the yellow-necked mouse (*Apodemus flavicollis*), as well as the variation in TBE seroprevalence and the feeding distribution of the sheep tick on this host, to model the temporal variation in infection hazard. Our hypotheses were: (i) the number of co-feeding tick groups is affected by specific climatic (precipitation and temperature) variables; (ii) the number of co-feeding tick groups is affected by specific ecological variables (i.e. rodent and ungulate density and rodent host features); (iii) the number of co-feeding groups positively affects TBEv seroprevalence in rodent hosts the following year, therefore predicting TBEv circulation as well as the TBE hazard.

2. Material and methods

2.1. Study area

Our study was carried out in a well-documented TBE focus in the Province of Trento, Italy, in the municipality of Cavedine (50°56′15″N, 16°31′13.8″E, 750 metres above sea level; Fig. 1). The rodent trapping area is an isolated calcareous ridge covered with mixed beech (*Fagus sylvaticus*) mature coppice woodland and scattered meadows. Despite differences in abundance in time and space, *I. ricinus* ticks in the study area showed a similar temporal pattern of life stages among years i.e. a bimodal pattern for nymphs, with a main peak of activity in May and a secondary peak in October, and a unimodal pattern for larvae, with a peak in August (Tagliapietra et al., 2011). Roe deer abundance for the Cavedine hunting district was obtained by annual censuses (Simonetta and Dessì-Fulgheri, 1998; Raganella Pelliccioni et al., 2013) carried out by the local hunters’ association (L. Luchesa, Trentino Hunters’ Association, Italy, personal communication).

2.2. Rodent and tick sampling

The population dynamics of yellow-necked mice was monitored on four permanent trapping grids (labelled A, B, C, D; Fig. 1) from 2000 to 2008 and from 2010 to 2014. Each trapping grid consisted of an 8 × 8 square array of trap stations set 15 m apart (with a total grid covering an area of 1.1 ha). One multiple-capture Ugglan live trap model n. 2 (Grahnab, Sweden) was set at each station and standard capture-mark-recapture techniques (CMR) were adopted (Lawson et al., 1992; Amstrup et al., 2005). Overall, 168 capture sessions (3 days/2 nights) were carried out, every 2 weeks from 2001 to 2008 and every month from 2010 to 2014. In 2009, no monitoring was carried out. The population density was estimated using the standard open population Jolly-Seber method (Schwarz and Neil Arnason, 1996). Grid sizes and locations remained constant from 2000 to 2014 (14,400 sqm) and the density was expressed as mice per hectare. At first capture, each mouse was individually marked with a s.c. implanted Passive Integrated Transponder (PIT) tag (ID100 Trovan Ltd., UK). At each sampling session, date, grid, trap station, body mass, sex and the PIT tag number were recorded, and a blood sample was collected from the retro orbital sinus using a hematocrit microcapillary (100 µl). The numbers and life stages of feeding ticks were also recorded. When feeding nymphs were present their distance (mm) from the nearest larva was estimated. A co-feeding tick group has been defined as an aggregation of ticks feeding at a perceptible distance, excluding larval-only associations as these are likely to be pathogen-free (Labuda et al., 1993b, 1996; Randolph et al., 1996). According to this definition, we considered a co-feeding group as the simultaneous presence of one or more larvae and one or more nymphs feeding at a maximum distance of 5 mm from one another. Animals were then released unharmed at the site of capture. All blood samples were centrifuged at 12,320*g* for 12 min in the laboratory to separate serum from blood. All trapping and sampling procedures were approved by the Wildlife Committee of the Autonomous Province of Trento (Prot. n. S044-5/2015/277268/2.4).

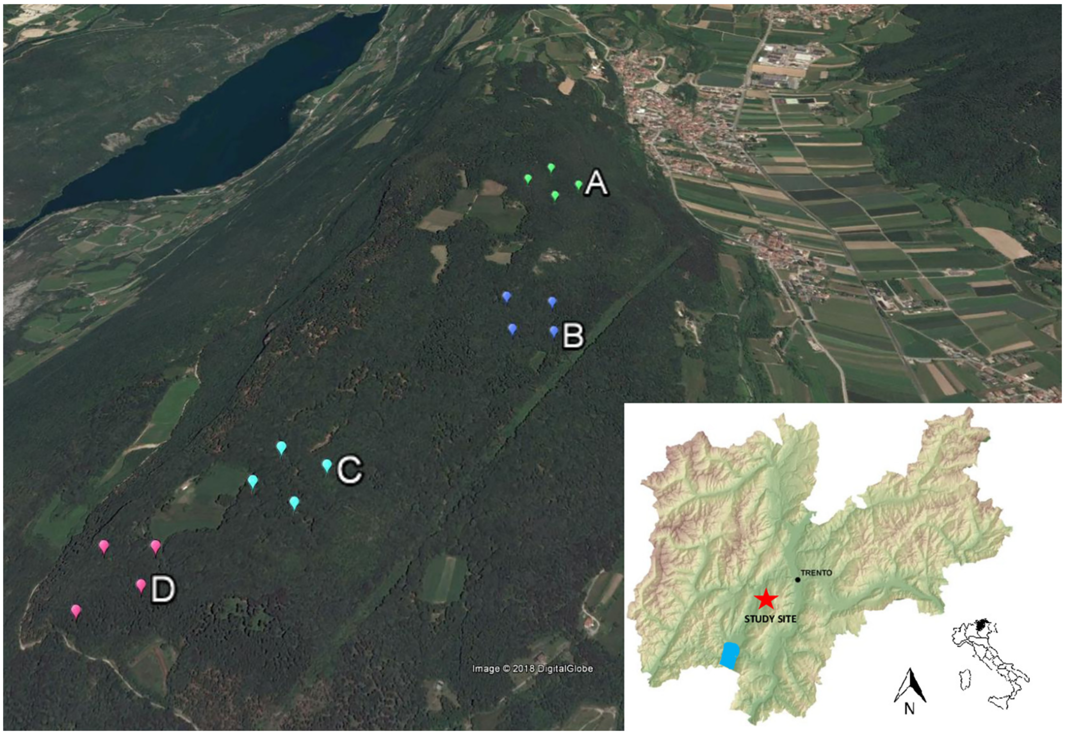

Fig. 1. Map of the study area in Cavedine (Province of Trento, Italy). Trapping grids are labelled with A, B, C, D.

2.3. TBEv antibody detection in *A. flavicollis*

The presence of antibodies to TBEv in *A. flavicollis* serum was tested using a standard ELISA method on samples collected from 2001 to 2006 (Kalon Biological Ltd., UK). TBEv coated plates were prepared from a purified TBEv strain (supplied by Dr. Ernie Gould, Centre for Ecology and Hydrology, Oxford, UK). The recorded antibody titre for each sample was compared with that from a population of *A. sylvaticus* from Ireland, which has never been exposed to TBEv or other flaviviruses (Kalon Biological Ltd., UK, internal report, available on request). Samples that had an antibody dose unit (ADU) level higher than the highest level observed in the TBEv-free population (ADU = 6.0, sample size = 61) were taken to be seropositive. From 2007, an IFA test using slides provided by the Department of Virology, University of Helsinki, Finland, was performed. Rodent sera was diluted 1/20, according to the manufacturer's protocol (HaartBio Ltd., Helsinki, Finland).

2.4. Autumnal cooling

Following Randolph et al. (2000), we calculated the autumnal cooling rate using temperature values from a weather station of the Fondazione Edmund Mach located in Cavedine (Trento, Italy; http://meteo.fmach.it/meteo/mappa.php; 45°59′06.3″N 10°58′51.5″E), near our study area. We calculated the autumnal cooling from 2000 to 2013, applying a linear regression to the average daily temperature against the Julian day in the period 1st August – 31st October. These values of autumnal cooling were the offset and the slope (that is negative by definition) of the linear regression. Lower values of the slope correspond to a more rapid decrease in temperature (steeper regression line).

2.5. Statistical analysis

All quantitative independent variables were standardised (by subtracting their mean and dividing by their standard deviation). Statistical analyses were carried out using the R software 3.5.1 (R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing) and packages *MuMIn* (Barton, 2018) and *tidyverse* (Wickham, 2017).

2.5.1. Co-feeding model

We investigated the association between the number of co-feeding groups recorded on each mouse and a range of ecological and climatic variables from 2001 to 2012 (except 2009 when no data were available). The ecological and climatic predictors were the annual roe deer abundance, the autumnal cooling rate of the previous year (both offset and slope of the linear regression were used), the density of *A. flavicollis* (and its squared function) recorded within the grid for each trapping session, weight and sex of individual mice, the number of larvae recorded on each mouse and the trapping grid. Pearson correlation pairplots and the Variance Inflation Factor (VIF > 3) were used to test for collinearity between all explanatory variables (Zuur et al., 2007). Following exclusion of collinear variables, we developed a Poisson Generalised Linear Model (GLM), named as the full model. Starting with the full model, we carried out a model selection by ranking all sub-models on the basis of the second-order Akaike information criterion (AICc). The model with the lowest AICc was selected as the best model (Burnham and Anderson, 2002). Data collected in 2013 and 2014 (15% of the whole dataset) were used for a model validation process where we carried out a simulation study (Zuur and Ieno, 2016) to assess if the simulated distribution of co-feeding groups generated by the best model was in compliance with observed values.

2.5.2. TBE model

We investigated the association between the proportion of yellow-necked mice that were seropositive for TBEv (per grid per year) and a range of ecological variables during the period 2002–2011 (except 2009 when we have no data). Blood samples were collected from animals at every session and infection was assessed for each captured individual independently if it was a first capture or a recapture in a previous session (recaptured mice in the same session were tested only one time). For this model, data were aggregated by grid and year to have a reasonable sample size to compute a reliable proportion of the infected mice on the total number of mice trapped, for which sera were tested for TBEv-antibody positivity. For this analysis we considered only the period from May-September as mice were sampled and tested in all grids and years only for these months. The explanatory variables included annual roe deer abundance, mouse density (and its squared function), the mean number of nymphs per individual mouse aggregated by grid and year, the total number of co-feeding groups recorded in the same grid during the previous year and trapping grid. Pearson correlation pairplots and the Variance Inflation Factor (VIF > 3) were used to test for collinearity between all explanatory variables (Zuur et al., 2007). Following exclusion of collinear variables, we developed a Binomial Generalised Linear Model (GLM). Starting from this full model we carried out a model selection by ranking all sub-models on the basis of the second-order Akaike information criterion (AICc). The model with the lowest AICc was selected as the best model (Burnham and Anderson, 2002). Data collected in 2012–2013 (20% of collected data) were used for model validation, in which a simulation study (Zuur and Ieno, 2016) was carried out to assess if the simulated distribution of TBEv-seropositive rodents generated by the best model matched the observed values.

2.6. Data accessibility

Data related to this study are available in Mendeley Data, V1 https://doi.org/10.17632/728g9w4rf9.1.

3. Results

3.1. Rodent density, tick burden and TBEv antibodies in rodents

A total of 5458 captures from 2320 individual *A. flavicollis* were recorded from 2001 to 2014. Other species such as *A. sylvaticus*, *Myodes glareolus* and *Sorex* spp. were only occasionally caught during the study period and will not be considered further in the study.

The overall mean rodent density was 13.9 animals/ha (Table 1) with minimums of 4.3 and 4.8 in 2003 and 2010, respectively, and a maximum of 20.3 and 20.5 in 2005 and 2007, respectively. Over the whole period, a total of 54,078 feeding larvae, 667 feeding nymphs and 388 co-feeding groups were counted. The mean number of feeding larvae on *A. flavicollis* was 9.8 (ranging from 0 to 137), while for nymphs it was 0.12 (ranging from 0 to 15) (Table 1). As expected, no feeding adults were found on captured rodents. Distributions of feeding larvae and nymphs on rodents were both strongly aggregated as shown by their variance to mean ratios (larvae = 15.6; nymphs = 2.1).

A total of 4145 sera samples, taken form 1801 *A. flavicollis*, were analysed and a mean TBEv seroprevalence of 3.5% was estimated (Table 1). Rodent densities, roe deer abundance, tick burdens and TBEv seroprevalence recorded per sampling year are reported in Table 1.

3.2. Co-feeding model

Following the collinearity analysis, the explanatory variables included in the full model were: autumnal cooling rate (slope of linear regression) of the previous year, annual roe deer abundance, rodent density (and its squared function) recorded within the grid for each trapping session, weight and sex of rodents, number of larvae recorded on each rodent and trapping grid.

The results of the Poisson GLM best model (Table 2 and Fig. 2A) showed that the number of co-feeding tick groups on mice was negatively affected by the autumnal cooling rate (slope) recorded in the previous season (i.e. a rapid decrease in late summer temperatures in the previous year is associated with an increase in the number of co-feeding tick groups). The number of co-feeding groups on mice was also positively influenced by the total number of feeding larvae (Fig. 2B), annual roe deer abundance (Fig. 2C) and rodent density observed during the season (Fig. 2D). While the first two factors increase linearly with the number of co-feeding groups, rodent density showed a humped relationship, with the number of co-feeding groups initially increasing with rodent density, reaching a maximum around 10 individuals/ha, and followed by a decrease for any further increase in rodent density. In addition, the number of co-feeding groups was affected by host sex (Fig. 2E) and body mass (Fig. 2F) with higher numbers of co-feeding groups recorded on heavier male animals. The number of co-feeding groups was not homogeneously distributed among the grids, with the highest values recorded in Grid A. Finally, the predicted relative frequency distributions of co-feeding tick groups based on 2001–2012 data

Table 1
Average rodent density, roe deer abundance, feeding larvae and feeding nymphs, and total number of co-feeding groups and tick-borne encephalitis seroprevalence (%) detected in rodents in different years.

Year	Average rodent density/ha (±S.E.)	Annual roe deer abundance[a]	Average feeding larvae (±S.E.)	Average feeding nymphs (±S.E.)	Total co-feeding groups	TBE seroprevalence (%) (±S.E.)
2001	9.55 ± 0.87	320	17.18 ± 1.20	0.26 ± 0.04	62	6.13 ± 1.65[b]
2002	7.70 ± 0.22	320	11.48 ± 0.73	0.24 ± 0.07	42	4.05 ± 1.25[b]
2003	4.30 ± 0.20	320	15.27 ± 1.15	0.16 ± 0.03	20	5.52 ± 1.90[b]
2004	7.02 ± 0.24	280	15.58 ± 1.05	0.43 ± 0.06	49	3.31 ± 1.33[b]
2005	20.31 ± 0.21	240	7.33 ± 0.28	0.05 ± 0.01	37	6.75 ± 0.84[b]
2006	7.44 ± 0.16	240	11.54 ± 0.64	0.19 ± 0.03	40	3.07 ± 1.07[b]
2007	20.48 ± 0.30	185	5.17 ± 0.21	0.03 ± 0.01	14	2.49 ± 0.49[c]
2008	5.48 ± 0.13	185	11.49 ± 0.69	0.10 ± 0.02	23	1.05 ± 0.52[c]
2009	NA	185	NA	NA	NA	NA
2010	4.84 ± 0.47	225	13.75 ± 1.35	0.23 ± 0.05	14	2.91 ± 1.66[c]
2011	8.52 ± 0.27	225	11.40 ± 0.69	0.14 ± 0.03	14	0[c]
2012	7.46 ± 0.23	225	14.56 ± 0.99	0.25 ± 0.06	40	3.04 ± 1.06[c]
2013	8.10 ± 0.63	225	13.51 ± 1.11	0.23 ± 0.05	11	0[c]
2014	17.77 ± 0.31	225	9.41 ± 0.49	0.05 ± 0.01	22	NA

NA, not available.
[a] In relation to the hunting district i.e. 3759 h.
[b] Elisa test.
[c] IFA test, see Section 2.3.

Table 2
Output of the best Poisson Generalised Linear Model for predicting co-feeding groups on rodents. Reference levels are for Sex (female) and for Grid (A).

Variable	Coefficient	S.E.	*z* value[a]	Pr(>|z|)[b]
Intercept	−2.893	0.162	−17.808	<0.0001
Autumnal cooling (slope)	−0.284	0.049	−5.780	<0.0001
Roe deer abundance	0.404	0.058	6.968	<0.0001
Feeding larvae	0.446	0.028	15.774	<0.0001
Rodent density	−0.134	0.091	−1.480	0.1390
Rodent density^2	−0.283	0.088	−3.216	0.0013
Weight	0.314	0.067	4.672	<0.0001
Sex_Male	0.540	0.134	4.019	0.0001
Grid_B	−0.581	0.163	−3.573	0.0004
Grid_C	−0.617	0.155	−3.970	0.0001
Grid_D	−0.512	0.151	−3.386	0.0007

[a] Estimate to S.E. ratio.
[b] Two-tailed *P* values for Z statistics.

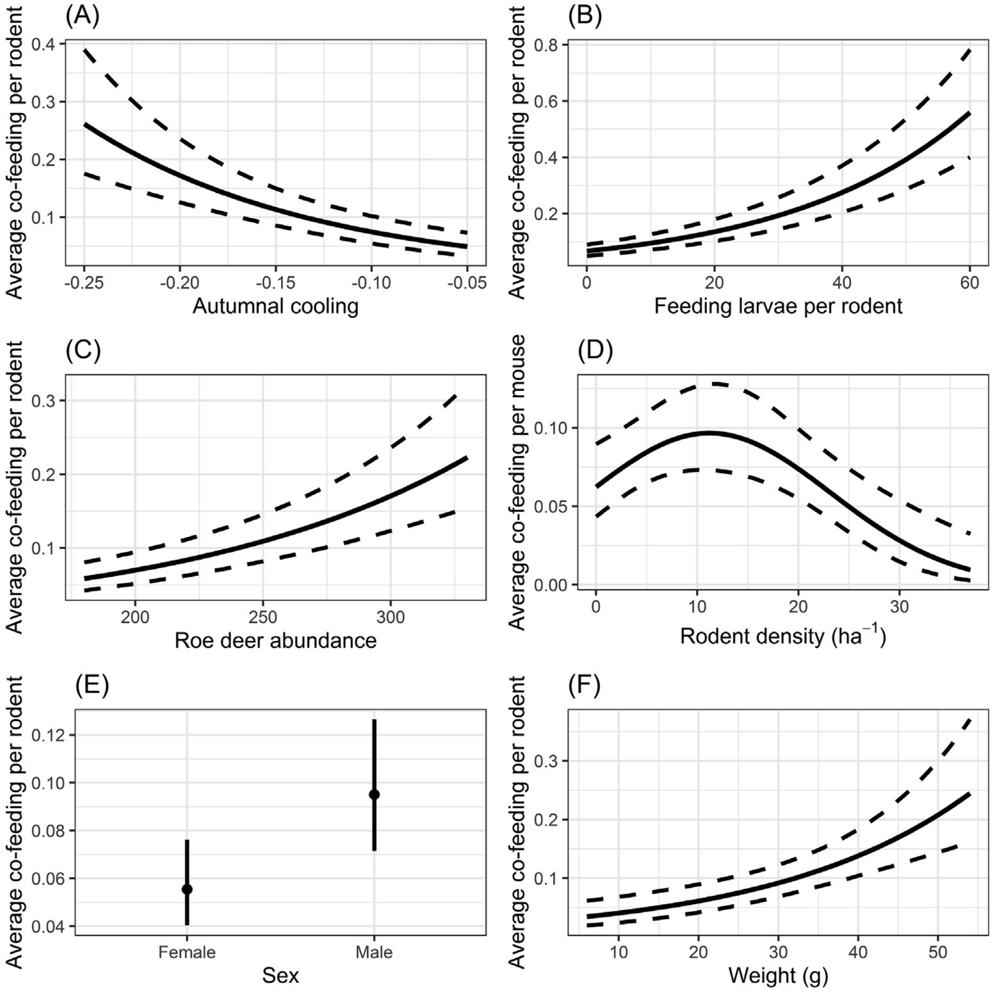

Fig. 2. Relationship between the average co-feeding per rodent and autumnal cooling (A), feeding larvae on rodents (B), roe deer abundance (C), rodent density (D), sex and weight (E and F). Black lines (and dots for sex) are the average values while dashed lines (and bars for sex) represent the 95% confidence intervals. Panels have different *Y*-axis ranges to assist visualisation.

and generated by the Poisson GLM (see vertical bars in Fig. 3) are in good agreement with the values observed in trapping grids during 2013 and 2014 (red dots in Fig. 3). In fact, except for grid C in 2013, the observed number of co-feeding groups recorded in 2013 and 2014 falls within the 95% confidence interval of the expected number of co-feeding tick groups (red dashed lines in Fig. 3) predicted by the model.

3.3. TBE model

Following collinearity analysis, the explanatory variables included in the full model were the number of co-feeding tick groups recorded on rodents trapped in the same grid during the previous year, and rodent density with its squared function. The output of the best Binomial GLM model (Table 3 and Fig. 4) showed

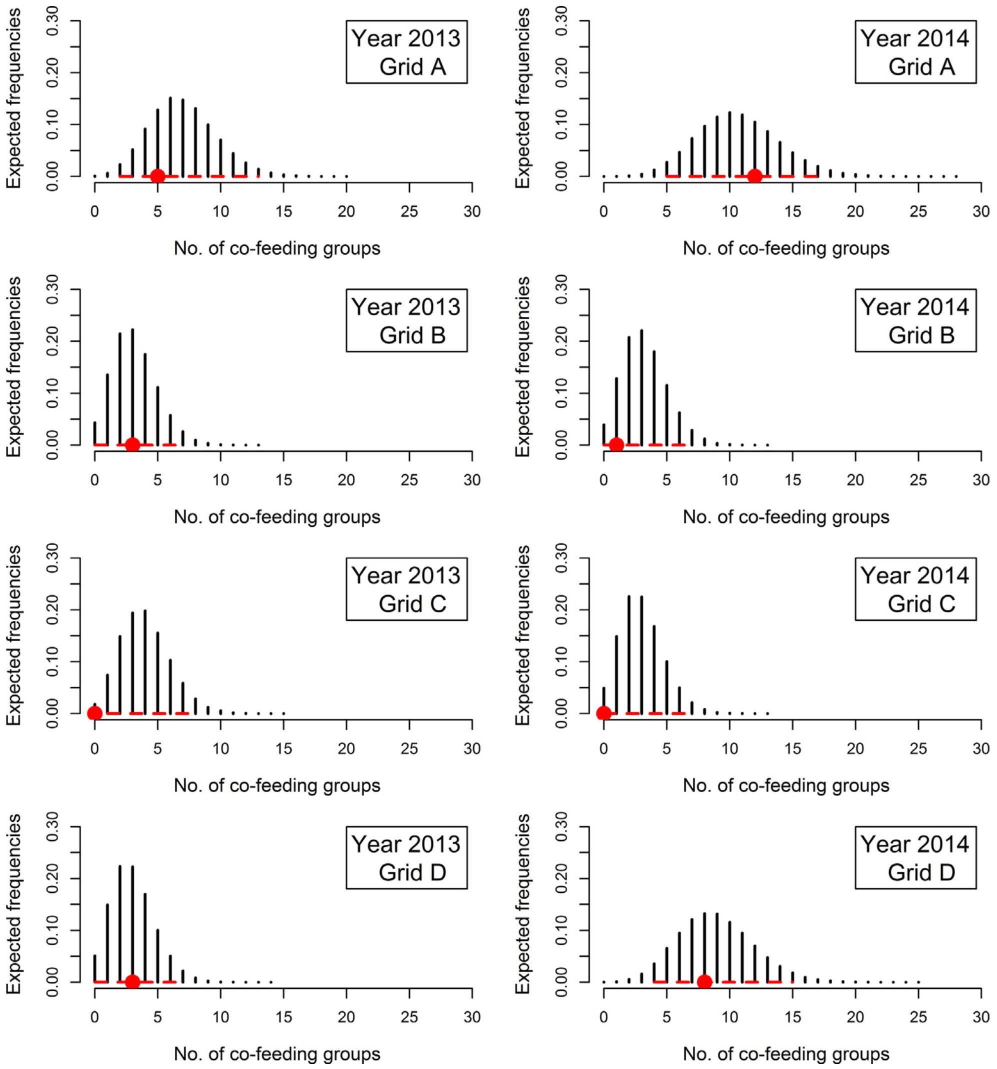

Fig. 3. Comparison between predicted and observed co-feeding groups per grid during years 2013 and 2014. Vertical bars are the expected frequencies obtained from 100,000 model simulations and red (black) dashed lines are the 95% confidence intervals of simulated co-feeding groups per grid. Red (black) dots are co-feeding groups observed in different grids in 2013 and 2014. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Output of the best Binomial Generalised Linear Model for the proportion of tick-borne encephalitis seropositive rodents (per grid per year).

Variable	Coefficient	S.E.	*z*-value[a]	Pr(>|*z*|)[b]
Intercept	−3.302	0.149	−22.088	<0.0001
Total co-feeding in the previous year	0.318	0.091	3.478	0.0005
Rodent density	0.747	0.229	3.265	0.0011
Rodent density^2	−0.340	0.123	−2.764	0.0057

[a] Estimate to S.E. ratio.

[b] Two-tailed *P* values for Z statistics.

that the proportion of TBE seropositive rodents within a specific grid and year was positively influenced by the total number of co-feeding tick groups counted on rodents trapped in the same grid during the previous year (Fig. 4A). Similar to the co-feeding model, the proportion of TBE seropositive rodents and rodent density observed during the season showed a humped relationship (Table 3 and Fig. 4B). Finally, the predicted relative frequency distributions of the number of TBE-infected rodents generated by the Binomial GLM based on 2001–2011 data (see vertical bars in Fig. 5) are in good agreement with the values observed during 2012 and 2013 (red dots in Fig. 5).

4. Discussion

TBE incidence and distribution in humans is increasing throughout Europe and Asia even though TBEv prevalence in ticks is very low compared with other tick transmitted pathogens and the transmission cycle is very fragile (Randolph, 2001). This increase has been shown to be associated with the expansion of tick populations, resulting from changes in climatic and land use factors, changes in human behaviour which affect the human-tick contact rate, an increasing abundance of ungulates supporting tick populations and an increased recognition and reporting of TBE

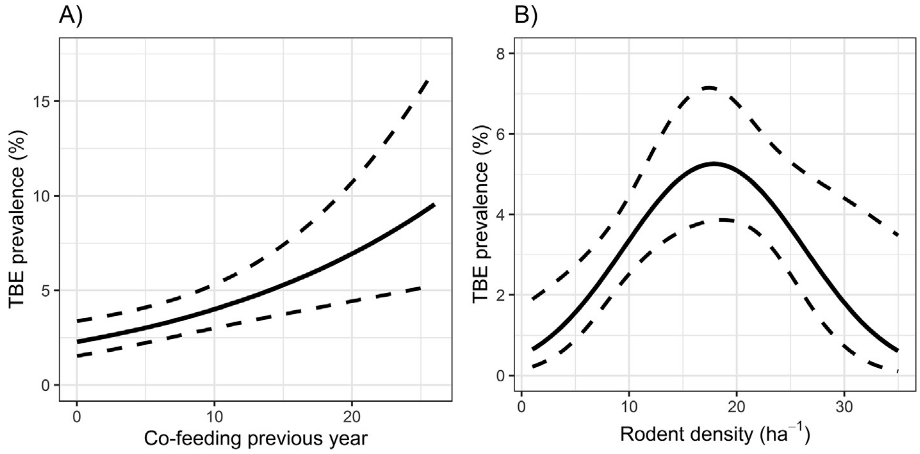

Fig. 4. Relationship between the tick-borne encephalitis (TBE) prevalence (proportion of infected rodent per year and grid) and total co-feeding groups recorded in the same grid and previous year (A) and the yearly average rodent density estimated in the same grid (B). Black lines are the average values while dashed lines represent the 95% confidence intervals. Panels have different *Y*-axis ranges to assist visualisation.

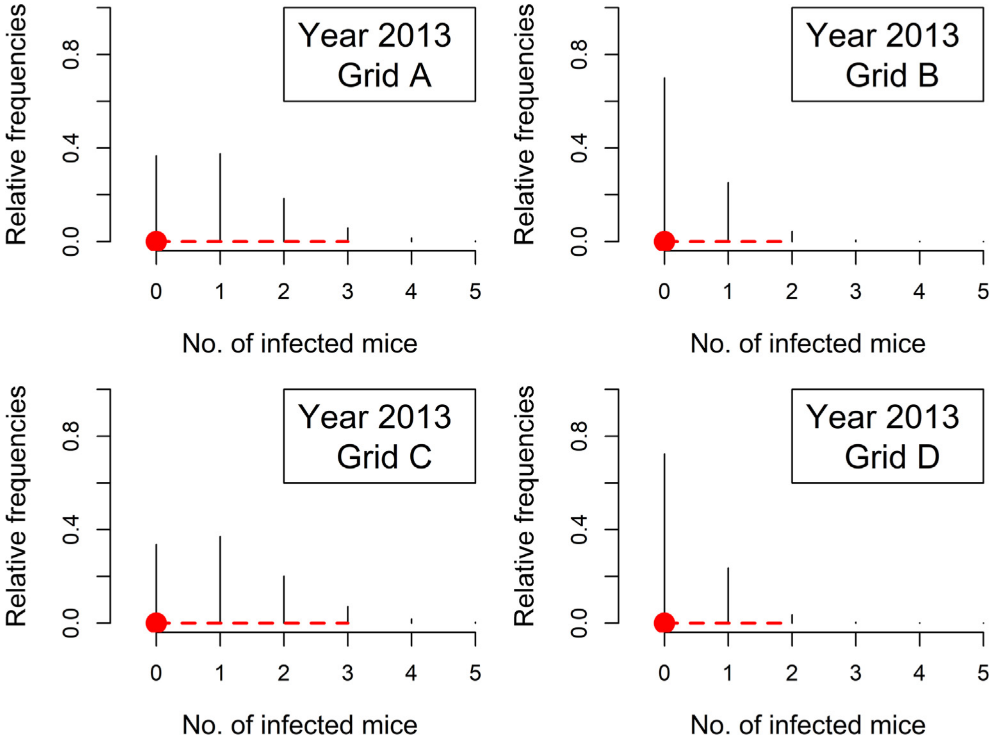

Fig. 5. Comparison between predicted and observed tick-borne encephalitis infected rodents per grid for 2013. Vertical bars are the expected frequencies obtained from 100,000 model simulations and red (black) dashed lines are the 95% confidence intervals of the simulated number of infected rodents. Red (black) dots are the observed number of infected rodents observed in different grid during 2013. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cases (Rizzoli et al., 2009; Randolph et al., 2010, Kunze and ISW-TBE, 2019) However, identifying the factors that explain the risk of TBE maintenance and circulation in the environment would be useful for developing early warning indicators for public health authorities.

Most of the modelling studies on ticks and tick-borne diseases have focused on assessing or forecasting tick distribution and related pathogens by modelling the presence and availability of suitable habitat, climate and hosts (Brownstein et al., 2003; Estrada-Peña, 2006; Alkishe et al., 2017; Rousseau et al., 2017; Watson et al., 2017). However, across the wide TBEv range of distribution from Central Europe to the Pacific Ocean, the risk of infection is not homogeneous, and current models fail as a result of the peculiar distribution of this virus in localised foci. Since climatic and ecological factors favouring the occurrence of ixodid ticks and the distribution of their bloodmeal vertebrate hosts are not enough to predict the occurrence of a TBEv focus, another approach must be considered.

The 'risk' of infection is defined as the product of the hazard (number of infected ticks) and the contact rate between ticks and humans (Dobson et al., 2011). In the case of TBEv, as for many tick-borne viruses, questing nymphal and adult ticks are responsible for virus transmission to humans; however, the overdispersed distribution of ticks in the environment and their very low TBEv prevalence (usually below 1%) cannot be directly translated into human risk. In addition, although the total number of ticks in the environment is usually quite stable, host density varies among years. Monitoring TBEv or TBEv-specific antibodies in sentinel hosts could be a reliable method to define areas with active TBEv circulation. However, the long-term persistence of antibodies in hosts other than rodents makes it difficult to reliably date back to the exact time and site of the primary infection. In contrast, rodents, especially *A. flavicollis* and *M. glareolus*, are susceptible to TBEv and develop high levels of viremia as well as supporting the non-viraemic transmission of TBEv (Labuda et al., 1993b, 1996, 1997). Furthermore, they are particularly well-suited as sentinel hosts since they are among the most important vertebrate reservoirs for TBEv, are widespread in most ecological systems, have a small home range (maximum 2–3 ha), are heavily parasitized by ticks and are easy to trap and monitor. Finally, once infected with the virus, they develop a persistent infection even though no TBEv antibodies are detected, and the viraemic state lasts only 2–3 days (Kozuch et al., 1990; Tonteri et al., 2011). Despite numerous studies investigating the effect of environmental conditions either on questing tick activity or on deer and rodent abundance, this is to our knowledge the first long-term longitudinal field study that simultaneously investigates local abiotic and ecological conditions and tick feeding dynamics on rodent hosts, which are believed to play fundamental roles in non-viraemic transmission of TBEv.

Tick development is synchronised with seasonal climatic conditions through both morphogenetic diapause, during which metamorphosis of engorged larvae and nymphs is delayed, and behavioural diapause, which corresponds to a cessation of questing activity (Belozerov, 1982; Gray et al., 2016). This study confirms that seasonal differences in tick abundance are associated with climatic factors which in turn favour the synchronous presence of immature stages necessary for the formation of co-feeding groups that is a prerequisite for efficient TBEv circulation. Specifically, we show that autumnal cooling has a major impact on the seasonal synchrony of ticks in the following year. That is, when temperatures rapidly decrease in autumn, they induce a behavioural diapause in larval and nymphal ticks, which overwinter as unfed. These larvae and nymphs will start their questing activity the following spring at the same time (overwintering engorged larvae will moult the following year and thus their questing activity will begin in the summer; Randolph et al., 2002).

Other factors which support TBE hotspot maintenance include the availability of roe deer to feed adult ticks during reproduction, and rodent hosts that feed immature stages. While roe deer feed ticks and act as an amplifier host of the total tick population, a higher number of larvae on rodents apparently also enhances the probability of this tick stage aggregating on rodents.

There is a positive effect of roe deer abundance on the number of co-feeding tick groups observed on rodents, which is not surprising since roe deer are known to be amplifier hosts of the total tick population in this environment (Rizzoli et al., 2009). However, the relationship between tick demography and rodent density is much more complex and sometimes contradictory. A negative effect of rodent density on the mean larval intensity per host has been previously observed (Perkins et al., 2003. Transmission dynamics of tick-borne diseases associated with small mammals. Phd Thesis, University of Stirling, UK; Kiffner et al., 2011). On the other hand, in another study, an increase in the total number of feeding larvae on rodents recorded in a year was positively correlated with rodent density (Rosà et al., 2007). In our study, we show that after an initial increase in the number of co-feeding tick groups with *A. flavicollis* density and after a rodent density threshold of approximately 10 mice/ha, the total number of co-feedings on mice declines. This effect is probably related to the discrete nature of tick demography (Rosà et al., 2007) which means that there is a limit to the tick population size within any one season. Thus, if the rodent population keeps expanding and reaches higher and higher densities, the tick per host ratio (and in particular co-feeding groups) will actually decrease. This complex relationship also explains why previous studies have noted different correlations between rodent density and the number of feeding ticks. Interestingly, male mice carried more co-feeding groups than females. This sex-biassed pattern already has been observed within host-parasite systems and has been related to sexual differences in home range (Perkins et al., 2003), behaviour (Boyer et al., 2010) and immunocompetence (Klein and Nelson, 1997; Hughes and Randolph, 2001). In addition, our study supports the 'body size' hypothesis (Perkins et al., 2003), i.e. heavier animals carry more ticks. The role of heavier males is probably particularly important since they tend to be those animals that successfully overwinter and are present in spring when the peak of larval and nymphal ticks is occurring.

Transmission of TBEv from infected nymphs to co-feeding uninfected larvae on rodents is considered the most efficient route for this virus, and the number of co-feeding ticks on rodent hosts seems to be one of the factors that separate a TBEv focus from a non-TBEv area (Burri et al., 2011).

According to the co-feeding transmission concept, larvae belonging to co-feeding groups with an infected nymph have more chances to acquire infection and, as observed in Rosà et al. (2007), would probably moult into infected nymphs the following year. These infected nymphs are responsible for the viraemic infection detected in rodents through the assessment of TBEv antibodies in rodent sera. Here, we observed that TBE seroprevalence in *A. flavicollis* is associated with the number of co-feeding groups recorded on mice in the same area during the previous year, confirming such a mechanism.

Finally, we found that, similar to the relationship with co-feeding groups, the proportion of TBEv seropositive mice and mouse density was hump-shaped (Table 3 and Fig. 4B). This result could be related to the fact that the number of infected nymphs is bounded within a season. In terms of seroprevalence, an initial increase in rodent density would lead to a higher probability of contacts between rodents and infected nymphs. However, a further increase in rodents will dilute the proportion of infected nymphs feeding on rodents, thus lowering the proportion of TBEv seropositive rodent individuals. We are aware that in this study we did not measure the infection in ticks, although we think that the seroprevalence in rodents is a good proxy for this information.

In conclusion, in this study we highlighted that the autumnal cooling rate and the presence of roe deer and mice are to be considered crucial ecological drivers for co-feeding transmission of TBEv and, in turn, in the maintenance of a TBE hotspot. These results contributed to further insights on important aspects of TBE ecology that can be used to provide an early warning signal for a TBE hazard and to address in advance public health interventions for TBE prevention and control.

Acknowledgments

We thank all field assistants for their hard work in gathering data. We thank the Wildlife Service of the Province of Trento, Italy and Mr. Lucio Luchesa of the Trentino Hunters' Association for providing roe deer abundance data. We also would like to thank Roberto Zorer for providing temperature data. The study was supported by EDENext (European Union grant FP7-261504) and is catalogued by the EDENext Steering Committee as EDENext 378 (http://www.edenext.eu).

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