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ABSTRACT

Key parasite transmission parameters are difficult to obtain from elusive wild animals. For *Echinococcus multilocularis*, the causative agent of alveolar echinococcosis (AE), the red fox is responsible for most of the environmental contamination in Europe. The identification of individual spreaders of *E. multilocularis* environmental contamination is crucial to improving our understanding of the ecology of parasite transmission in areas of high endemicity and optimising the effectiveness of prevention and control measures in the field. Genetic faecal sampling appears to be a feasible method to gain information about the faecal deposition of individual animals. We conducted a 4 year faecal sampling study in a village that is highly endemic for *E. multilocularis,* to assess the feasibility of individual identification and sexing of foxes to describe individual infection patterns. Individual fox identification from faecal samples was performed by obtaining reliable genotypes from 14 microsatellites and one sex locus, coupled with the detection of *E. multilocularis* DNA, first using captive foxes and then by environmental sampling. From a collection of 386 fox stools collected between 2017 and 2020, tested for the presence of *E. multilocularis* DNA, 180 were selected and 124 samples were successfully genotyped (68.9%). In total, 45 unique individual foxes were identified and 26 associated with at least one sample which tested positive for *E. multilocularis* (*Em* (+)). Estimation of the population size showed the fox population to be between 29 and 34 individuals for a given year and 67 individuals over 4 years. One-third of infected individuals (9/26 *Em*(+) foxes) deposited 2/3 of the faeces which tested positive for *E. multilocularis* (36/60 *Em*(+) stools). Genetic investigation showed a significantly higher average number of multiple stools for females than males, suggesting that the two sexes potentially defecated unequally in the studied area. Three partially overlapping clusters of fox faeces were found, with one cluster concentrating 2/3 of the total *E. multilocularis*-positive faeces. Based on these findings, we estimated that 12.5 million *E. multilocularis* eggs were produced during the study period, emphasizing the high contamination level of the environment and the risk of exposure faced by the parasite hosts.

1. Introduction

Wildlife parasites are increasingly studied due to their importance in the ecology of mammals (Marathe et al., 2002) and their possible impact on public health as causative agents of zoonosis (Thompson, 2013). However, because wild animals can sometimes be nocturnal, secretive, and wide ranging (Prugh et al., 2005; Liccioli et al., 2015), accurate estimations of crucial characteristics such as parasite prevalence and host abundance or density are difficult to obtain (Pedersen et al., 2005; Wobeser, 2007).

Alveolar echinococcosis (AE) is a potentially fatal zoonotic disease caused by the parasite *Echinococcus multilocularis*. The parasite life cycle is primarily sylvatic in Europe and depends on predator–prey interactions between red foxes as definitive hosts (Hegglin et al., 2015) and small mammals as intermediate hosts (Oksanen et al., 2016; Beerli et al., 2017). Although domestic dogs and cats can also be infected, the red fox (*Vulpes vulpes*) is currently the main definitive host in the palearctic Eurasian region (Eckert

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and Deplazes, 2004) and responsible for most environmental contamination with *E. multilocularis* eggs in both rural and urban environments in Europe (Hegglin and Deplazes, 2013; Knapp et al., 2018). Foxes are recognised for their propensity to disperse, often over long distances exceeding tens of kilometres and more (Allen and Sargeant, 1993). Such spatiotemporal variation in dispersal patterns is influenced by factors such as population density, individual differences in age and sex, and the level of human activity and constraints on the foxes' home ranges (Walton et al., 2018; Hagenlund et al., 2019; Zecchin et al., 2019). Juvenile foxes are more likely to disperse and disseminate *E. multilocularis* because they generally harbour a substantially higher worm burden than adults (Fischer et al., 2005; Robardet et al., 2008; Soulsbury et al., 2008). Both fox populations and the prevalence of *E. multilocularis* infection in foxes are expanding in endemic and previously non-endemic areas (Torgerson and Budke, 2003; Deplazes et al., 2017). Thus, understanding the structure and dynamics of the fox population to develop reliable estimates of parasite prevalence in this primary host may aid in predicting *E. multilocularis* dispersal patterns. This may allow for the development and implementation of appropriate control or prevention measures for both animal and human exposure to the parasite. *Echinococcus multilocularis* contamination of the environment in fox faeces is generally clustered within micro-foci, with seasonal variations likely due to changes in fox activity patterns and defecation behaviour (Giraudoux et al., 2002; Robardet et al., 2011; Raoul et al., 2015; Knapp et al., 2018; Da Silva et al., 2020). Distribution of the parasite among foxes is highly heterogeneous (Raoul et al., 2001; Fischer et al., 2005). Thus, knowing the specific defecation behaviour of only a few individuals (e.g., faecal over-marking of the home range, extensive foraging) in the local fox population may be critical in understanding the spatial aggregation of *E. multilocularis*.

The gold standard methodologies for the detection of *E. multilocularis* in foxes include post-mortem diagnosis by necropsy and methods to examine the intestinal content, such as the intestinal scraping technique (IST) or sedimentation and counting technique (SCT), followed by morphological identification of the adult parasitic stage (Rausch et al., 1990; Eckert, 2003). However, environmental contamination with *E. multilocularis* eggs occurs via faecal deposition. Thus, it is appropriate to analyse such biological material to trace the primary source of contamination for wild intermediate hosts and humans. In addition, this approach is non-invasive and does not disturb animal populations (Conraths and Deplazes, 2015; Knapp et al., 2018; Da Silva et al., 2020). Combining individual faecal genotyping and parasitological analysis allows individual determination of the parasitic status without confusion between samples from the same animal corresponding to the same infection event (Marathe et al., 2002; Zhang et al., 2011). Approaches based on host genetics enable determination of the size, sex ratio, genetic diversity, and structure of the population (Prugh et al., 2005; Valière et al., 2006; Liccioli et al., 2015). Although this integrated approach has several pitfalls (Taberlet, 1996; Taberlet and Luikart, 1999; Valière et al., 2006), it was successfully used in a Canadian urban setting with high *E. multilocularis* prevalence in coyotes to assess temporal patterns of infection and individual re-infection (Liccioli et al., 2015).

According to estimates, the prevalence of *E. multilocularis* in foxes in historically endemic areas in France is over 50% (Raoul et al., 2001; Combes et al., 2012).

In this study, we used a non-invasive faecal genetic sampling method to gather data on individual stools from foxes and track spatial and temporal variations in *E. multilocularis* infection. We aimed to understand how a red fox population is structured at the most local level in terms of population size and genetic structure, focusing on a rural village in the historic endemic area of AE in eastern France. Then, we attempted to link this structure with the distribution of *E. multilocularis* DNA-positive faeces. Because we conducted this study over 4 years, we used faecal genotyping and capture-recapture methods to assess inter-annual variations in the presence of *E. multilocularis*.

2. Materials and methods

2.1. Study area and field sampling

The study was conducted from March 2017 to January 2020 in a grassland area of 3.18 Km^2 in the village of Les Alliés (006°26′46″E, 46°56′53″N, Fig. 1) in eastern France, at an average altitude of 971 m above sea level. This location is in a historically endemic area for *E. multilocularis* (Combes et al., 2012; Piarroux et al., 2013, 2015). The study site is characterized by a proportion of permanent grassland to total land of 52% (agreste.agriculture.gouv.fr) and a continental climate with long, cold, snowy winters. The mean annual temperatures range from −2.2 to 15.1 °C, with average precipitation of 1317 mm/year (https://www.meteofrance.com).

Faecal samples were systematically collected every 2 months throughout a predetermined circuit (Fig. 1) for a total of 17 sampling sessions (Table 1). All carnivore-like faeces found on the circuit were collected and geolocated as previously described (Knapp et al., 2018).

Fox faeces were identified using a host faecal quantitative PCR technique from total copro-DNA extracts, as previously described (Knapp et al., 2016). Based on 5′-3′ *Taq* polymerase activity and the TaqMan probe technology, PCR products can be quantified with high specificity. The smaller the amount of DNA in the sample, the greater the number of amplification cycles. A cycle threshold value (Cq) is obtained, that is inversely proportional to the DNA template present at the start of the PCR. Multiplex quantitative PCRs (qPCRs) were performed, using several primers and TaqMan probe sets (simultaneous detection of red fox, dog, cat DNA and PCR inhibitor presence), to reduce the cost of analysis. Confirmation of faeces from fox origin was validated for a Cq value < 29 PCR cycles (Knapp et al., 2016). Faecal DNA extracts from foxes were tested for the presence of *E. multilocularis* (*Em*(+)) using a specific *E. multilocularis* qPCR as previously described (Knapp et al., 2014, 2016). The presence of *E. multilocularis* DNA in faecal samples was confirmed when Cq < 45 cycles (Knapp et al., 2014).

2.2. Fox genotyping and sexing

Hair and faecal samples from captive red foxes were used for DNA extraction and the resolution of microsatellite technical issues. The ANSES laboratory (France) has had ethical authorization for animal experimentation since 2013 (Decree n° 2013–118 of February 1, 2013) from the national ethics committee. Field faecal DNA was extracted using the QIAmp Fast DNA Stool Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with modifications, as detailed in Supplementary Data S1, to maximize DNA recovery. DNA was eluted in a volume of 200 µl ultra-pure water and stored at – 20 °C until molecular analysis.

Fourteen microsatellite markers (AHT121, AHT137, C01.424, INU055, AHTh171, C08.618, CPH2, FH2010, C04.140, CXX0279, FH2848, REN169O18, CPH11, FH2457) and one sex marker (K9-AMELO), all previously published (Ostrander et al., 1993; Fredholm and Winterø, 1995; Holmes et al., 1995; Breen et al., 2001; Ichikawa et al., 2002; Moore et al., 2010), were used for individual genotyping (Table 2). The primer pairs that amplify the microsatellite loci were pooled into three multiplex assays using the program Multiplex Manager 1.2 (Holleley and Geerts, 2009) and a simplex reaction was used for the sex marker

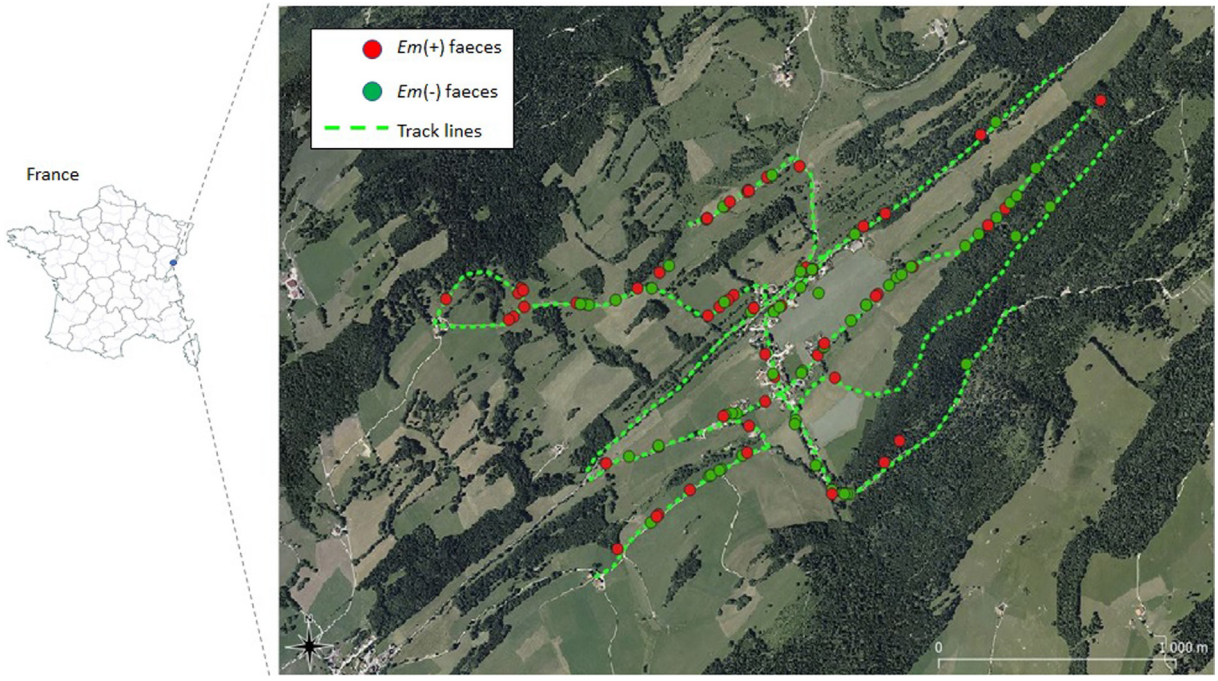

Fig. 1. A map showing the location of the study area of Les Alliés in the east of France, and the location of fox faeces tested for genotyping and the presence of *Echinococcus multilocularis* DNA from March 2017 to January 2020.

Table 1
Number of fox faecal samples collected and successfully genotyped by sampling date, in the study site of Les Alliés in France, from March 2017 to January 2020.

Sampling date	No. of stools sampled	No. of stools identified as fox	No. of stools tested for genotyping	No. of fox stools successfully genotyped	Genotyping success rate(%)	No. of foxes genotyped
March 2017	110	41	38	22	57.9	12
May 2017	56	30	21	13	61.9	8
July 2017	59	32	19	11	57.9	7
September 2017	63	18	5	4	80	4
November 2017	59	25	13	11	84.6	9
January 2018	97	34	16	12	75	9
April 2018	160	34	10	8	80	6
May 2018	23	9	2	1	50	1
July 2018	48	18	11	7	63.6	5
September 2018	60	21	10	8	80	8
November 2018	79	27	14	14	100	9
April 2019	103	21	10	7	70	5
May 2019	44	8	1	1	100	1
July 2019	48	13	1	0	0	1
September 2019	99	40	3	1	33.3	1
November 2019	26	2	2	0	0	0
January 2020	57	13	4	4	100	4
Total	1191	386	180	124	68.9	

(Table 2). PCRs were performed using a C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) in a 10 µL final reaction volume containing 1 µL of DNA extract (0.1 to 56 ng/µL), 1 × Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.1 µM of each primer, and 3% DMSO. PCRs were conducted for 15 min at 95 °C, followed by 40 cycles at 94 °C for 30 s, 57 °C for 90 s, and 72 °C for 60 s, and a final step at 60 °C for 30 min. After fragment size analysis, allele sizes were quantified using a SeqStudio Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genetic profiles were determined using the Microsatellite Analysis module available on the Thermo Fisher cloud (https://apps.thermofisher.com/editor-web/#/app/app-microsatellites-web).

A comparative multiple-tube approach (Taberlet, 1996) was used to determine multilocus genotypes and find a consensus genotype by reducing genotyping errors (Supplementary Data S1).

2.3. Data analysis

2.3.1. Individual identification and genotyping error rates

The success of genotyping was compared between collection periods using Chi-square tests to assess homogeneity. Values of $P < 0.05$ were considered statistically significant. Genotyping error rates between amplifications (allelic dropout, ADO; false allele, FA) (Broquet and Petit, 2004) were checked using GIMLET software (Valière, 2002).

Fox faecal samples with identical multilocus genotypes or genotypes differing by one or two alleles were considered to originate from a single individual. The R package allelematch (Galpern et al., 2012) was used with R 3.5.1 (R Development Core Team, 2018) software to perform multiple comparisons between genotypes. A Venn diagram was plotted using the R package VennDiagram (Chen and Boutros, 2011) to visualise individual resampling over time.

Table 2
Genetic diversity of the 14 selected microsatellite and sex markers used for individual genotyping and molecular sexing of 45 red fox genotypes in the present study performed in the town of Les Alliés in France.

Loci	Primer sequence	bp	N_A	H_O	H_E	*P*(HWE)
Multiplex set 1						
AHT121	F: [FAM] 5′-TATTGCGAATGTCACTGCTT-3′R: 5′-ATAGATACACTCTCTCTCCG-3′	81–109	7	0.89	0.81	0.054
AHT137	F: [FAM] 5′-TACAGAGCTCTTAACTGGGTCC-3′R: 5′-CCTTGCAAAGTGTCATTGCT-3′	135–147	6	0.87	0.78	0.233
CPH11	F: [PET] 5′-GTTAATGAAACTCCGATGTTTACA-3′R: 5′-GAAAGCCAAGCATGACTAGG-3′	116–128	4	0.38	0.36	0.558
REN169O18	F: [NED] 5′-TAGCAAAACCCCCAACTCAC-3′ R: 5′-ACTGTGTGAGCCAATCCCTT-3′	168–194	11	0.78	0.83	0.006
FH2010	F: [VIC] 5′-GAATGGAACAGTTGACGATGC-3′R: 5′-CCCCTTACAGCTTCATTTTCC-3′	216–228	5	0.71	0.67	0.087
Multiplex set 2						
CO1.424	F: [FAM] 5′-AGCCTAGCTTACTGCCCTGG-3′R: 5′-TCCTTTGGTTTTTAGCAGGG-3′	169–187	6	0.67	0.62	0.085
INU055	F: [FAM] 5′-CCAGGTCTGCCTATCCATCT-3′R: 5′-GCACCACTTTGGGCTCCTTC-3′	200–222	10	0.76	0.84	0.062
AHTh171	F: [VIC] 5′-AGGTGCAGAGCACTCACTCA-3′R: 5′-CCTCAAACCCAGGTGAAGC-3′	151–161	2	0.00	0.04	0.010
FH2848	F: [NED] 5′-CAAAACCAACCCATTCACTC-3′R: 5′-GTCACAAGGACTTTTCTCCTA-3′	216–242	12	0.76	0.87	0.143
FH2457	F: [PET] 5′-AACCTGGTACTTTGAATTTGCA-3′R: 5′-AGAATGGATGGAAGCAAGGA-3′	170–218	17	0.71	0.78	0.017
Multiplex set 3						
C08.618	F: [VIC] 5′-CAACCCAGGGTGGAAGC-3′ R: 5′-GCCCAGAATCCATTGAGAAA-3′	173–187	6	0.60	0.68	0.268
CPH2	F: [VIC] 5′-TTCTGTTGTTATTGGCACCA-3′R: 5′-TTCTTGAGAACAGTGTCCTTCG-3′	93–97	3	0.56	0.61	0.481
C04.140	F: [NED] 5′-CAGAGGTGGCATAGGGTGAT-3′R: 5′-TCGAAGCCCAGAGAATGACT-3′	138–158	10	0.80	0.76	0.479
CXX0279	F: [NED] 5′-TGCTCAATGAAATAAGCCAGA-3′R: 5′-GAATTTGATTACTCTGGAAAACTCC-3′	90–110	8	0.89	0.82	0.010
Sex marker						
K9-AMELO	F: [NED] 5′-GTGCCAGCTCAGCAGCCCGTGGT-3′R: 5′-TCGGAGGCAGAGGTGGCTGTGGC-3′	201215	2	-	-	-

F, forward; R, reverse; in square parentheses, primer dye; bp, observed allele size in this study; N_A, number of observed alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *P*(HWE), *P*-value associated with deviations from Hardy-Weinberg equilibrium (according to an exact test based on Monte Carlo permutations of alleles).

The analysis of molecular variance (AMOVA) is a statistical method used to assess if the genetic diversity among groups is significantly similar to what would be expected if the individuals derived from the same group (Excoffier et al., 1992). AMOVA was conducted using the poppr package in R (Kamvar et al., 2014) to assess the consistency of genetic variations across different years. This analysis excluded geographic data structure and focused on decomposing the total genetic variation between sampling years.

The probability of identity (P_{ID}) was calculated using allele frequencies obtained from genotyped samples, which is an indication of the ability of the 14 microsatellite markers to distinguish between individuals (Waits et al., 2001). The P_{IDrand} for populations of individuals with random mating (Paetkau and Strobeck, 1994) and the P_{IDsibs} for populations of related individuals (Taberlet and Luikart, 1999) were determined following the equations provided by Waits et al. (2001), as implemented in GIMLET software.

2.3.2. Estimation of the population size

The size of the fox population was estimated using the DNA-based mark-recapture R package CAPWIRE (Miller et al., 2005; Pennell et al., 2013). This method is a maximum likelihood-based approach, designed for the estimation of population size with non-invasive sampling, involving multiple observations of the same individuals per sampling session. Two models are implemented in CAPWIRE. These were the even capture probability model (ECM), which allows estimation of the population size in situations of equal probability of individual capture and the two innate rates model (TIRM), which estimates the size of a population including individuals with different capture probabilities. The performance of the two models was first compared using maximum likelihood estimates using the CAPWIRE-inherent likelihood ratio test (LRT). The 95% confidence interval (95% CI) of the population size estimates was calculated by running 1000 parametric bootstraps.

2.3.3. Genetic diversity

Genetic variation was estimated over all loci by assessing the number of alleles observed per locus (N_A) and the expected and observed heterozygosity (H_E and H_O). Conformation to the Hardy-Weinberg equilibrium (HWE) was estimated using a paired t-test. Values of $P < 0.05$ were considered statistically significant. Linkage equilibrium across all pairs of loci was tested using the R package genepop 1.1.7 (Rousset et al., 2020). Bonferroni corrections for multiple tests were performed to adjust the probabilities. The inbreeding coefficient (F_{IS}) and its statistical significance were assessed for all multilocus genotypes using the R package HierFstat (de Meeûs and Goudet, 2007).

2.3.4. Genetic structure of the population

Based on the multilocus genotypes of the individual foxes sampled, a Bayesian approach implemented in STRUCTURE 2.3 software (Pritchard et al., 2000) was used to infer whether foxes were likely to be structured into K genetic clusters. Twenty independent runs with 1,000,000 Markov chain Monte Carlo (MCMC) repetitions and a 100,000 burn-in period were performed for each value of K (1–10) using no prior information and assuming correlated allele frequencies and admixture. K was identified using the maximum values of lnP(D) (the log probability of the data for a given K), returned by the software, and ΔK based on the rate of change in the likelihood of K (Evanno et al., 2005). The posterior probability q_i, which is the proportion of each individual genotype derived from each of the K genetic clusters, was estimated using this model (Pritchard et al., 2000). The q_i-values were used to assign each individual genotype to the K retained genetic clusters and averaged across the 20 runs.

Principal Component Analysis (PCA) was performed using the R package adegenet (Jombart and Ahmed, 2011) and putative genetic clusters. The genetic differentiation between genetic clusters was quantified by computing the Weir and Cockerham estimator of F_{ST} using the hierfstat package (de Meeûs and Goudet, 2007).

2.3.5. Relatedness among red foxes

Relatedness among individual red foxes was assessed using the R package related (Pew et al., 2015). First, simulations were used to compare the performance of six relatedness estimators to select the best estimator given the genotype data. Four moment estimators (Queller and Goodnight, 1989; Li et al., 1993; Lynch and Ritland, 1999; Wang, 2002) and two likelihood estimators were used (Milligan, 2003; Wang, 2007). Data sets of 100 pairs were estimated for four relatedness categories (parent-offspring (PO),

full-sibling (FS), half-sibling (HS), and unrelated (UR)), with allele frequencies calculated from values observed in the field dataset. Pearson's correlation coefficients were used to compare the relatedness estimates from simulations with the expected relatedness values (PO = 0.5, FS = 0.5, HS = 0.25, and UR = 0.0). For the following analyses, the estimator with the highest correlation coefficient was chosen.

Relatedness was assessed for all individuals in the dataset and we tested whether individuals within genetic clusters were more related than expected if these subgroups simply represented random clusters of individuals (Pew et al., 2015). A permutation test with 100 iterations was used to compare the relatedness between genetic clusters.

2.3.6. Assessment of the contribution of individual foxes to E. multilocularis spatial dispersion

The cumulative percentage of fox faeces that tested positive for *E. multilocularis* DNA was plotted against the cumulative number of unique fox genotypes with at least one faecal sample testing positive for *E. multilocularis*. The mean, minimum, and maximum distances (m) between faeces positions were determined. Convex polygon surfaces (ha) were calculated for genotyped foxes with at least three faecal samples collected using the R packages sp v1.4–5 (Pebesma, E., Bivand, R., Rowlingson, V., Gomez-Rbio, R., Hijmans, R., Sumner, M., MacQueen, J., Lemon, F., Lindgren, F., O'Brien, J., O'Rourke, J., 2021. Sp: Classes and Methods for Spatial Data) and rgeos v0.5–5 (Bivand, R., Rundel, C., Pebesma, E., Stuetz, R., Hufthammer, O., Giraudoux, P., David, M., Santilli, S., 2020. Rgeos: Interface to Geometry Engine - Open Source ('GEOS')) to assess the area covered by each fox over the study area and during the study period. The distribution of *E. multilocularis* DNA-positive faeces was compared across genetic clusters using a $\chi 2$ test. We searched for a potential spatial autocorrelation in the distribution of Cq values of positive faeces using Moran's I autocorrelation test. Values of $P < 0.05$ were considered statistically significant.

2.3.7. Estimation of the minimum number of individual infection events by E. multilocularis

There is no accurate data about the life span of adult *E. multilocularis* worms in fox intestines (Thompson et al., 2017). However, according to data from experimental infections, the worm burden in foxes decreases rapidly after 35 days p.i. and the infection is likely cleared within 6 months (Nonaka et al., 1996; Kapel et al., 2006; Thompson et al., 2006). Thus, according to our collection frequency, two *E. multilocularis*-positive fecal samples collected six months apart would be attributed to at least two different infection events. This minimum number of infection events can be estimated for each fox from which at least two fecal samples were collected during the study. The frequency of infection can be calculated for the foxes with samples collected at least 4 months apart by dividing the minimum number of infection events by the number of periods of 4 months (considered here as the average time of an infection). The total number of *E. multilocularis* eggs expelled by an infected fox was previously estimated to be 346,473 based on experimental infections (Kapel et al., 2006). Considering that all infections were similar in terms of the biotic potential (i.e., number of eggs produced), it is possible to estimate the environmental contamination from the studied fox population over 4 years.

2.4. Data accessibility

The datasets including the hair and faecal samples from captive red foxes and the field sample collection are available from MendeleyData (https://data.mendeley.com/): "Individual genotyping of caged red foxes from the analysis of 14 microsatellite targets and one sex marker", https://doi.org/10.17632/hppjm4vs3b, "Individual genotyping of red fox field stools from the analysis of 14 microsatellite targets and one sex marker and the research of *Echinococcus multilocularis* DNA presence", https://doi.org/10.17632/vcg2xx4pnc.2.

3. Results

3.1. Individual fox genotyping and assignment of faecal spreading

We first evaluated the performance of the 14 microsatellites and sex marker K9-AMELO on hair and faecal samples from captive red foxes (Supplementary Data S1). Among 1,191 carnivore faecal samples collected during the study period, 386 were identified as fox by the qPCR host faecal test. Of the 180 fox faecal samples selected and analysed from the field, 124 were successfully genotyped using a multi-tube approach (Navidi et al., 1992). We excluded 37 samples that failed to be amplified from the analysis and 19 with incomplete genotyping results. The success rate of amplification ranged from 80.6% for fox faeces collected during the cold period (September – January) to 63.4% for those collected in the spring (March – May) and 58.1% for those collected in the summer (July). The difference was statistically significant (Chi-square test, $\chi^2 = 7.13$, df = 2, $P = 0.028$). The success of genotyping did not significantly vary between years (Chi-square test, $\chi^2 = 4.98$, df = 2, $P = 0.083$). In total, 45 unique multilocus genotypes (i.e., individuals) were identified from the 124 successfully genotyped fox faeces. Among these 45 distinct individuals, 27 were identified as male and 18 as female, for an overall sex ratio of 0.6, which was not significantly different from 0.5 (Test of given proportions, $\chi^2 = 1.42$, df = 1, $P = 0.233$). The genotyping error was assessed through the average per-locus per-replicate ADO rate and estimated to be 0.056 (±0.039), whereas the FA rate was null.

The number of faecal-samples per individual ranged from one to 15, with an average number of 2.76 (±2.67), which is suitable to achieve estimates within 10% of the true population size (Miller et al., 2005). Throughout the course of this study, 24 foxes were identified in 2017, 24 in 2018 and 11 in 2019–2020. There were 24 genetically confirmed re-encounters (i.e., individuals sampled repeatedly, which are considered to be recaptures) (Fig. 2). Among the individuals with at least two samples, the mean number of multiple occurrences significantly differed between females (mean = 6 ± 3.71, $n = 10$) and males (mean = 3.07 ± 1.21, $n = 14$) (Welch two sample t-test, t = 2.41, df = 10, $P = 0.036$). The recapture probability was significantly different between individuals throughout the study period (LRT, $P < 0.001$).

The AMOVA results showed most of the genetic variation to be within individuals (97.44%), with only minor variations between individuals (1.91%) and between years of sampling (0.65%). The

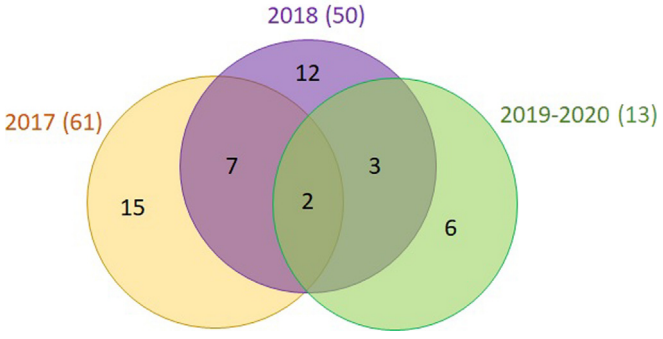

Fig. 2. Venn diagram of fox genotypes identified by year of sampling. The numbers of successfully genotyped fox faeces are shown in parentheses.

genetic diversity across years was deemed minor, hence the genetic profiles were grouped together. Based on 45 individuals, the probability of two individuals sharing the same multilocus genotype across all 14 loci was low, with P_{IDrand} = 3.87 × 10^{-14} and P_{IDsibs} = 8.68 × 10^{-6}, indicating the high discriminatory power of this microsatellite panel and necessity to prevent the shadow effect (i.e., underestimation of the real number of unique genotypes (Mills et al., 2000)).

3.2. Estimation of the population size on the study area

TIRM was then selected as the best-fitting estimation model, showing a population of 29 to 34 individuals per year and 67 individuals (95% CI: 56 – 78) for the whole study (Supplementary Table S1). In terms of the area of our study site (3.18 km^2), this population size represents a density of nine to 10 foxes/km^2/year or 21 foxes/km^2 over the 4 years.

3.3. Genetic diversity of the fox population

The 14 microsatellite loci used for the genotyping analyses were polymorphic, with an average of N_A = 7.6 alleles per locus (range 2 – 17, Table 2). AHT137 showed significant linkage disequilibrium with two loci (FH2457 and CXX0279). To assess deviation from HWE, all 45 individual genotypes were regrouped. Deviation from HWE was not detected when considering the whole microsatellite panel (Paired t-test, t = 0.51, df = 13, P = 0.616), with an average observed (H_o) and expected (H_E) heterozygosity of 0.67 and 0.68, respectively (Supplementary Table S1). The overall inbreeding coefficient was low and did not significantly differ from zero (F_{IS} = 0.011; P = 0.544).

3.4. A marked genetic structure among the foxes

The 20 independent runs of the Bayesian analysis implemented in the STRUCTURE software showed maximum average lnP(D) and ΔK values for K = 3 (Supplementary Fig. S1). A PCA plot of the individual genotypes indicated clear structuration among these three genetic clusters (Fig. 3) and an F_{ST} = 0.08 indicated moderate genetic differentiation. The spatial distribution of the 124 faecal samples from the three genetic clusters partially overlapped (Fig. 4), which, combined with the observed moderate genetic differentiation, suggests a single sub-structured population rather than several fox populations. The sex ratio in the genetic clusters was 0.55 in cluster 1 (11/20), 0.73 in cluster 2 (11/15), and 0.5 in

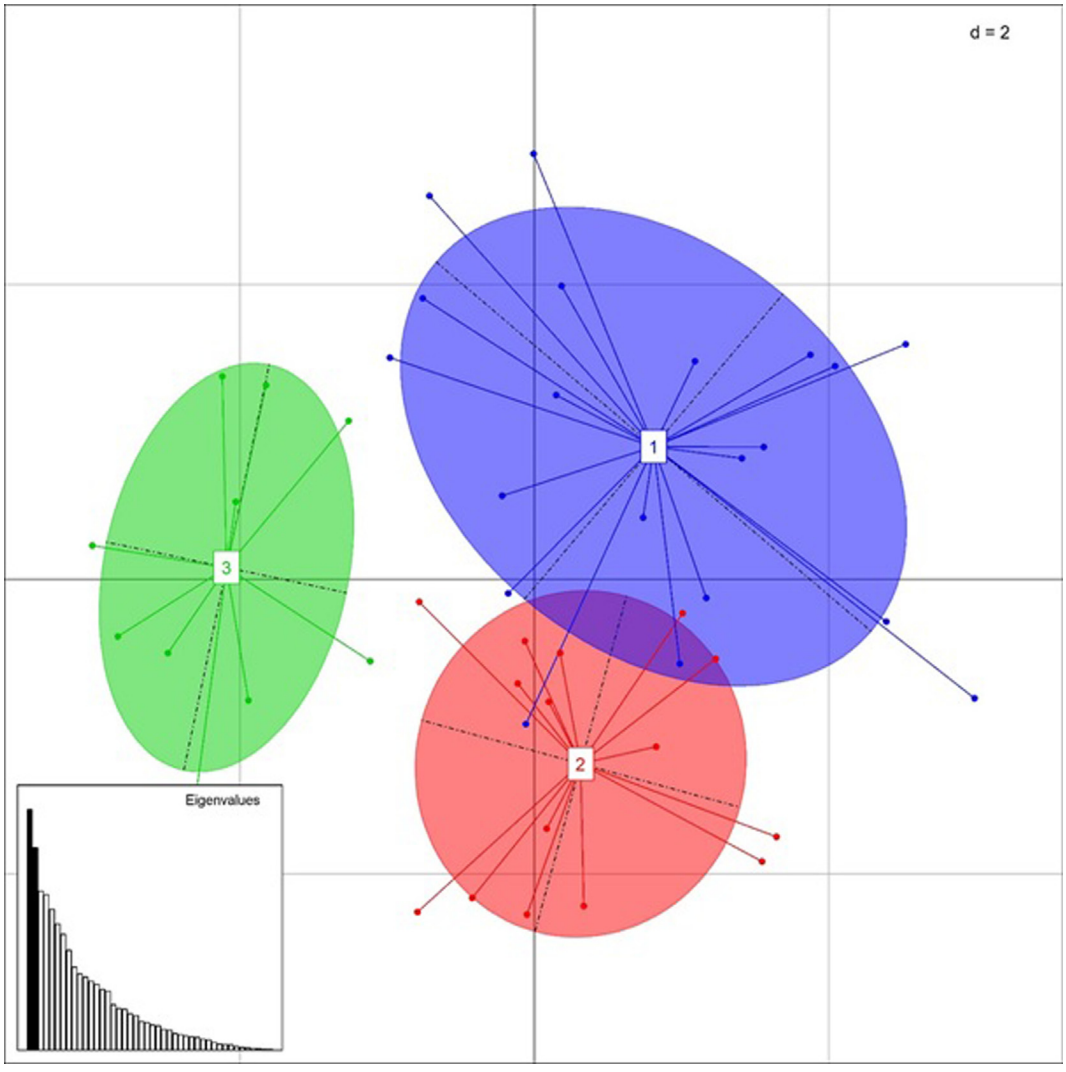

Fig. 3. Principal component analysis (PCA) of genetic clusters in the studied red fox population based on the allelic frequencies of 14 microsatellite loci. The genetic clusters 1, 2, 3 are shown as inertia ellipses of different shades of colour (grey), while dots represent individuals. The eigenvalues for the represented components are shaded in black, with the first axis explaining 10.9% of the variance and the second axis explaining 9.2%.

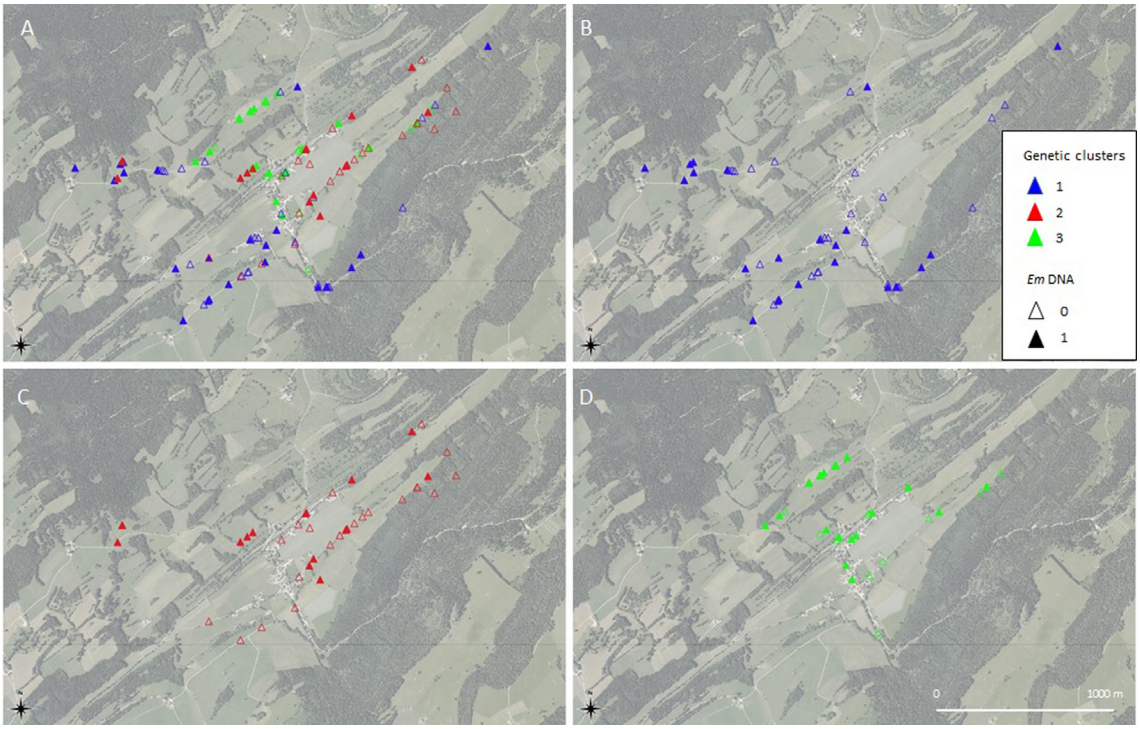

Fig. 4. Distribution of red fox faeces samples in the village of Les Alliés (eastern France) according to genetic cluster, as defined by STRUCTURE Bayesian clustering. The coloured (grey) triangles show the genetic clusters 1, 2, 3, while empty shapes represent the absence (0) and filled shapes the presence (1) of *Echinococcus multilocularis* DNA in the faecal samples. (A) Spatial distribution of all 124 faeces samples with genetic clusters 1, 2, and 3. (B) Spatial distribution of faeces from cluster 1. (C) Spatial distribution of faeces from cluster 2. (D) Spatial distribution of faeces from cluster 3. In B, C, and D, faeces samples positive for *Echinococcus multilocularis* are represented by filled triangles, whereas negative samples are represented by empty triangles.

cluster 3 (5/10) and did not significantly differ between the three clusters (Fisher's exact test for count data, $P = 0.462$).

3.5. Relatedness among red foxes and clusters

The relatedness estimate described by Wang (2002) showed the highest correlation coefficient between simulated and expected values (r = 0.84) and was thus used for all subsequent analyses (Supplementary Fig. S2 and Supplementary Table S2). Pairwise relatedness differed significantly between genetic clusters (Permutation test, $P < 0.001$). Relatedness was higher within clusters, with high values of relatedness between individuals in clusters 2 (average relatedness of 0.19) and 3 (average relatedness of 0.25). Relatedness did not differ between females and males (Permutation test, $P = 0.800$).

3.6. Echinococcus multilocularis in foxes and individual spreading

Among 386 fox faecal samples 79 were found positive for *E. multilocularis* DNA (*Em*(+)) and subjected to fox genotyping. For financial reasons, not all fox faeces negative for *E. multilocularis* (*Em*(-)) were genotyped. From the 307 *Em*(-) faeces, 101 (33% of *Em*(-) samples) were randomly selected from each of the 17 sampling sessions to study *E. multilocularis* infection-re-infection in the fox population over the time period (Table 1). The overall faecal occurrence of *E. multilocularis*, without considering individual genotyping, was 20.47% (79/386, 95%CI: 16.74 – 24.77, Table 3).

From the 45 sampled individuals, 26 foxes (15 males and 11 females) were identified with at least one *E. multilocularis*-positive scat, to a maximum of 10, with an average number of 2.31 (±1.87) positive faecal samples per individual. Only 34.6% (9/26) of positive individuals deposited 60% of the faeces positive for *E. multilocularis* DNA (Fig. 5). One of the clusters showed a large

Table 3
Collection of fox stools performed during the study period and *Echinococcus multilocularis* DNA detection in faeces (*Em*(+)) in the study area of Les Alliés in France.

Sampling session	No. of fox stools	No. of *Em*(+) faeces	No. of *Em*(+) faeces genotyped
2017	146	44	32
2018	143	30	25
2019–2020	97	5	3
Total	386	79	60

number of faeces from females (cluster 3, 27 faeces from females and 12 from males), with 2/3 of *Em*(+) faeces produced by these females (24 *Em*(+) of 39 sampled, including 16 *Em*(+) from females).

From qPCR analysis, Cq values varied greatly between and within individuals. Moran's I autocorrelation test showed there to be a spatial autocorrelation in the Cq distribution of DNA-positive faeces (I = 0.11, S.D. = 0.06, $P = 0.033$). The distribution of *E. multilocularis*-positive faeces depending on the Cq values is shown in Fig. 6. The distribution of positive faeces did not differ significantly between the three clusters ($\chi^2 = 4.78$, df = 2, $P = 0.092$).

The minimal size of the area covered by individuals during the study period was assessed using convex polygons for identified foxes with more than one deposited faecal sample over the 4 years. Based on the locations and distances between the collected faeces, the area that each individual covered throughout the study period differed greatly within the borders of the study area (1.32 ha to 35.01 ha) (Table 4). However, an insufficient number of individuals with an estimated area covered were studied, even if 45 distinct individuals were identified, to properly link this data to individual variables (for example, age or sex). Indeed, only two of the

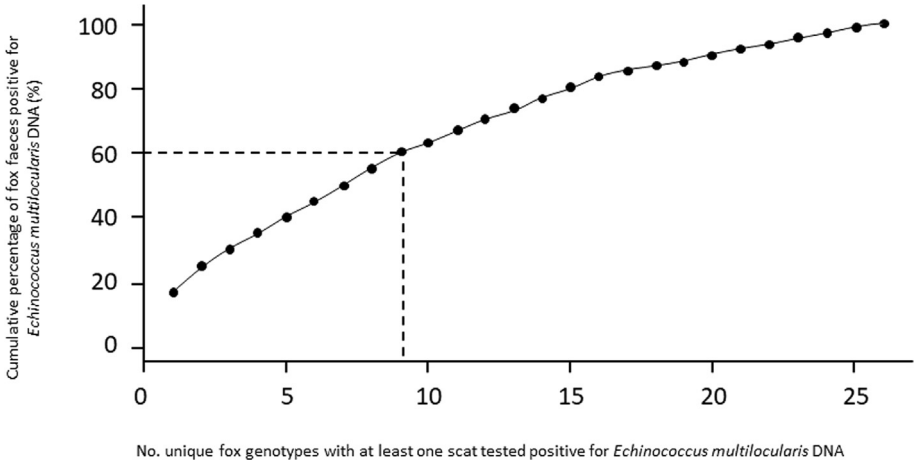

Fig. 5. Cumulative percentage of *Echinococcus multilocularis*-positive fox faeces according to the number of foxes genotyped in the study area of Les Alliés (eastern France).

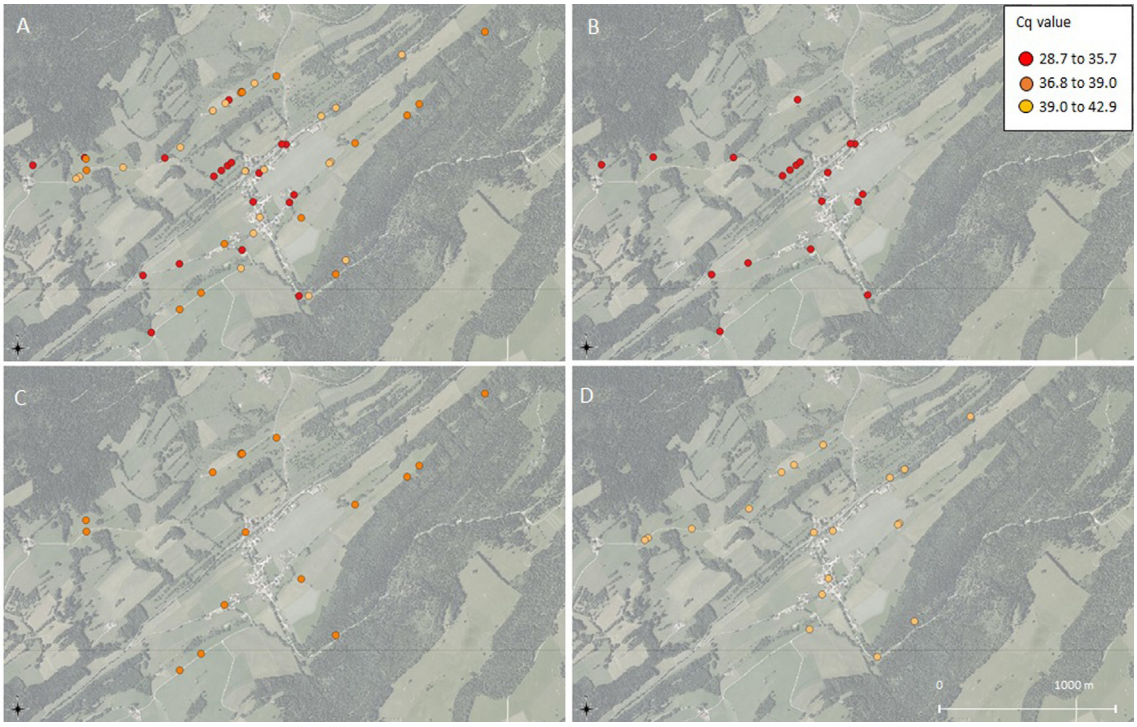

Fig. 6. Spatial distribution of cycle threshold (Cq) values from quantitative PCR for *Echinococcus multilocularis* DNA detection. Three interval classes were arbitrarily selected for graphical representation. (A) Spatial distribution of 57 *E. multilocularis*-positive (*Em*(+)) faecal samples (three samples having no geographical coordinates available). (B) Cq values from 28.7 to 35.7 cycles for 20 *Em*(+) faecal samples. (C) Cq values from 36.8 to 39 cycles for 18 *Em*(+) faecal samples. (D) Cq values from 39.01 to 42.9 cycles for 18 *Em*(+) faecal samples.

19 individuals with at least two faecal samples over several sampling sessions were negative for *E. multilocularis* DNA throughout the study period (Fig. 7).

3.7. *Echinococcus multilocularis* egg production assessment

According to the copro-qPCR for the detection of *E. multilocularis* DNA, 26 individuals were identified as being infected, with the minimum number of infection events ranging from one to three during a period of two to 34 months (Table 5). Among the 45 genotyped foxes, 30 (12 positive and 18 negative) were excluded to estimate the frequency of infection, as only one faecal sample was collected, except for four foxes with two to four faecal samples but within a period inferior to the minimum of 4 months. Considering all 15 foxes (14 positive and one negative) for which faecal samples were collected at least in two sessions, the average frequency of infection was 0.6. Considering all included foxes, the total minimum number of infection events estimated for the studied population was 36, corresponding to an estimated production of 12.5 million *E. multilocularis* eggs released into the home range of these foxes over 4 years.

4. Discussion

The present study combined individual fox genotyping with assessment of the occurrence of *E. multilocularis* from

Table 4
Distances (in meters) between the locations of faeces for genotyped foxes with multiple occurrences of faeces tested for the presence of *Echinococcus multilocularis* DNA in the study site of Les Alliés in France.

Fox genotype ID	No. stool available	No. *E. multilocularis*-positive stools	Distance (m) between faecal samples			Area covered (ha)
			Min.	Max.	Mean (±S.D.)	
1	7	5	79.63	1000.44	446.61 (±305.61)	21.52
2	8	3	82.25	1168.35	543.10 (±288.33)	32.98
4	15	10	0.58	816.35	381.42 (±199.79)	30.5
5	3	3	47.64	105.12	70.08 (±30.74)	-
7	6	3	17.43	561.01	299.66 (±176.92)	6.3
9	4	1	82.58	470.96	334.72 (±160.08)	5.55
10	6	0	3.528	973.778	519.11 (±317.50)	10.78
11	5	3	131.7	1283.2	811.76 (±443.06)	35.01
12	3	1	22.76	526.93	354.88 (±287.69)	-
15	2	1	-	649.1	-	-
16	2	0	-	609.78	-	-
17	2	2	-	504.3	-	-
19	3	2	2.69	119.61	79.75 (±66.74)	-
20	6	3	17.34	411.25	197.23 (±127.05)	1.32
23	2	1	-	744.9	-	-
24	2	0	-	633.81	-	-
25	5	3	53.1	981.7	614.48 (±309.12)	21.96
26	3	2	135.2	618.1	432.75 (±260.29)	3.28
30	5	2	53.44	361.73	173.45 (±99.78)	-
31	3	3	221	370.7	275.17 (±82.97)	2.44
32	2	2	-	423.56	-	-
33	4	0	8.49	254.47	134.4 (±109.57)	-
38	2	2	-	496.4	-	-
39	2	2	-	NA	-	-

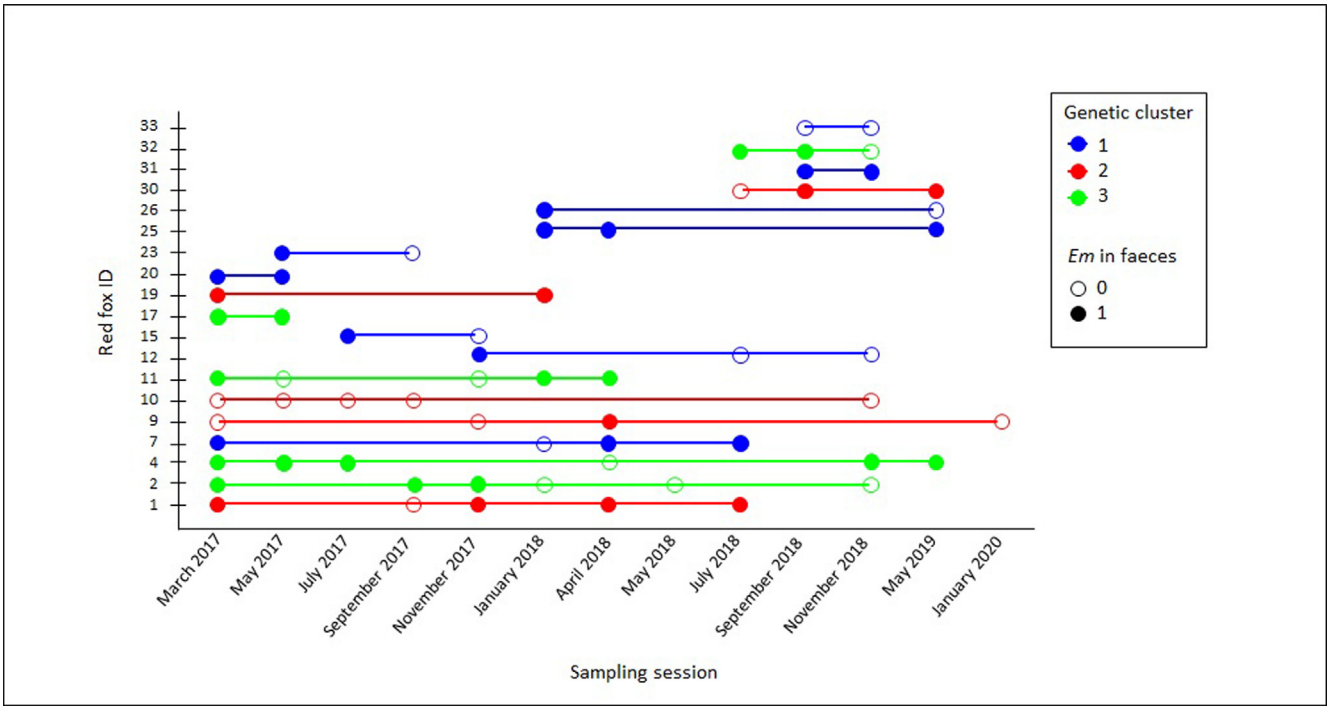

Fig. 7. Individual status for the presence of *Echinococcus multilocularis* DNA in stools (as determined by quantitative PCR) for each genotyped red fox re-sampled at least one time throughout the sample sessions. The coloured (grey) points show the genetic clusters 1, 2, 3, while empty points represent the absence (0) and filled shapes the presence (1) of *E. multilocularis* DNA in the faecal samples.

geo-localised stool sampling. Liccioli et al. (2015) reported consistent results between the faecal occurrence of *E. multilocularis* for genotyped coyotes and those obtained from non-genotyped faeces in the Calgary Metropolitan Area (Canada). The probability of sampling faeces from the same individual certainly varies depending on the behaviour of wild animals (e.g., social dominance, territorial faecal marking (Barja, 2009; Liccioli et al., 2015) in different landscapes). Indeed, according to our data, approximately 2/3 of *Em*(+) faeces were produced by only 1/3 of the infected individuals.

According to the ratio of the frequency of infection observed, individuals in the population were considered to be infected for an average of 60% of their life span in the study area. To the best of our knowledge, no study reporting longitudinal monitoring of fox infection at an individual level is available in the literature. However, this value is in accordance with the prevalence of 56.8% obtained using genotyping analyses on the three first sessions, for which almost all available samples where genotyped. Concerning the number of infection events, it was not possible to

Table 5
List of the 15 genotyped red foxes, with an estimation of the minimum number of infection events with *Echinococcus multilocularis* and when possible (i.e., minimum period of 4 months between the first and last faecal sample collected), the frequency of infection related to their period of exposure in the study area of Les Alliés in France.

Fox genotype ID	Minimum No. of infection events	No. of stools available	No. of sampling sessions	Month available	Frequency of internal infection (No. infections/4 months)
10	0	6	5	20	0.0
15	1	2	2	4	1.0
23	1	2	2	4	1.0
32	1	3	3	4	1.0
12	1	3	3	12	0.3
26	1	3	3	14	0.3
9	1	4	4	34	0.1
30	2	5	3	8	1.0
19	2	3	2	10	0.8
11	2	5	5	12	0.7
25	2	5	3	14	0.6
7	2	6	4	16	0.5
2	2	8	6	20	0.4
1	3	7	5	16	0.8
4	3	15	6	24	0.5

account for successive infections during a 6 month period, whereas simultaneous infections have been frequently reported, notably in highly endemic areas in France, using the EmsB microsatellite tool (Knapp et al., 2008; Umhang et al., 2014). Thus, based on the calculation of the ratio, a maximum value of 1 is possible, which was reached for four foxes. These foxes probably had an important role in environmental contamination and human exposure to *E. multilocularis* (Liccioli et al., 2015). Moreover, with a reported defecation rate of eight faeces per fox per day (Webbon et al., 2004), the 45 individuals identified during this study may have defecated a large number of times during the study period, representing high environmental contamination on a local scale close to human housing and kitchen gardens, which we estimate to be 12.5 million eggs. According to Hegglin and Deplazes (2013), this corresponds to a highly endemic rural scenario, with close to 100% environmental contamination (Hegglin and Deplazes, 2013).

It appears that faecal samples collected during the months favourable for *E. multilocularis* infection (Combes et al., 2012; Liccioli et al., 2015) are more suitable for genotyping. Non-invasive studies to monitor *E. multilocularis* could thus be optimized for cost and efficiency by conducting frequent faecal sample collection during the cold seasons. Future studies focusing on the dynamics of *E. multilocularis* egg excretion by both wild and domestic hosts need to be conducted to confirm these seasonal variations in parasite burden and permit assessment of the subsequent zoonotic transmission risk (Liccioli et al., 2015).

Here, we show that a few individuals are likely to spread *E. multilocularis* over time into the environment through multiple contamination events, even in a relatively small area of high parasite prevalence in foxes (Hofer et al., 2000; Raoul et al., 2001). This information is crucial for improving our understanding of the ecology of *E. multilocularis* transmission in highly endemic areas and optimising the effectiveness of prevention or control measures such as baiting programmes in the field, recognised to be more efficient in limited treated areas (Hegglin et al., 2003; Hegglin and Deplazes, 2013).

Based on a multi-tube approach and 14 microsatellite markers, the overall genotyping success rate (68.9%) in our study is comparable to that obtained in previous studies using fox faecal DNA (average success rates of approximately 33.4 to 78%) (Oishi et al., 2010; Liccioli et al., 2015; Amaike et al., 2018). Moreover, Liccioli et al. (2015) reported the highest rate during cold periods, as found in our study, probably due to better DNA conservation during this period.

Non-invasive genetic estimates require a sufficient sample size to reflect the structure and size of the population studied. In total, 45 individuals were studied, ranging from 24 in the first 2 years, respectively, to only 11 in the third and fourth years combined, with 12 individuals being observed in more than 1 year. This may be partially due to a sampling bias, because the selection of samples for genotyping depended on the number of positive faeces detected. As recommended by Miller et al. (2005), a suitable average number of samples per individual for a population with an equal sex ratio were available to estimate the fox population parameters in the study area, even though a significantly higher average number of females deposited multiple stools than males, suggesting that the two sexes defecated unequally. This is likely due to specific behaviours such as seasonal foraging for pup feeding (Smith et al., 2006; Murdoch et al., 2010). Fox densities estimated using night roadside counts and distance sampling in a nearby area approximately 10 km from the study area (ZELAC project: https://zaaj.univ-fcomte.fr/spip.php?article118) were between 1.2 and three individuals/km^2 (Giraudoux et al., 2020). In our study, between 29 and 34 individuals visited the area over a year, hence between 9.1 to 10.7 individuals/km^2 (67 over 4 years, 21 individuals/km^2). Distance sampling carried out over a short duration of 2 to 3 h in one night is more likely to underestimate the number of foxes visiting the area than genetic methods based on faecal sampling. The relationship between results obtained by roadside counting, and distance sampling and genetic estimation over longer periods is still unclear and requires specific studies. From the structural analysis of the fox population, three clusters were found, constituting subgroups rather than different populations. The social organization of foxes is well documented, with clusters consisting of related individuals, potentially associated with unrelated animals (Zabel, C., 1986. Reproductive behavior of the red fox (*Vulpes vulpes*): a longitudinal study of an island fox population. University of California, USA; Cavallini, 1996; Baker et al., 1998, 2000). Population structure parameters such as sex and age of foxes certainly influence the spread of *E. multilocularis* eggs in the environment. Indeed, juveniles could be more frequently infected than adults, their dispersion behaviour reinforcing the parasite spreading phenomenon (Hofer et al., 2000; Comte et al., 2017).

CRediT authorship contribution statement

Abdou Malik Da Silva: Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Visualization, Formal analysis, Investigation, Methodology. **Eve Afonso:** Conceptualization, Writing – review & editing, Visualization, Validation, Formal analysis, Methodology, Supervision, Software. **Francis Raoul:** Conceptualization, Writing – review & editing,

Visualization, Formal analysis, Software. **Patrick Giraudoux:** Writing – review & editing. **Marina Mergey:** Conceptualization. **Gérald Umhang:** Writing – review & editing, Resources. **Sandra Courquet:** Data curation. **Dominique Rieffel:** Data curation. **Laurence Millon:** Funding acquisition, Writing – review & editing, Supervision. **Jenny Knapp:** Conceptualization, Funding acquisition, Data curation, Writing – review & editing, Visualization, Validation, Methodology, Supervision, Resources, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2024.03.003.

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