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eDNA metabarcoding reveals a core and secondary diets of the greater horseshoe bat with strong spatio-temporal plasticity

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Abstract

Dietary plasticity can be a determining factor allowing species to cope with environmental changes. Consequently, it is an important issue to consider in conservation biology. Despite this, it remains rarely addressed in the literature, potentially due to methodologies which were until recently rather limited. The advent of molecular approaches now makes it possible to get a precise picture of diet and its plasticity, even for endangered and elusive species. Here, we focused on the greater horseshoe bat (*Rhinolophus ferrumequinum*) in Western France, where this insectivorous species has been classified as “Vulnerable” on the Regional Red List in 2016. We applied an eDNA metabarcoding approach to 1986 fecal samples collected in six maternity colonies on three sampling dates. We described the diet and investigated whether the landscape surrounding colonies and the different phases of the maternity cycle influenced the diversity and the composition of this diet. We showed that *R. ferrumequinum* feed on a much more diverse prey spectrum than expected from previous studies, highlighting how eDNA metabarcoding can improve our knowledge on the dietary habits of elusive species. Our approach also revealed that the diet of *R. ferrumequinum* seems to be composed of two distinct features: the core diet consisting of a few preferred taxa shared by all the colonies (25% of the occurrences) and the secondary diet consisting of numerous rare prey taxa that were highly different between colonies and sampling dates (75% of the occurrences). Constraints associated with the greater horseshoe bat life cycle, as well as insect phenology and landscape features, strongly influenced the diversity and composition of both the core diet and the diet as a whole. Further research should now explore the relationships between *R. ferrumequinum* dietary plasticity and fitness, to better assess the impact of core prey decline on *R. ferrumequinum* population viability.

KEYWORDS

bat, environmental DNA, insectivorous diet, spatio-temporal variation

Pontier and Charbonnel equally contributed to this work.

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1 | INTRODUCTION

Food resources constitute a major environmental factor for animal populations (Hutchinson, 1957; Schoener, 1974). Both the quantity and quality of food resources are known to strongly impact the fitness of individuals (Eeva et al., 1997; Serrano-Davies & Sanz, 2017; Sorensen et al., 2009) and in turn, the dynamics and viability of populations (Johnsen et al., 2017; Taylor & Schultz, 2008; Vickery et al., 2001). Understanding a species' dietary requirements is therefore of critical importance when designing conservation strategies (Brown et al., 2014; Clare, 2014; Cravens et al., 2018; Groom et al., 2017). Several studies have even shown that some dietary characteristics could be related to elevated risk of species extinction. In particular, species in higher trophic levels face greater exposure to the cumulative effects of pollutants along the food chain (Careddu et al., 2015; Mann et al., 2011; Purvis et al., 2000). Furthermore, a narrow and specialized trophic niche (i.e., the range of possible prey) can also increase the vulnerability of species: specialists can face greater constraints when responding to environmentally driven changes in resource availability than generalist species (Boyles & Storm, 2007; Clavel et al., 2011; Owens & Dittman, 2003; Pratchett et al., 2006; Twining et al., 2019). However, foraging can be a flexible activity. According to the optimal foraging theory, predators exploit resources that maximize the net energy intake while minimizing energetic costs through a trade-off between food profitability and searching time (Emlen, 1966; MacArthur & Pianka, 1966). Such dietary plasticity is crucial to cope with environmental changes, including seasonal fluctuations (Bergmann et al., 2015; Kartzinel & Pringle, 2015), climate change (Durant et al., 2007; Oliveira & Val, 2017) or anthropogenic pressure (Hempson et al., 2017; Quéméré et al., 2013; Smith et al., 2018). However, a suboptimal diet can have negative impacts on individual fitness (Sasakawa, 2009). Dietary plasticity, despite its potential ecological and evolutionary importance, remains scarcely addressed in the literature (Sousa et al., 2019; but see Shutt et al., 2020), potentially because dietary studies have long been constrained by methodological limits (e.g., low taxonomic level, nondetection of soft-bodied prey, and limited number of processed samples; Nielsen et al., 2018). The development of molecular approaches for identifying prey DNA contained in feces, in particular environmental DNA (eDNA) metabarcoding, has overcome most of the limitations associated with traditional methods (Clare, 2014; Khanam et al., 2016). Metabarcoding of fecal samples has become a commonly applied approach for studying bat diet (Sousa et al., 2019). Indeed, bats are nocturnal, elusive, highly mobile and most of them are threatened, making it challenging to gather detailed prey information based on direct observations (IUCN, 2019; Kunz et al., 1995, 2011). Insectivorous bats display a large range of foraging strategies, from specialists (e.g., mountain long-eared bat, *Plecotus macrotullaris*; Alberdi et al., 2012) to generalists (e.g., big brown bat *Eptesicus fuscus*; Clare et al., 2014). Bat species qualified as generalists can show preference toward certain prey (e.g., *Myotis daubentonii*; Vesterinen et al., 2016) and

be more selective when their favorite prey are available in the environment (e.g., *Eptesicus fuscus*; Agosta et al., 2003).

The greater horseshoe bat (*Rhinolophus ferrumequinum*) is an insectivorous bat species whose diet and foraging behavior have been previously explored, especially in Northern Europe where it has experienced severe declines this last century as the result of anthropogenic changes (Kervyn et al., 2009; Mathews et al., 2018; Pir, 2009). Previous studies based on microscopy analyses have shown that *R. ferrumequinum* feeds mainly on three orders of arthropods: Lepidoptera, Coleoptera, and Diptera (Flanders & Jones, 2009; Jones, 1990). They have also revealed that the proportion of each order of prey in the feces varied throughout the year with a preference for Lepidoptera when these were abundant in summer. An experimental study under controlled conditions showed that *R. ferrumequinum* can discriminate and select prey based on the size and relative abundance of the prey in the environment, using its very precise echolocation system (long constant-frequency calls enabling Doppler shift compensation) (Koselj et al., 2011). Altogether these studies suggested that *R. ferrumequinum* might have a plastic foraging strategy to maximize energy intake while minimizing energy costs (optimal foraging theory; Emlen, 1966; MacArthur & Pianka, 1966). Yet, energy costs could fluctuate between and within seasons as previously shown for several bat species (e.g., summer peak of *Myotis lucifugus* energy expenditure during lactation; Kurta et al., 1989). The decrease in flight maneuverability of pregnant bat females, as well as the decrease in flight distance and the increase in returns to the roosts of bat females during lactation period (Dietz & Kalko, 2007; Henry et al., 2002) could decrease bat selectivity during gestation and lactation. In addition, *R. ferrumequinum* is very sensitive to the landscape surrounding its colonies, in particular to the vertical vegetation elements (Froidevaux et al., 2017; Pinaud et al., 2018; Wang et al., 2010), because of its short-distance echolocation system (up to 10 m; Ortega et al., 2016). They connect *R. ferrumequinum* foraging areas to colonies and they also provide protection against wind and predators (Forman & Baudry, 1984; Holland & Fahrig, 2000; Lewis, 1969; Verboom & Spoelstra, 1999). Acoustic and radio-tracking studies conducted around colonies (radius up to 10 km) indicated that deciduous woodlands and pastures are preferential foraging areas for *R. ferrumequinum* (Dietz et al., 2013; Flanders & Jones, 2009; Jones, 1990; Pinaud et al., 2018). Such landscapes are known to harbor rich insect communities, notably thanks to livestock and reduced soil modification. Yet, landscape effects on *R. ferrumequinum* diet and dietary plasticity have never been explored. There is thus a growing need to simultaneously examine the temporal and spatial variations of *R. ferrumequinum* diet, coupling improved taxonomic resolution, and greater sampling to overcome the detection and identification biases associated with traditionally used morphology-based microscopy analyses. Such studies should enable us to better understand the influence of energetic constraints associated with the life cycle and the foraging landscape on dietary plasticity, thereby helping to improve the design

of conservation strategies (e.g., preservation of key landscape and prey; Arrizabalaga-Escudero et al., 2015).

In this study, we analyzed the diet of *R. ferrumequinum* in Western France, an area with a strong responsibility for the conservation of this species (Leuchtman et al., 2019; Vincent & Bat Group SFEPM, 2014) classified as “Vulnerable” on the Regional Red List. This area is dominated by an agricultural landscape and has experienced important changes in landscape features due to agricultural intensification since the 1960s (e.g., decrease of meadows, grasslands, and hedges; increase in the average size of cultivated fields, increase in pesticides use; Agreste, 2016). We focused on the maternity season, as it corresponds with a period of high energy expenditure and foraging constraints for female bats due to gestation and lactation (Henry et al., 2002; Hughes & Rayner, 1993; Kurta et al., 1989; Mclean & Speakman, 1999). We specifically explored whether *R. ferrumequinum* diet varied between the different phases of the maternity cycle and/or was associated with landscape features surrounding the colonies. First, we expected that *R. ferrumequinum* diet should be less diverse in June and July—because of higher energy expenditures and constraints associated with the gestation and lactation—than in August when the young start to feed by

themselves (Anthony & Kunz, 1977; Czenze et al., 2018; Whitaker et al., 1996). We then hypothesized that a favorable environment (semi-open habitat composed of hedgerows and permanent meadows; Flanders & Jones, 2009; Froidevaux et al., 2017) should favor a more diverse diet than a less favorable environment where foraging could be more constrained by local insect richness and profitability.

2 | MATERIALS AND METHODS

2.1 | Collection of guano samples

We collected 95 fecal pellets once a month from June to August 2018 beneath seven maternity colonies of *R. ferrumequinum* in Western France (Figure 1). We selected the freshest pellets with regard to their appearance (the least dry) and position on the plates (the most isolated ones from the other pellets) to limit degradation and cross-contamination between samples.

The three sampling dates coincided with gestation (end of May to mid-June), lactation (mid-July to end of July), and postlactation (mid-August to end of August) of *R. ferrumequinum*.

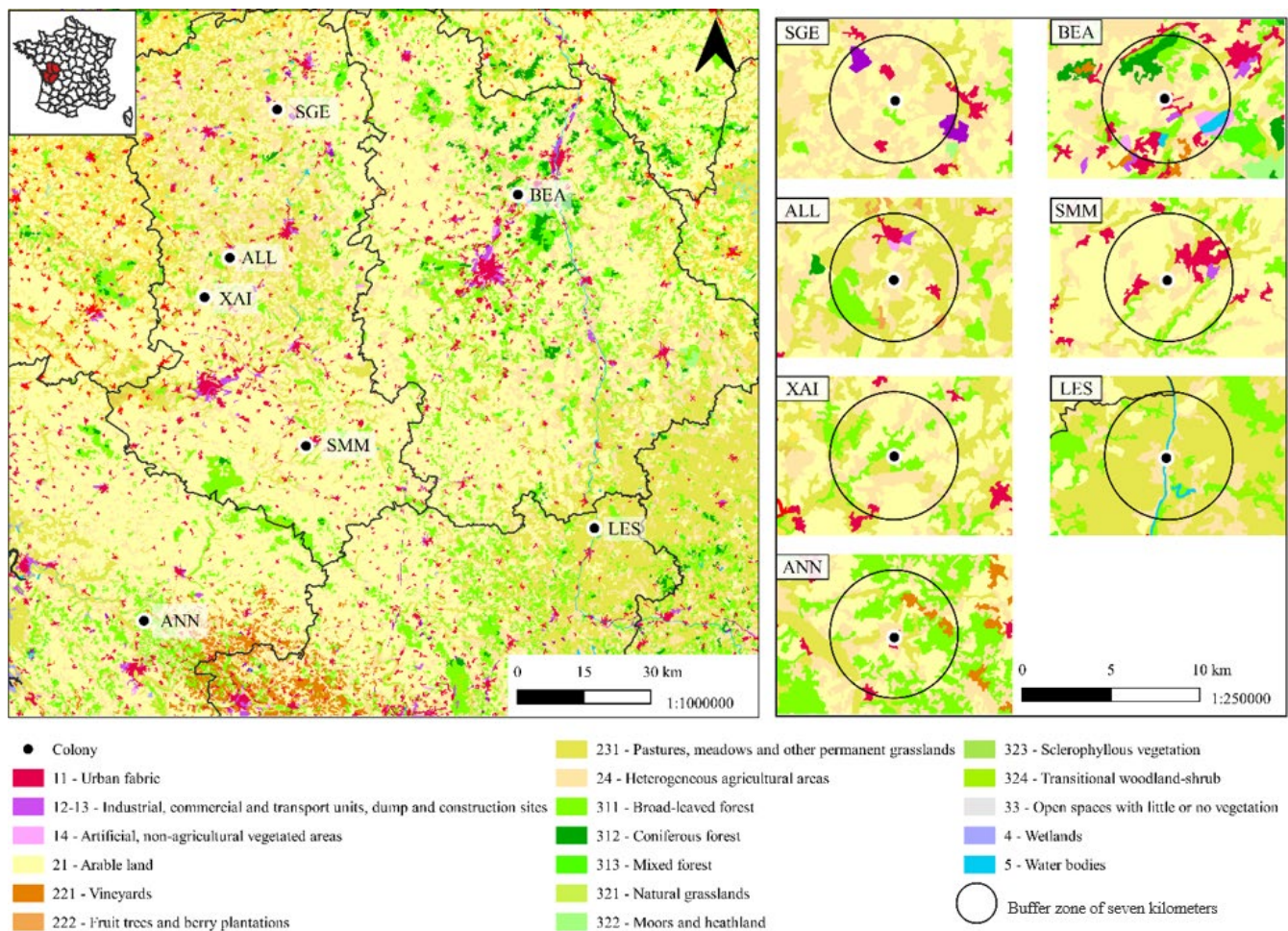


FIGURE 1 CORINE land cover map of the colonies included in this study: Allonne (ALL), Annepont (ANN), Beaumont (BEA), Lessac (LES), Sainte-Gemme (SGE), Saint-Martin-les-Melle (SMM), and Xaintray (XAI). Buffer zone of seven kilometers corresponds to the maximum hunting distance of *R. ferrumequinum* females during the lactation period

As *R. ferrumequinum* often shares its maternity colonies with the Geoffroy's bat (*Myotis emarginatus*) and as their droppings are indistinguishable, the high number of fecal samples collected ensured a sufficient number of *R. ferrumequinum* samples for diet analyses. Each pellet was retrieved from paper plates, which had been left on the ground for 10 days, using single-usage forceps, and pellets were placed individually within a 96-well microplate. The 10-day time period is likely to lead to sample and DNA degradation (Oehm et al., 2011), but it could not be reduced because of logistical constraints and of the necessity to limit the disturbance of the colonies. We selected this sampling interval in accordance with previous bat studies which detected high levels of nuclear and mitochondrial DNA amplification, and high-quality genotypes (up to 338 bp) from bat feces after 10 to 15 days (Puechmaille & Petit, 2007; Zarzoso-Lacoste et al., 2018). Paper plates were renewed at each collection date. This protocol ensured that contaminations were minimized between sampling dates. Samples were stored at -20°C until DNA extraction.

2.2 | Characterization of the landscape surrounding bat colonies

We described 12 landscape variables around *R. ferrumequinum* colonies within a buffer zone of seven kilometers, which corresponds to the average maximum hunting distance of *R. ferrumequinum* females during the lactation period (Pinaud et al., 2018): woodland, closed coniferous forest, closed deciduous forest, closed mixed forest, open forest, hedgerow, woody moor, poplar, orchard, permanent meadow, temporary meadow, and vineyard. Data were extracted from the two French "Institut Géographique National" databases: "BDTopo" database (2018 version) for permanent vegetation (hedgerows, forests, etc.) and "Registre Parcellaire Graphique" (Graphic Parcel Register, GPR, 2017 version) for agricultural land use (crops). Hedge proportions corresponded to the sum of the hedgerow areas around the colonies. For GPR crops, the categories "Permanent meadow—predominant grass" and "Long-rotation meadow (six years or more)" were combined to form the variable "Permanent meadows." The categories "Other temporary meadow of five years or less" and "Ray-grass of five years or less" were clustered into the variable "Temporary meadows." Spatial operations on geographic entities were carried out using QGIS 3.4.6 Geographic Information System (QGIS Development Team, 2019). We then applied a Principal Component Analysis (PCA) on these variables to characterize the landscape surrounding colonies (sum of the surface occupied in hectares). We used the *FactoMineR* R package to perform this analysis (Lê et al., 2008).

2.3 | DNA extraction, PCR, and library construction

DNA extraction was performed on pellet samples according to Zarzoso-Lacoste et al. (2018). Briefly, pellets were frozen at -80°C

then bead-beaten for 2×30 s at 30 Hz on a TissueLyser (Qiagen) using a 5-mm stainless steel bead. DNA was extracted using the NucleoSpin 8 Plant II kit (Macherey Nagel) with the slight modifications recommended in Zarzoso-Lacoste et al. (2018). We amplified a 178-bp COI minibarcode using the primer set proposed by Vamos et al. (2017) (fwhF1: 5' YTCHACWAAYCAYAARGAYATYGG 3'; fwhR1: 5' ARTCARTTWCRAAHCCCHCC 3'). These primers offer the best compromise to (a) maximize the detection and identification of arthropod prey and (b) identified the bat species to separate *R. ferrumequinum* and *M. emarginatus* diets (Tournayre et al., 2020). We used the metabarcoding protocol described in Galan et al. (2018) with the PCR programs optimized in Tournayre et al. (2020). We included a negative control for extraction (NC_{ext}), a negative control for PCR (NC_{PCR}), and a negative control for indexing (NC_{index}) in each 96-well microplate. Amplifications (PCR_1 and PCR_2) were carried out three times per DNA extract (i.e., triplicates) using different dual-indexes per replicate. PCR_1 , corresponding to the gene-specific amplification, was performed in 10 μl reaction volume using 5 μl of $2\times$ Qiagen Multiplex Kit Master Mix (Qiagen), 2.5 μl of ultrapure water, 0.5 μl of each mix of forward and reverse primers (final concentration: 0.5 μM each), and 1.5 μl of DNA extract. The PCR_1 conditions consisted of an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 45 s, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The PCR_2 consisted of a limited-cycle amplification step to add multiplexing indexes i5 and i7 (8 bases each) and Illumina sequencing adapters P5 and P7 at both ends of each DNA fragment from PCR_1 . PCR_2 was carried out in a 10 μl reaction volume using 5 μl of Qiagen Multiplex Kit Master Mix (Qiagen) and 2 μl of each indexed primer i5 and i7 (final concentration: 0.7 μM each). Then, 2 μl of PCR_1 product was added to each well. The PCR_2 , corresponding to the sample-specific dual indexing, started by an initial denaturation step of 95°C for 15 min, followed by 8 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min followed by a final extension step at 72°C for 10 min. PCR_2 products for the same Illumina run were pooled with equal volumes and put on a low-melting agarose gel (1.25%) for excision. We used the PCR Clean-up Gel Extraction kit (Macherey-Nagel) to purify the excised bands. The DNA pool was quantified using the KAPA library quantification kit (KAPA Biosystems), normalized at 4 nM before loading 14 pM and 5% of PhiX control on a MiSeq flow cell with a 500 -cycle Reagent Kit v2 (Illumina). We sequenced a total of five Illumina runs.

2.4 | Bioinformatics and taxonomic assignments

We used the R preprocessing script from Sow et al. (2019) to merge pair sequences into contigs using FLASH v.1.2.11 (Magoč & Salzberg, 2011) and to trim primers using CUTADAPT v.1.9.1 (Martin, 2011). We then used the FROGS pipeline ("Find Rapidly OTU with Galaxy Solution," Escudé et al., 2018) to create an abundance table for Operational Taxonomic Units (OTUs). The FROGS

pipeline enabled to the following steps: (a) filter sequences by length (± 20 bp from the expected length), (b) cluster in very resolute OTUs the sequences using a maximum aggregation distance of one mutation with the SWARM algorithm (Mahé et al., 2014), (c) remove chimeric sequences using VSEARCH with de novo UCHIME method (Edgar et al., 2011; Rognes et al., 2016), and (d) filter by keeping only OTUs present in at least two libraries. In addition, we used *isBimeraDenovo* from *dada2* (Callahan et al., 2016) to remove the residual chimeric sequences which were not detected using the FROGS pipeline. We also used the T_{CC} and T_{FA} thresholds approach proposed by Galan et al. (2016) to filter cross-contaminations during the laboratory procedure and to filter the false assignments of reads to a PCR product due to the generation of mixed clusters during the sequencing, respectively. Lastly, we considered that a sample was positive for a particular OTU if all replicates (3 out of 3) were positive for this taxon. This procedure enabled the removal of inconsistent OTUs due to PCR or sequencing errors, and reduced the number of putative false-positive results (Alberdi et al., 2018; Tournayre et al., 2020). The expected mean number of reads after merging the three technical PCR replicates is around 20,000 reads per sample.

Taxonomic assignments were carried out using the NCBI BLAST + automatic affiliation tool available in FROGS pipeline, with the Public Record Barcode Database (data related to BOLD database <http://v3.boldsystems.org> in February 2019, with maximum 1% of N). Taxonomic assignments were considered for taxa with an identity percentage $\geq 97\%$, according to the confidence levels described in Galan et al. (2018) and a coverage percentage $\geq 90\%$. Arthropod species that were not referenced in Europe according to Fauna Europea (Jong et al., 2014) or INPN (Muséum national d'Histoire naturelle, 2003), as well microscopic species (e.g., Macrochelidae, Uropodidae, and Cheyletidae), were discarded from the dataset. Finally, among the identified prey, we looked for agricultural pests according to the Arthemis database (<http://arthemisdb.supagro.inra.fr/>; 2,185 species of insects listed as pests on the date of database extraction—04/10/2019).

2.5 | Diet analyses

Diet analyses were only conducted on *R. ferrumequinum* samples that were not contaminated by other vertebrate species (other bats, birds, or rodents).

2.5.1 | Reliability of the data

We checked for appropriate sequencing depth per sample to ensure reliable comparisons across samples using the function *depth.cov* from the R package *hilldiv* (Alberdi and Gilbert, 2019). This function gives the percentage of estimated diversity covered in each sample. Then, we assessed our efficiency in describing prey diversity with respect to sampling effort by generating taxa accumulation curves

for each colony, sampling date, and considering each taxonomic rank (order, family, genus, and species). We used the function *iNEXT* of the R package *iNEXT* (Chao et al., 2014; Hsieh et al., 2016). Diversity measures based on Hill numbers were calculated for $q = 0$ and $q = 1$. For all curves, 95% confidence intervals were calculated based on a bootstrap method with 1,000 replications.

2.5.2 | Diet diversity and composition analyses

Alpha diversity

Alpha diversity analyses were carried out using Hill numbers in the R package *hilldiv*. Hill numbers enable to modulate the relative weight of abundant and rare OTUs through a single parameter q (the order of diversity). Alpha diversities were computed for (a) $q = 0$ (corresponding to prey richness; the same weight is attributed to all OTUs) and (b) $q = 1$ (corresponding to Shannon diversity which considers both richness and evenness). For each level of taxonomic resolution, we tested the effects of sampling date, landscape (described as the coordinates of the colonies on the main axes of the PCA) and their interaction on alpha diversity using quasi-Poisson Generalized Linear Models (GLMs). We used the false discovery rate (FDR) to account for multiple testing (Benjamini & Hochberg, 1995). The adjusted p -value thresholds after FDR correction ($p_{critical}$) were estimated following Castro and Singer (2006).

Beta diversity

The spatio-temporal variation of *R. ferrumequinum* diet composition was first explored using histograms of the frequency of prey occurrence, computed with the *ggplot2* R package (Wickham, 2016). For each level of taxonomic resolution, we built Bray–Curtis distance matrices using the *vegdist* function from the R package *vegan* (Oksanen et al., 2019). Then we applied the permutational multivariate analysis of variance (perMANOVA; 999 permutations) using the *adonis* (Analysis of variance using distance matrices) function to investigate whether diet composition differed between sampling dates and colonies. Homogeneity of variance was tested using the *betadis-per* function of the package *vegan*. The adjusted p -value thresholds after FDR correction ($p_{critical}$) were estimated following Castro and Singer (2006). Dissimilarities between prey communities (Bray–Curtis matrix) were visualized using the Nonmetric Multidimensional Scaling (NMDS) with the *metaMDS* function of the R package *vegan* (try = 20, trymax = 5,000). The quality of the solution was evaluated based on the stress value: stress values lower than 0.05 indicate that the solution is of excellent quality and stress values higher than 0.2 indicate solution of poor quality (Kruskal, 1964). Finally, as the landscape could influence the prevalence of taxa in the environment (e.g., presence or absence of prey ecologic requirements), we investigated whether diet composition between colonies was correlated with landscape composition using Mantel test (999 permutations) between diet and landscape dissimilarity matrices. Mantel tests were computed using the *mantel.rtest* function from the R package *ade4* (Dray & Dufour, 2007).

3 | RESULTS

3.1 | Pretreatment of high-throughput sequencing data

3.1.1 | Data filtering

We obtained 26,737 OTUs (44,801,072 sequences) after applying the FROGS pipeline. 8,839 supplementary OTUs were ruled out because they were considered as chimera by *iBimeraDenovo* tools from *dada2* (33.05% of the OTUs). After filtering using the controls and triplicates, we obtained 7,103 OTUs (41,216,468 sequences) for 1,986 samples. Of the 7,103 OTUs 2,110 could not be taxonomically assigned due to low confidence level (< 97% identity and/or <90% coverage; 13% of the total number of reads) and 3,204 were absent from database (*i.e.*, blast produced no result; 8% of the total number of reads). Among the 1,178 assigned OTUs, 72 OTUs were discarded because they were not referenced in Europe, were assigned by error to insect species in BOLD but correspond to bacteria (*e.g.*, *Wolbachia* and Rickettsiales), or were likely to be environmental contaminations (*e.g.*, mites).

3.1.2 | Identification of the predator

Among the 1,986 samples obtained after the filtering steps, 1,194 corresponded to *R. ferrumequinum* (60.1%), 381 to *M. emarginatus* (19.2%), 32 to the greater mouse-eared bat (*Myotis myotis*; 1.6%), and one to the genus *Serotinus* (0.05%). 29 samples were identified as a mix of two bat species, including *R. ferrumequinum*, *M. emarginatus*, *Serotinus* sp., the long-eared bat (*Plecotus auritus*), the Bechstein's bat (*Myotis bechsteinii*), and the Natterer's bat (*Myotis nattereri*). Twelve samples were identified as rodents or a mix of rodents and bats (0.6%), 15 samples as birds (*Hirundo* sp.; 0.7%), and 91 samples as a mix of birds and bats (4.5%). Finally, we could not identify any predator for 178 samples (8.9%) and neither predator nor prey identification for 52 samples (2.6%), probably because OTUs of these samples were below the filtering thresholds used to clean the data (*i.e.*, false negatives). The colony of Saint-Martin-les-Melle has been ruled out from the analyses because of an insufficient number of *R. ferrumequinum* sampled in June ($N = 0$) and in July ($N = 1$). Then, 82 samples among the 1,115 from the six remaining colonies were discarded because of an absence of prey detection following the application of previous filters.

3.2 | Landscape characterization

Principal component analysis of the sum of the vegetation surface surrounding the colonies (ha) clearly separated two types of landscapes with the first two PCA axes explaining the largest part of the total inertia (Table S1). The first axis (Axis.1, 48.34%) represented a landscape gradient running from a habitat dominated by

forests (Beaumont) to a semi-open habitat dominated by meadows and hedgerows (Lessac, Sainte-Gemme, Allonne, and Xaintray), with an intermediate situation in Annepont (Figure 2). The second axis (Axis.2, 25.90%) separated the colony of Annepont—characterized by vineyards and deciduous forests—from all other colonies (Figure 2).

3.3 | Diet analyses

3.3.1 | Reliability of the data

Our results revealed an appropriate sequencing depth per sample, with the estimated diversity covered in each sample at $q = 0$ (*i.e.*, richness) and $q = 1$ (*i.e.*, Shannon diversity; both richness and evenness) ranging between 95% and 100%.

The more precise the taxonomic level, the larger the sampling effort needed to recover all the diversity (Figure S1). At $q = 0$, the accumulation curves almost reached the plateau at the order level (around $N \approx 75$ samples) but not at the family, genus, and species levels (Figure S1). At $q = 1$, the accumulation curves reached the plateau at the order ($N \approx 25$ samples) and family ($N \approx 50$ samples) levels and almost reached the plateau at the genus ($N \approx 75$ samples) and species levels ($N > 75$ samples) (Figure S1), except in Annepont where the plateau was reached at the order level only.

3.3.2 | Variability of whole and core diets

The final complete dataset included 1,033 *R. ferrumequinum* samples corresponding to six colonies collected at three sampling dates. Most fecal pellets contained between one and four prey taxa (median = 4, min = 1, max = 19, mean = 4.44; Figure S2).

We identified 679 taxa from 17 arthropod orders, 124 families, 434 genera, and 519 species (Figure 3). Three main orders were detected: Lepidoptera (57% of the occurrences), Diptera (23%), and Coleoptera (13%). We identified an important number of agricultural pests with 133 species listed in the Arthemis database (Table S2) representing 31.86% of the occurrences. Pest species were mainly Lepidoptera (70.9%; *e.g.*, *Thaumetopoea pityocampa* and *Archips podana*), Coleoptera (13.9%; *e.g.*, *Curculio elephas* and *Melolontha melolontha*), Diptera (12.2%; *e.g.*, *Tipula lateralis* and *Nephrotoma appendiculata*) and Hemiptera (2.7%; *e.g.*, *Adelphocoris lineolatus* and *Fieberiella florii*).

We also noticed that the complete dataset included a large proportion of rare prey: 59% of prey taxa were represented by only two (103 taxa) or even only one (300 taxa) occurrence in the 1,033 samples. We therefore considered a smaller dataset (hereafter called "core diet") composed of the most frequent prey species (frequencies of occurrences > 5%). They represented 2.50% of all the taxa detected in the 1,033 samples, 24.89% of the occurrences, and 46.26% of the total number of reads. In this core diet, we identified 17 taxa from three orders (Lepidoptera, Diptera, and Coleoptera), 10

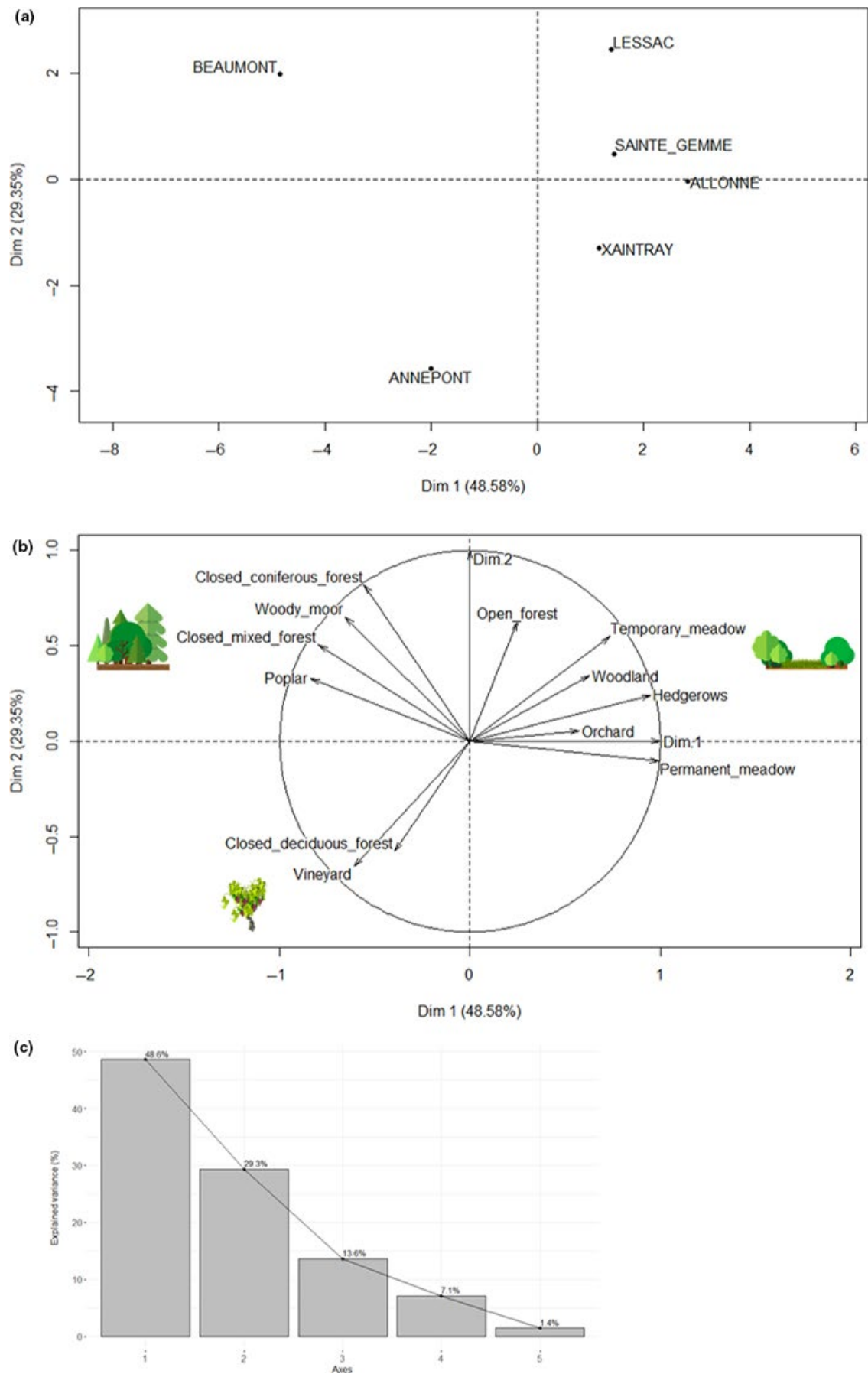


FIGURE 2 Principal Component Analysis (PCA) of twelve features describing the landscape surrounding the colonies (radius = 7 km, surface in hectare). Representation of (a) the six colonies, (b) the landscape variables, and (c) the eigenvalue graph which indicates the percentage of variance explained by each axis of the PCA

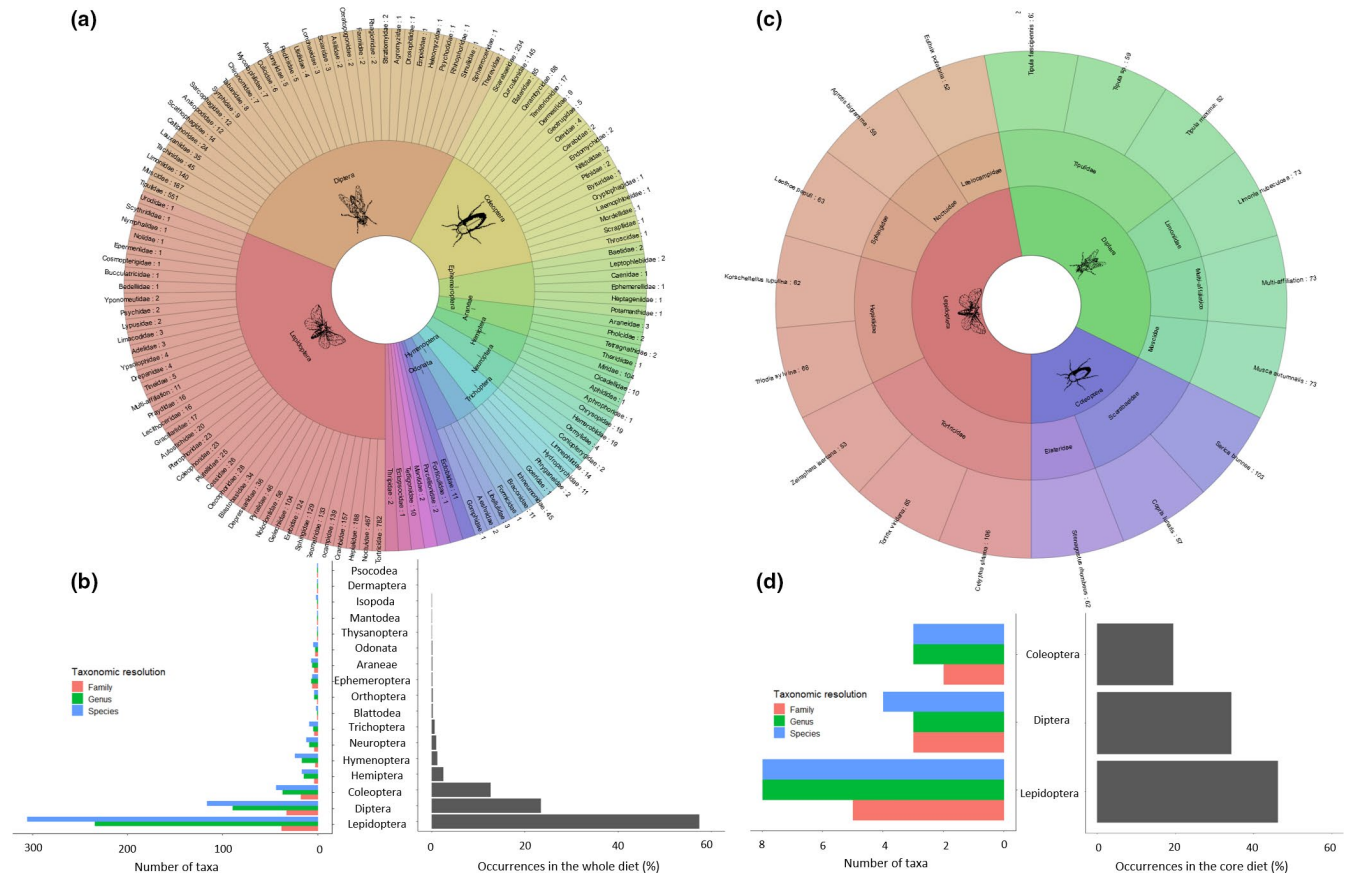


FIGURE 3 Representation of the prey taxonomic diversity detected from *R. ferrumequinum* guano, considering six colonies and three sampling dates. (a) The two circles represent taxonomic ranks from the families outwards to the order in the center. Numbers correspond to the number of occurrences of each taxonomic family in the whole diet. (b) Taxonomic diversity of the arthropod orders expressed as the number of prey families, genus, species (left), and percentage of occurrences (right) in the whole diet. (c) The circles represent taxonomic ranks from the species outwards to the order in the center considering only the prey with an occurrence frequency > 5%. Numbers correspond to the number of occurrences of each taxonomic family in the core diet. (d) Relative representation of the frequent prey (core diet) expressed as the number of prey families, genus, species (left), and percentage of occurrences (right)

families, 14 genera, and 15 species (Figure 4; Table S3). Three agricultural pest species and one vector of livestock disease were also identified: *Serica brunnea*, *Tortrix viridana*, *Tipula maxima*, and *Musca autumnalis*, respectively. Further analyses of alpha and beta diversity were performed both on the complete (whole diet) and restricted (core diet) datasets.

3.3.3 | Alpha diversity

Considering the whole diet, we found a significant effect of the first PCA axis (Axis.1) and of the sampling date (month) whatever the taxonomic level and q value considered.

The interaction between Axis.1 and sampling date was significant for $q = 0$ and all taxonomic levels except family, and for $q = 1$ and the order level only (Table 1, Figure S3). Alpha diversity increased along Axis.1 in June and July, then slightly decreased in August at the order, family, and genus level but remained stable at the species level (Table 1). Alpha diversity remained almost constant throughout the

summer in the two colonies located in landscapes dominated by forests (left of Axis.1) while it was lower in August in the four colonies located in landscapes dominated by meadows and hedgerows (right of the Axis.1). At the family ($q = 0$ and $q = 1$), genus ($q = 1$) and species ($q = 1$) levels, we observed a positive effect of Axis.1 on alpha diversity and a peak of alpha diversity in July (Table 1).

Considering the core diet, the effects of sampling date and landscape (Axis.1) were always significant whatever the taxonomic levels and q values considered (Table 1). We observed a negative effect of sampling date and a positive effect of Axis.1 on alpha diversity.

All results are detailed in Table 1 and Figure S3.

3.3.4 | Beta diversity

Considering the whole diet, the relative occurrence of Lepidoptera and Diptera showed opposite patterns of temporal variations with an increase and a decrease over the summer, respectively (Figure 4a). The core diet showed different patterns. The colonies of Annepont

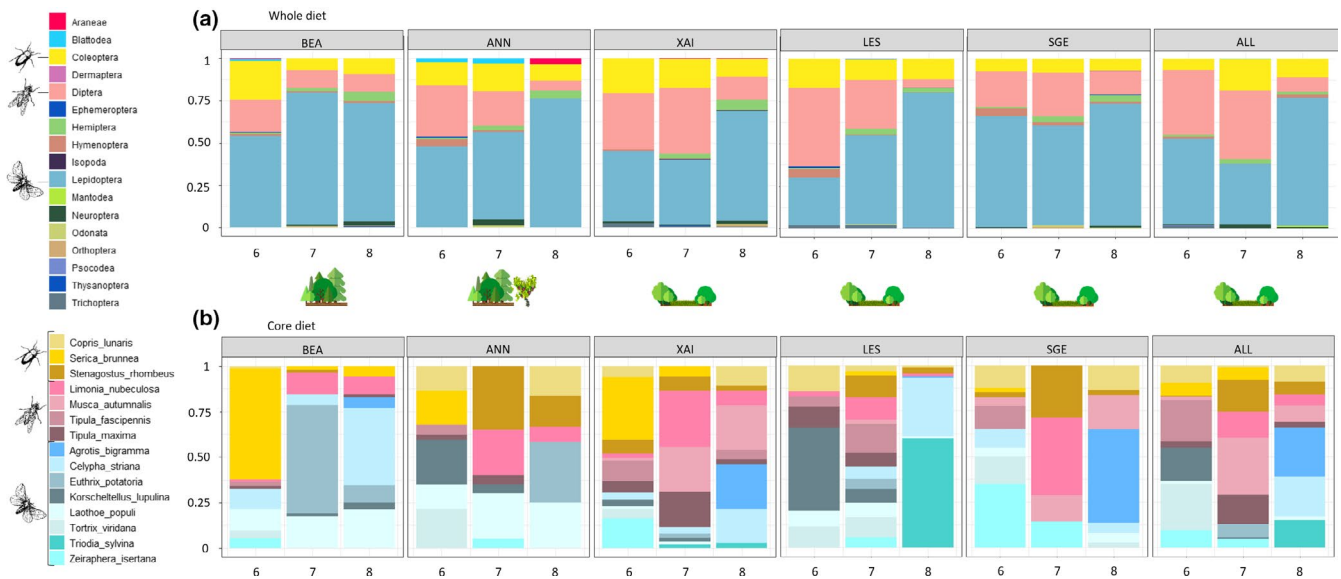


FIGURE 4 Frequency of occurrences in each of the six colonies—sorted according to their coordinates on the Axis.1 of the PCA (Allonne “ALL,” Annepont “ANN,” Beaumont “BEA,” Lessac “LES,” Sainte-Gemme “SGE,” Xaintray “XAI”)—for the three dates of sampling (“6” = June, “7” = July, and “8” = August). (a) The whole diet considered at the order level of taxonomic resolution. (b) The core diet considered at the species level of taxonomic resolution. In both figures, the three most occurrent orders (Lepidoptera, Diptera, Coleoptera) are represented by an icon. The dominant landscapes—as determined by the axes 1 and 2 of the PCA—are indicated for each colony

(landscape dominated by vineyards and forest), Lessac, Allonne, Sainte-Gemme, and Xaintray (all characterized by hedgerows and meadows) exhibited a peak of Diptera and a minimum of Lepidoptera in July (Figure 4b). The colony of Beaumont (landscape dominated by forests) showed a singular pattern: whatever the sampling date considered, the prey community was dominated by a single order (Coleoptera in June, Lepidoptera in July and August) and a maximum of two species (Figure 4b).

PerMANOVA analyses revealed significant variations of *R. ferrumequinum* diet between sampling dates and colonies, for both the whole and core diets (Table 2). Note however that the variance between groups (colonies or sampling dates) was not homogeneous and R_2 values were low ($0.035 < R_2 < 0.167$; Table 2). NMDS plots built on the whole diet highlighted the presence of rare prey that exacerbated the dissimilarity between colonies and sampling dates (Figure S4; see Figure 5a and b at the family and species levels). When considering the core diet, NMDS plots showed a high overlap of prey between sampling dates and colonies (Figure 5c and d). At the species level, NMDS plots revealed a high overlap between colonies but also suggested a pattern corresponding to the summer progression (from June to August), with less prey species shared between June (e.g., *Tortix viridana* and *Zeiraphera isertana*) and August (e.g., *Triodia sylvina* and *Agrotis bigramma*) (Figure 5d).

Finally, we observed a positive relationship between the beta diversity (differentiation between diet composition) and the dissimilarity of the landscape surrounding the colonies when considering the whole diet (family: $p = .006$ and $r = 0.015$, genus: $p = .003$ and $r = 0.015$, species: $p = .002$ and $r = 0.012$) or the core diet (order: $p = .012$ and $r = 0.024$, family: $p = .029$ and $r = 0.013$, genus: $p = .001$

and $r = 0.025$, species: $p = .001$ and $r = 0.020$), except at the order level for the whole diet ($p = .478$ and $r = 3.32e-05$).

4 | DISCUSSION

Using eDNA metabarcoding, we have shown that *R. ferrumequinum* diet is much more diversified than previously described by microscopic (Flanders & Jones, 2009; Jones, 1990) and molecular analyses (Alberdi et al., 2020; Aldasoro et al., 2019; Galan et al., 2018). We revealed a broader ecological and taxonomic variety of prey from the order to species levels of taxonomic resolution (519 species; 76% of the taxa affiliated at the species level). Diet included insects of varying size (from 1 mm to >70 mm), insects that emerge from water (e.g., Ephemeroptera, Odonata, or Trichoptera), hard and soft-bodied insect species as well as spiders.

We could not exclude that among the 519 prey species, some detections might be due to environmental contaminants (Galan et al., 2018). For example, we were not able to filter the presence of coprophagous insect DNA contaminating guano before sample collection. However, environmental contaminants may only have a limited impact on our results due to the stringent filtering steps carried out before statistical analyses. We could not exclude either that some detections might be due to indirect predation (e.g., spider prey; Sheppard et al., 2005). Indirect predation could not be distinguished from direct predation but should not be ignored in conservation perspectives. Indeed, indirectly consumed prey may exert a bottom-up control on *R. ferrumequinum*, through a direct impact on *R. ferrumequinum* prey (Frederiksen et al., 2006). Furthermore, our

TABLE 1 GLM results testing the effect of landscape variables (Axis 1 and 2 of the PCA), sampling months (June, July, August) and their interactions (represented by **), on the estimates of alpha diversity for $q = 0$ and $q = 1$ at each taxonomic level: order, family, genus and species. Significant p -values after correction for FDR multiple tests are represented in bold. (A) On the whole diet, (B) on the core diet

		$q = 0$			$q = 1$		
		Df	F	p	Df	F	p
(A)							
Order	Axis 1	1	18.040	2.360e-05	1	14.322	.0002
	Month	2	35.875	8.7290e-16	2	25.973	1.077e-11
	Axis 2	1	4.191	.041	1	4.190	.041
	Axis 1 * Month	2	15.771	1.796e-07	2	12.853	3.132e-06
	Axis 2 * Month	2	0.131	.877	2	0.0004	.999
Family	Axis 1	1	18.001	2.408e-05	1	16.603	5.015e-05
	Month	2	25.019	2.466e-11	2	30.548	1.457e-13
	Axis 2	1	0.548	.459	1	1.944	.164
	Axis 1 * Month	2	4.383	.013	2	1.814	.164
	Axis 2 * Month	2	1.133	.323	2	0.962	.383
Genus	Axis 1	1	19.247	1.269e-05	1	15.919	7.151e-05
	Month	2	12.139	6.166e-06	2	25.249	2.152e-11
	Axis 2	1	0.065	.799	1	1.693	.193
	Axis 1 * Month	2	8.588	.0002	2	3.055	.048
	Axis 2 * Month	2	4.232	.015	2	2.156	.116
Species	Axis 1	1	23.805	1.238e-06	1	16.593	5.048e-05
	Month	2	10.608	2.759e-05	2	17.081	5.259e-08
	Axis 2	1	0.169	.681	1	1.503	.220
	Axis 1 * Month	2	6.452	.002	2	2.179	.114
	Axis 2 * Month	2	3.864	.021	2	1.670	.189
(B)							
Order	Axis 1	1	19.486	1.173e-05	1	17.848	2.743e-05
	Month	2	21.638	7.617e-10	2	13.346	2.102e-06
	Axis 2	1	0.107	.744	1	0.110	.741
	Axis 1 * Month	2	2.266	.104	2	1.265	.283
	Axis 2 * Month	2	3.592	.028	2	0.846	.429
Family	Axis 1	1	38.443	9.663e-10	1	40.056	4.686e-10
	Month	2	13.463	1.834e-06	2	10.945	2.126e-05
	Axis 2	1	1.278	.259	1	1.918	.166
	Axis 1 * Month	2	4.112	.017	2	1.513	.221
	Axis 2 * Month	2	0.642	.526	2	0.178	.837
Genus	Axis 1	1	36.904	2.042e-09	1	37.617	1.521e-09
	Month	2	15.794	1.958e-07	2	11.086	1.854e-05
	Axis 2	1	2.227	.136	1	3.221	.073
	Axis 1 * Month	2	4.022	.018	2	1.451	.235
	Axis 2 * Month	2	0.989	.372	2	0.002	.998
Species	Axis 1	1	32.795	1.533e-08	1	33.239	1.286e-08
	Month	2	12.358	5.338e-06	2	7.265	0.0007
	Axis 2	1	0.461	.497	1	0.400	.527
	Axis 1 * Month	2	3.781	.023	2	1.246	.288
	Axis 2 * Month	2	1.943	.144	2	0.468	.626

TABLE 2 Results of (A) permANOVA analyses and (B) homogeneity of variance analysis by permutation (999 permutations), based on the Bray–Curtis dissimilarity for each taxonomic rank tested. The interaction effect between sampling month and colonies is represented by ******. Significant *p*-values after correction for multiple FDR tests are represented in bold. These analyses were performed on the whole diet and on the core diet

			permANOVA			
(A)			<i>Df</i>	<i>R</i> ²	<i>F</i>	<i>p</i>
Whole diet	Order	Month	2	0.081	51.160	.001
		Colony	5	0.042	10.527	.001
		Month * Colony	10	0.074	9.401	.001
	Family	Month	2	0.062	38.527	.001
		Colony	5	0.047	11.620	.001
		Month * Colony	10	0.064	7.827	.001
	Genus	Month	2	0.049	28.939	.001
		Colony	5	0.037	8.7538	.001
		Month * Colony	10	0.062	7.38	.001
	Species	Month	2	0.043	24.992	.001
		Colony	5	0.035	8.207	.001
		Month * Colony	10	0.064	7.460	.001
Core diet	Order	Month	2	0.167	91.334	.001
		Colony	5	0.081	17.592	.001
		Month * Colony	10	0.118	12.845	.001
	Family	Month	2	0.098	46.668	.001
		Colony	5	0.082	15.561	.001
		Month * Colony	10	0.095	8.993	.001
	Genus	Month	2	0.112	54.269	.001
		Colony	5	0.076	14.853	.001
		Month * Colony	10	0.104	10.056	.001
	Species	Month	2	0.112	53.585	.001
		Colony	5	0.074	14.235	.001
		Month * Colony	10	0.106	10.181	.001
			permTest			
(B)			<i>Df</i>	<i>F</i>	<i>p</i>	
Whole diet	Order		17	11.066	.001	
	Family		17	8.819	.001	
	Genus		17	9.928	.001	
	Species		17	10.419	.001	
Core diet	Order		17	6.782	.001	
	Family		17	4.554	.001	
	Genus		17	4.5064	.001	
	Species		17	4.749	.001	

sampling effort did not enable a complete description of the whole prey diversity of *R. ferrumequinum* for each sampling date and colony surveyed, despite the high number of pellets analyzed. The reason was probably the high number of rare prey species (300 taxa with only one occurrence in the entire dataset) (Clare et al., 2009; Razgour et al., 2011; Vesterinen et al., 2013). This result suggested that the diversity of *R. ferrumequinum* diet could be even higher than described, and a greater sampling effort would be required to

capture its full diversity (Mata et al., 2018). In this study, we captured most of the prey diversity at the order, family, and genus taxonomic levels. Accumulation curves suggest that about 25 samples might be sufficient to describe the main diversity at the order level, 50 samples at the family level, and between 75 and more than 100 samples at the genus and species levels. Because it is important to minimize disturbance and stress for bats, the number of samples is critical to assess prey diversity when using eDNA metabarcoding. Future bat

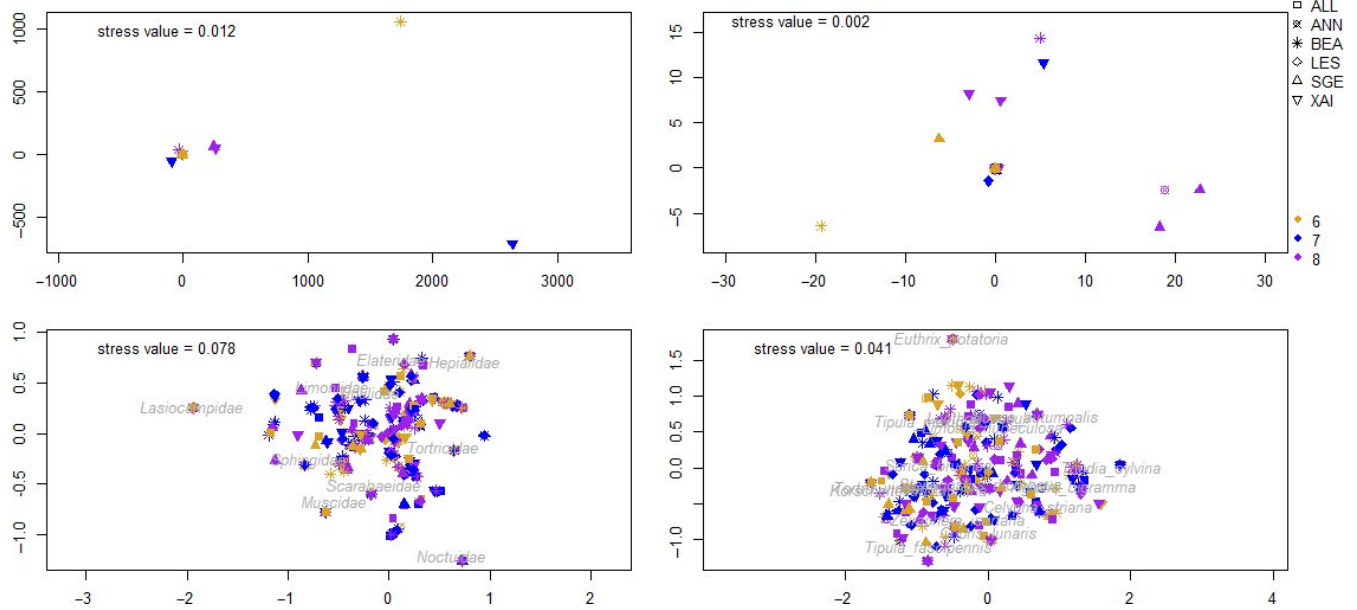


FIGURE 5 Representation of the Bray–Curtis dissimilarity using NMDS. (a) Whole diet at the family level of taxonomic resolution (top left), (b) Whole diet at the species level of taxonomic resolution (top right), (c) Core diet at the family level of taxonomic resolution (bottom left), (d) Core diet at the species level of taxonomic resolution (bottom right). Due to the high overlap of points, family and species names are not represented in 5a and 5b. Abbreviation: 6 = June (yellow), 7 = July (blue), 8 = August (purple), ANN = Annepont (circle cross), ALL = Allonne (square), BEA = Beaumont (star), LES = Lessac (diamond), SGE = Sainte-Gemme (triangle point up) and XAI = Xaintray (triangle point down)

diet studies should rather rely on a large number of guano collected beneath colonies rather than on feces collected from captured bats (usually < 30 samples; Alberdi et al., 2020; Aldasoro et al., 2019; Bohmann et al., 2018; Galan et al., 2018; Vallejo et al., 2019).

Our results also suggested the existence of a core diet potentially constituting the foundation of *R. ferrumequinum* diet, and of a secondary diet composed of numerous rare prey taxa mainly occurring once at all colonies and sampling dates. *Rhinolophus ferrumequinum* core diet included 15 common prey species shared by all the colonies, from medium to large size (10 to 40 mm long for Coleoptera and Diptera, and 15 to 100 mm width for Lepidoptera). A previous study based on microscopic analyses of *R. ferrumequinum* feces from England suggested that various moths, beetles, tipulids, and ichneumonids could be considered as “key prey” or “secondary prey” (Ransome & Priddis, 2005). However, these terms were not clearly defined and this classification was mainly based on the prey abundance estimated from the percentage of prey volume in the feces. Most of the key prey described by Ransome and Priddis (2005) were also found in the core diet (e.g., moth, beetles, and tipulids), but some differences were observed. For example, we did not detect *Geotrupes* sp. and ichneumonids in the core diet, although *Geotrupes spiniger* (36 occurrences) and ichneumonids (19 taxa with one to 12 occurrences) were detected in the secondary diet. Conversely, in the core diet we found dipteran families (Limoniidae, Muscidae) that were not identified by Ransome and Priddis (2005). The differences between the two studies probably arise from two biases. First, the inability of the microscopic approach to detect and identify some prey of the core diet with precise taxonomic resolution: it

may bias the relative importance of each prey in *R. ferrumequinum* diet (Andriollo et al., 2019; Nielsen et al., 2018). Second, delineating the contours of the core and secondary diet, based on occurrence frequencies, still remains arbitrary. This issue deserves further dedicated studies that could provide objective criteria to discriminate between the core and secondary diets.

Our results corroborated the assumption that prey availability might be an important factor influencing the diversity, composition, and spatio-temporal variations of *R. ferrumequinum* diet. Previous studies suggested that food availability is a main factor regulating the timing of parturition in insectivorous bats, and, as a consequence, reproductive success in bats is highly dependent on synchronizing reproduction with peaks of food availability (Arlettaz et al., 2001; Nurul-Ain et al., 2017). The ability to adjust foraging behavior with reproductive conditions, and hence energy requirements, has already been shown in some bat species including *R. ferrumequinum* (Dietz & Kalko, 2007). In this study, the peak of prey diversity observed in the whole diet in July might be explained by the variations of insect abundance over the summer. Indeed a peak of insect diversity is often observed at, or near, the middle of this season (Wolda, 1988). Moreover, the presence of some prey species in the core diet well reflected the phenology of these insects (e.g., *Agrotis bigramma* and *Zeiraphera isertana*). Consequently, the composition of the core and secondary diets might strongly reflect the natural availability and abundance of insects in the foraging range of *R. ferrumequinum*. The ensuing decrease of diet diversity in late summer has already been observed for several bat species, for example, *Plecotus auritus* (brown long-eared bat; Andriollo et al., 2019) and

Myotis lucifugus (the little brown bat; Clare, Symondson, Broders, et al., 2014). All these bat species seemed to consume a greater volume of a limited number of prey taxa, which can lead to a decrease in diet diversity. Bats need to rapidly accumulate body reserves before the onset of winter and hibernation. Therefore, the feeding strategy in August should target abundant and profitable prey species (e.g., rich in fatty acids; Krüger et al., 2014; Levin et al., 2013) and be associated with hormonal, metabolic and gut microbial composition/diversity changes (Kronfeld-Schor et al., 2000; Levin et al., 2013; Srivastava & Krishna, 2008; Xiao et al., 2019).

However, the composition of the core diet and its temporal variations did not seem to be explained by insect availability only. For example, *Copris lunaris* was scarcely detected in July, although flying adults are naturally abundant from spring to autumn. Selective feeding and seasonal variations in selectivity have previously been described for insectivorous bat species (e.g., *Myotis lucifugus*: Anthony & Kunz, 1977; *Myotis daubentonii*; Vesterinen et al., 2016). Some studies have shown that bats selectively ate their favorite prey when these were available in the environment (e.g., *Eptesicus fuscus*; Agosta et al., 2003; *R. ferrumequinum*; Jones, 1990), and were less selective when these favorite prey were rare. *Rhinolophus ferrumequinum* exhibits a great capacity for prey selection, linked to its particular echolocation call structure. This enables to accurately discriminate insects, their speed, and trajectory while compensating for the Doppler shifts induced by its own flight (Barataud, 2015; Emde & Menne, 1989). Based on previous encounter experiences with prey, *R. ferrumequinum* is able to link prey-specific echo information with prey profitability, and to use this information for informed hunting decisions from its perch (Koselj et al., 2011). *Rhinolophus ferrumequinum* could hence exploit some preferred taxa (core diet) while simultaneously developing an opportunistic strategy and consuming a wide range of available prey (secondary diet). This could rely on the two hunting tactics used by *R. ferrumequinum*, that is, prey searching in flight and prey searching from a perch (Jones & Rayner, 1989). Because the first tactic induces a greater metabolic cost than the second one, *R. ferrumequinum* is expected to be more selective on size and/or prey behavior while perch-hunting (low cost–high yield tactic; Nadjafzadeh et al., 2016) compared to actively searching in flight (Koselj et al., 2011; Voigt et al., 2010).

In this context, the secondary diet might play a role of diet completion to enable survival when essential prey are scarce (e.g., Mirhosseini et al., 2015). The role of the secondary diet in energy completion is well known in human nutritional ecology (Fanelli & Steinhagen, 1985; Koehler et al., 1989; McGowan et al., 2012; Taylor et al., 2005) but remains unexplored in other animal species. Thus, further studies combining ecological and nutritional analyses will be of great interest to better assess the relative importance of a core and a secondary diet in providing energy and mineral intake of insectivorous bats. To what extent the core/secondary diet partition can be generalized to insectivorous bat species and whether the echolocation system and hunting tactics of bats are associated with such diet partition remain important issues that need to be addressed in the future.

Finally, our results suggested that landscape features are important drivers of prey availability, and as such, they seemed to influence the diversity, composition, and spatio-temporal variations of *R. ferrumequinum* diet. Insect species richness and abundance are strongly influenced by landscape features such as plant species richness or landscape heterogeneity (Rundlöf & Smith, 2006; Schuldt et al., 2019). For example, the lepidopteran *Laotloe populi* is known to be mainly associated with forests. As such it was much more detected in the two forested colonies (Beaumont and Annapont) than in the other ones. Conversely, the coleopteran *Copris lunaris*, which is associated with dung in meadows, was less abundant in the two forested colonies. We found that *R. ferrumequinum* diet diversity was lower in colonies surrounded by forests than by meadows and hedgerows. Thus the contrasting patterns of prey composition and diversity observed between the colonies dominated by forests and those by meadows and hedgerows were likely to reflect the adaptation of *R. ferrumequinum* to semi-cluttered foraging habitats (e.g., echolocation, hunting strategies; Dietz et al., 2013; Jones & Rayner, 1989). The large concentration and diversity of insects provided by hedgerows in agricultural fields should enable the consumption of a wider variety of local prey (Forman & Baudry, 1984; Holland & Fahrig, 2000; Lewis, 1969; Verboom & Spoelstra, 1999). Therefore, it is likely that these differences in prey communities associated with landscape features lead to the selection of different profitable prey (Clare et al., 2011; Danks, 2007; Kolkert et al., 2020).

Overall, we detected a significant positive correlation between differences of diet between colonies and differences in landscapes between these colonies. However, the colonies surveyed were heterogeneously distributed along a forest-meadow landscape continuum. These relationships between diet diversity and landscape may therefore potentially be driven by the two most differentiated colonies: Beaumont (mostly surrounded by forests) and Annapont (surrounded by forests and vineyards). Assessing *R. ferrumequinum* diet in more colonies that would better represent the landscape continuum could be particularly useful to deepen our understanding of this relationship between diet diversity and landscape.

At this point, we lack critical information on insect availability (abundance and nutritional composition) throughout the maternity period of *R. ferrumequinum*. Comparing such data with the prey identified in the *R. ferrumequinum* diet would help to confirm/refute the influence of the biological processes proposed above to explain dietary plasticity. In particular, it would enable the evaluation of the degree of selectivity in the *R. ferrumequinum* foraging strategy (Emlen, 1966; Koselj et al., 2011; Tracy et al., 2006; Vesterinen et al., 2016). Furthermore, analyzing inter-individual dietary variations in greater depth, by collecting a larger number of samples, would be of particular interest. Indeed, as described for other bat and mammal species, local patterns might be influenced by the specialization of different individuals within colonies (Bolnick et al., 2003; Johnston & Fenton, 2001; Thiemann et al., 2011). Differences in echolocation characteristics, flight and hunting performances between juveniles and adult bats may, for example, contribute to a wider spectrum of prey when young start

to feed themselves compared to adults (Arrizabalaga-Escudero et al., 2019; Czenze et al., 2018; Rolseth et al., 1994; Salsamendi et al., 2008).

Conservation perspectives

This study confirmed the high dietary plasticity as well as the wide spectrum of arthropods consumed by *R. ferrumequinum*. Hence, it might seem reasonable to consider that this bat species should not be at high risk when facing environmental changes affecting its prey distribution and abundance (Boyles & Storm, 2007; Owens & Dittman, 2003; Pratchett et al., 2006; Twining et al., 2019). However, our results have emphasized the existence of a core diet—potentially essential for optimizing *R. ferrumequinum* fitness—which could be threatened by the modification of the landscape, the indirect effect of cattle anti-parasite drugs on the beetles, and more globally the use of pesticides (Dietz et al., 2013; Finch et al., 2020; Froidevaux et al., 2019; Geiger et al., 2010; Gonzalez-Tokman et al., 2017; Pocock & Jennings, 2008). *Rhinolophus ferrumequinum* is also vulnerable as it is a long-lived species (up to 30 years) with a low reproductive rate (maximum of one pup per year) and a late sexual maturity (two to five years) (Caubère et al., 1984; Ransome, 1995; Wilkinson & South, 2002). Further studies are therefore needed to evaluate the effects of the core and secondary prey variations on bat life-history traits and fitness. In particular, it is critical to assess whether these variations might significantly impact the demography and viability of *R. ferrumequinum* populations (Schweiger et al., 2015). This is all the more important given that it has recently been shown that some populations at the edge of the *R. ferrumequinum* distribution might be at higher risk of extinction in the near future (Tournayre et al., 2019).

Our results support a growing literature illustrating the potential role of insectivorous bats in arthropod pest control (Aizpurua et al., 2018; Cohen et al., 2020; Kolkert et al., 2020). Some large and/or chitinized pests had already been identified in previous *R. ferrumequinum* diet studies, such as the Coleoptera *Serica brunnea* and *Melolontha melolontha* or the Diptera *Tipula maxima* (Aldasoro et al., 2019; Galan et al., 2018; Jones, 1990). However, no study has described as many deleterious or potentially deleterious insect species (one third of all occurrences) in the *R. ferrumequinum* diet. Thus our results confirmed that *R. ferrumequinum* may be not only be important as a sentinel of agricultural insect pests (chirosurveillance) but also as an efficient agent of pest control (Cohen et al., 2020; Maslo et al., 2017; Weier et al., 2019). This role may not only be important in the future because climate change is expected to favor the establishment and proliferation of many deleterious insects (Trumble & Butler, 2009). Our results also provide evidence of the presence of arthropods beneficial to agriculture (i.e., natural enemy of pests) in the insectivorous bat diet (Cohen et al., 2020; Kolkert et al., 2020). Beneficial arthropods are rarely addressed in bat diet literature, probably because of the scarcity of detailed databases, but it is likely that insectivorous bats eat both pests and beneficial

arthropods. Kolkert et al. (2020) showed that the diet of several Australian insectivorous bat included 1% of beneficial insects (predators, parasitoids, pollinators), emphasizing the service rather than the disservice these bats provide to agriculture. Assessing the impact of *R. ferrumequinum* on pest populations and determining which category of prey insectivorous bats eat, that is, the relative quantities of pest and beneficial arthropods consumed, will be needed to further evaluate the effectiveness of *R. ferrumequinum* as agent of pest control.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

The study was conceived and designed by O.T., M.L., O.F.C., M.G., D.P., and N.C. The sampling schemes were designed by M.L., M.G., O.T., O.F.C., D.P., N.C., and conducted by M.L. Landscape data were extracted by D. P. Laboratory protocols were designed by M.G. and performed by M.G., O.T. and M.T. O.T., S.P., M.G., and M.T. performed the bioinformatic analyses and O.T. carried out the statistical analyses. A first draft of the manuscript was written by O.T., M.G., D.P., and N.C. All authors contributed to the writing of the final version of this paper.

DATA AVAILABILITY STATEMENT

Supplementary data deposited in Zenodo (<https://www.doi.org/10.5281/zenodo.3819911>) include the following: (i) raw sequence reads of the five runs (fastq format), (ii) information on the samples, positive and negative controls, (iii) raw abundance table before and after filtering, (iv) final abundance table from the *R.*

ferrumequinum samples only, and (v) landscape data table used to build the PCA.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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