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To cite this version:

Eve Afonso, Pierline Tournant, Jean-Christophe Foltête, Patrick Giraudoux, Pierre-Emmanuel Baurand, et al.. Is the lesser horseshoe bat (Rhinolophus hipposideros) exposed to causes that may have contributed to its decline? A non-invasive approach. Global Ecology and Conservation, 2016, 8, pp.123-137. 10.1016/j.gecco.2016.09.002 . hal-01368326

HAL Id: hal-01368326

https://hal.science/hal-01368326v1

Submitted on 22 Mar 2022

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Global Ecology and Conservation 8 (2016) 123–137

Contents lists available at ScienceDirect

Global Ecology and Conservation

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

Original research article

Is the lesser horseshoe bat (*Rhinolophus hipposideros*) exposed to causes that may have contributed to its decline? A non-invasive approach

Eve Afonso [a,c,*], Pierline Tournant [a,b,c], Jean-Christophe Foltête [b,c], Patrick Giraudoux [a,c,d], Pierre-Emmanuel Baurand [a,c], Sébastien Roué [e], Victoria Canella [a], Danièle Vey [f], Renaud Scheifler [a,c]

[a] Chrono-Environnement UMR 6249 CNRS, Université Bourgogne Franche-Comté, Place Leclerc, F-25030 Besançon, France
[b] ThéMA UMR 6049 CNRS, Université Bourgogne Franche-Comté, 32, rue Mégevand, F-25030 Besançon, France
[c] MSHE USR 3124, CNRS, Univ. Bourgogne Franche-Comté, F-25000 Besançon, France
[d] Institut Universitaire de France, Paris, France
[e] Commission de Protection des Eaux, du Patrimoine, de l'Environnement, du Sous-sol et des Chiroptères (CPEPESC) Franche-Comté, 3 rue Beauregard, F-25000 Besançon, France
[f] Laboratoire de Toxicologie Vétérinaire (Biolytics), VetAgroSup, 1, avenue Claude Bourgelat, F-69280 Marcy l'Etoile, France

HIGHLIGHTS

- Twenty lesser horseshoe bat maternity colonies were measured for habitat availability, genetic differentiation, and exposure to chemical pollutants and a protozoan parasite.
- Colonies exhibited a low genetic differentiation, suggesting a non-fragmentedd population.
- 7 colonies were exposed to lindane at concentrations comparable to those reported in the literature for bat guano.
- Prevalence for the parasite *Eimeria hessei* ranged from 0% to 80% among colonies.
- Concentrations of cadmium and zinc in droppings were positively related to *E. hessei* prevalence.

ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 9 August 2016
Accepted 4 September 2016
Available online 19 September 2016

Keywords:
Rhinolophus hipposideros
Bat population genetics
Organochlorides
Metals
Eimeria hessei
Pollution/pathogen interaction

ABSTRACT

While the lesser horseshoe bat (*Rhinolophus hipposideros*) was common in most western and Central Europe, this species endured a dramatic decline in the 1950s–1970s. The causes are thought to comprise the extensive spread of chemical pollutants affecting both bat and prey populations, changes in landscape composition and structure leading to population fragmentation or roost deterioration, and infectious diseases. While lesser horseshoe bat populations recently increased after nearly thirty years of decline in the Franche-Comté region (eastern France), it is unclear whether bats are currently confronted with causes that may have contributed to former demographic disturbances. Although stressors are generally studied separately, we simultaneously measured several variables directly or indirectly reflecting the supposed causes of bat decline: availability of woodland habitats around bat roosts, genetic differentiation amongst colonies, and exposure to chemical pollutants and to a protozoan parasite, *Eimeria hessei*. Twenty maternity colonies were sampled using a non-invasive approach based on droppings. Maternity roosts were located in buildings mainly surrounded by woodland and shrubland (48% of a 2500 m radius

* Corresponding author at: Chrono-Environnement UMR 6249 CNRS, Université Bourgogne Franche-Comté, Place Leclerc, F-25030 Besançon, France. Fax: +33 381 665 797.
E-mail address: eve.afonso@univ-fcomte.fr (E. Afonso).

http://dx.doi.org/10.1016/j.gecco.2016.09.002
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buffer around the colonies), which are preferentially used by the lesser horseshoe bat as foraging areas. Low genetic differentiation ($F_{ST} = 0.023–0.028$, $D = 0.076–0.097$) and no genetic clusters were found among the 20 sampled colonies, in which 449 individuals were distinguished over two sampling sessions (2010 and 2011). This suggests that no obvious physical barrier acts as a constraint with respect to gene flow, and we conclude that all individuals sampled belong to a unique non-fragmented population. Among the 66 chemical substances measured in bat droppings, the insecticides endosulfan alpha (0.035 μg/g dm) and lindane (from 0.013 to 0.112 μg/g dm) were the only organic compounds found at detectable concentrations in one and 7 colonies, respectively. Fourteen metals were measured in the guano. Metal concentrations were lower than or of the same order of magnitude as concentrations reported in bats in the literature. The overall faecal prevalence of *E. hessei* was 42% (range: 0%–80%) and did not differ between the two sampling sessions. Faecal prevalence was not related to lindane concentrations in droppings. Despite the low concentrations of metals, a positive relationship was found between cadmium and zinc concentrations and *E. hessei* faecal prevalence. Although it is difficult to conclude on the risks of adverse effects due to the lack of both toxicological references and knowledge on *E. hessei* pathogenicity, this study stresses out the interest of systemic approaches in the study of ecology of wildlife species, especially via the investigation of multiple exposures.
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1. Introduction

The populations of many European bat species experienced a dramatic decline in the latter half of the twentieth century, and most of them are currently considered to be vulnerable or endangered species (IUCN, 2012). The lesser horseshoe bat (*Rhinolophus hipposideros*) is comparably the bat species that endured the most spectacular decline in most of western and Central Europe in the 1950s–1970s, becoming locally extinct (Bontadina et al., 2000; Stebbings, 1989). The causes of this decline have not formally been identified but bat experts attributed it to a combination of various factors, such as the extensive spread of chemical pollutants, habitat destruction, changes in landscape structure, disturbance and destruction of roost sites (through deforestation and/or urban expansion), climate change, declines in insect prey, competition for prey with pipistrelles, genetic inbreeding, and/or diseases (Arlettaz et al., 2000; Bontadina et al., 2000; Motte and Libois, 2002).

Since the 1990s, the number of hibernation sites of the lesser horseshoe bat has increased across European countries, suggesting that populations may have partly recovered from their former massive decline (Van der Meij et al., 2015). Van der Meij et al. (2015) argue that this trend could be a consequence of global environmental regulations like restrictions of pesticide use and to specific bat conservation efforts engaged in the 1990s, such as the protection of roost sites (EUROBATS, EU Habitats Directive). In western France, the number of maternity roosts and the effective size of maternity colonies of the lesser horseshoe bat have increased since 1998 (Farcy et al., 2009; Petit et al., 2014). In the Franche-Comté region (eastern France), the effective size of hibernating colonies has increased of almost 200% since 1990 after nearly thirty years of population decrease (Roué, 2005). More than 100 maternity roosts are currently recorded in the region, which might represent a population of approximately 3000 individuals (Commission de Protection des Eaux, du Patrimoine, de l'Environnement, du Sous-sol et des Chiroptères (CPEPESC) Franche-Comté, unpublished data). The exposure of lesser horseshoe bat populations in Franche-Comté to potential stressors is largely unknown. However, although the number of roosts and individuals has increased, it is not clear whether these colonies are currently confronted with causes that may have contributed to former demographic disturbances. In that context, the aim of the present study was to evaluate the potential exposure of lesser horseshoe bats to some of the stressors that may have led to their former decline. While the vast majority of studies on bat species focussed on one potential stressor, we simultaneously searched for population fragmentation, pollutant and pathogen exposure, as well as their potential interactions.

The lesser horseshoe bat is a sedentary species, with winter and maternity roosts located less than 30 kilometres apart (Gaisler and Chytil, 2002). Individuals hibernate during the winter in mixed-sex groups within roosts commonly located in caves (Crucitti and Cavalletti, 2002). Females join maternity roosts at the end of spring to give birth and raise their single offspring throughout the summer, while males generally live as isolated individuals or gathered in small groups of three or four individuals (Gaisler, 1963). Because this species is known to forage along linear wooded elements, such as tree lines and well-structured hedgerows, landscape connectivity is assumed to be a key-factor for population sustainability (Bontadina et al., 2002; Motte and Libois, 2002; Zahn et al., 2008). The loss of connectivity between maternity roosts and foraging areas due to the fragmentation of wooded habitats is thus expected to parcel out lesser horseshoe bat populations. Population fragmentation and restricted gene flow can lead to genetic differentiation between sub-populations, loss of genetic variation through genetic drift, and loss of fitness due to inbreeding, which are predicted to enhance the risk of local extinction, especially in populations that have recently declined in size (Freeland, 2009; Mayer et al., 2009). Yet, the distribution of maternity colonies of the lesser horseshoe bat in the Franche-Comté region does not depend on wooded elements connecting the maternity roost with the foraging areas, and instead depends on the spatial integration of maternity roosts into a connected network of wooded patches (Tournant et al., 2013). This suggests that individual movements are little

constrained during inter-seasonal periods, supporting the hypothesis of a non-fragmented population, which, however, has yet to be studied.

In addition to wooded habitat fragmentation, the effects of chemical pollutants, in particular pesticides, are believed to be one of the main causes of the population decline (Bontadina et al., 2000). Bats may be exposed to chemicals by dermal contact (e.g. when pesticides are spread in building wood frames in presence of bats), by inhalation, and, probably more chronically, by ingestion in their diet. Bats, especially insectivorous temperate-region microchiropterans, have characteristics that increase their exposure and their susceptibility to pollutants (Clark and Shore, 2001). Like other insectivorous mammals and birds, bats may be exposed to higher concentrations of cumulative chemicals than herbivorous organisms because of biomagnification, *i.e.* the increase of concentrations along the food chain. Moreover, bats exhibit high metabolite rates associated to small size and flight, which require high feeding rates that consequently lead to high exposure to pollutants. Bats are also susceptible of remobilization of lipophilic chemicals stored in their fat when they use it for energetic purposes during migration or hibernation. Finally, their relatively long lifetime expectancy makes bats exposed to pollutants over long periods and susceptible of cumulative effects (Clark and Shore, 2001). While organic chemicals including persistent organic pollutants (POPs) and pesticides have long been recognized as a risk for bat populations (see for instance Bayat et al., 2014), recent studies showed that exposure to metals like cadmium (Cd) or lead (Pb) can also represent a threat for bats (Zukal et al., 2015; Hernout et al., 2016). Exposure to chemical pollutants can have lethal or sub-lethal effects on bats, including increases in metabolic rate, immune suppression, disruption of the endocrine system and susceptibility to infectious diseases (Clark and Shore, 2001; Carravieri and Scheifler, 2013; Bayat et al., 2014). In the lesser horseshoe bat, studies reported cases of death due to organochlorines (lindane, DDT, PCBs) in the eighties (Hamon, 1990). While some pesticides, such as lindane and DDT, that were widely used in agriculture in the 1960s, were progressively limited then banned over the following decades, Lüftl et al. (2005) still detected residues of these substances in organs of different bat species collected in Austria (1996–2000), confirming the very high persistence of these chemicals in the environment.

Recently, mass mortality cases due to pathogenic organisms (including bacteria, virus, fungi and parasites) have been reported in bat populations generally, and some of them have drastically decreased different bat populations. For example, the white nose syndrome, caused in bats by the fungus *Geomyces destructans*, has recently decimated entire populations of various bat species in North America (Wibbelt et al., 2010). This fungus has only been reported occasionally in France and never in the lesser horseshoe bat, and it does not appear to be related to bat population mortality events in Europe (Puechmaille et al., 2011). However, it cannot be excluded that other pathogenic organisms might have contributed to population demographic disturbances. Except for some emblematic viruses, such as rabies or some Lyssaviruses (Wibbelt et al., 2010), little is known about pathogenic organisms in bats. In the lesser horseshoe bat, the very few parasitological studies focussed only on *Eimeria hessei*, a protozoan parasite (Lavier, 1924). Because of the difficulties inherent in studying small (10–40 μm) parasites, the occurrence, transmission and pathogenicity of *Eimeria* species in bats is poorly studied (Duszynski, 2002). *Eimeria* species, including *E. hessei*, are generally located in the small intestine of their hosts. Bats excrete oocysts in their faeces after an unknown prepatent period. The pathogenicity of *Eimeria* species is largely unknown in bats, but they have been shown to cause weakness, diarrhoea and emaciation (especially in young animals) in rodents, lagomorphs, ovids, and birds (Allen et al., 1973; Mesfin et al., 1978; Gomez-Bautista et al., 1987; Gregory and Catchpole, 1990). Even so, nothing is known about *E. hessei* prevalence and its possible impact on bat colonies.

An increasing amount of data, especially in the aquatic environment, suggests that pathogenic organisms may interact with anthropogenic pollution and that the two factors may have additive or even synergistic deleterious effects on animal health (Marcogliese and Pietrock, 2011). One emblematic example of such a phenomenon and its conservation consequences is the study of Rohr et al. (2008) showing that the herbicide atrazine was the best predictor of the abundance of larval trematodes in declining populations of the northern leopard frog *Rana pipiens*. A combined approach with both field data and mesocosm experiments showed that agrochemicals (herbicides and nutrients) increased frog exposure to larval trematodes through an increase of intermediate snail hosts and increased susceptibility of the frogs by suppressing their immunity. Such potential interactions between pathogenic organisms and pollution in terrestrial environments are poorly documented, and the few available results show both positive and negative relationships between concentrations of pollutants (i.e. metals) in bird host tissues and the presence or prevalence of various pathogens (Bichet et al., 2013; Provencher et al., 2014).

In this context, we studied 20 lesser horseshoe bat maternity colonies located in Franche-Comté. We first quantified landscape composition around roosts to appraise the relative importance of woodland compared to other landscape elements. Genetic differentiation between maternity colonies located all over the Franche-Comté region was assessed to investigate the hypothesis of a non-fragmented population. We measured the exposure of bat colonies to pesticides, trace metals, and to the parasite *E. hessei*, as well as their relationships. Taking advantage of recent developments in non-invasive approaches in wildlife studies, the bats were studied exclusively through sampling and analysis of approximately 900 dropping samples from 20 maternity roosts sampled in 2010 and 2011.

2. Material and methods

2.1. Dropping collection and storage

Faecal samples were collected from lesser horseshoe bat maternity roosts in Franche-Comté (Fig. 1). All roosts were located in buildings (churches, barns) in small villages. Eleven roosts were sampled in June 2010 and 18 in June–July 2011.

Fig. 1. Location of the 20 lesser horseshoe bat maternity roosts sampled in the Franche-Comté region.

In both sampling sessions, sampling was made when individuals were abundant within the roost and before the birth of offspring. Nine of the roosts sampled in 2010 were re-sampled in 2011. Hence, a total of 20 different maternity roosts were sampled. Plastic sheets were placed on the ground beneath the main clusters of bats and were left for 15 days. In each roost, droppings were randomly collected directly from the plastic sheets for parasitological analyses, placed individually in 2 mL microtubes containing 90° alcohol, and stored at room temperature. Using this methodology, 40 droppings were collected per roost in June 2010, and 24 droppings were collected per roost in June–July 2011. For pollutant analysis, a single pool of droppings (1.0–2.5 g, representing approximately 20–50 droppings) per colony was also collected from the plastic covers and stored at −20 °C until analysis.

2.2. Landscape characteristics

Landscape composition around maternity roosts was assessed to evaluate the importance of woodlands – considered as the main foraging areas – around the maternity roosts. A land cover map in a raster format was constructed by combining several land-cover databases. The forests, rivers, roads, motorways, railway lines (high-speed and standard) and buildings

were provided by the French land cover database (BD Topo IGN) with 1-m accuracy. Ponds were mapped from a specific database (BD Zones Humides DREAL). The agricultural census (BD Agreste 2010) was used to distinguish between grasslands and arable lands in open areas. Hedgerows, forest edges and forest core areas were dissociated by morphological spatial pattern analysis (MSPA) (Vogt et al., 2006). Finally, a landscape map composed of 13 classes was obtained. The spatial resolution was set at 10 m per grid cell to catch small elements, such as hedgerows. Landscape composition around each maternity roost was defined as the set of percentages p_i of each 13 land-cover class in a circular-shaped neighbourhood with a 2500 m radius around the roost, computed as follows: $p_i = \frac{a_i}{A}100$, where a_i is the area of the class i in the neighbourhood and A is the total of the neighbourhood. The neighbourhood size was chosen to reflect the mean distance of foraging of females from their maternity roost (Bontadina et al., 2002; Motte and Libois, 2002; Zahn et al., 2008; Farcy et al., 2009).

2.3. Pollutant concentrations in faecal samples

The exposure of lesser horseshoe bat colonies to chemical pollutants was measured for both metals and pesticides. Metals concentrations were measured at the Chrono-environnement department (Besançon, France). Organic compounds were analysed by the Veterinary Toxicology Laboratory (Biolytics), VetAgro Sup, Lyon (France).

In 2010, the concentrations of 3 metals (Cd, Pb, and Zn) were measured in pools of droppings by furnace (Cd, Pb) and flame (Zn) atomic absorption spectrometry (Varian 220Z and 220FS, respectively). In 2011, the concentrations of 16 elements (Al, As, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, Pb, Se, and Zn) were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES, ThermoFisher Scientific iCAP 6000). Pools of droppings were defrost and dried in an oven (60 °C) until constant mass. Digestion of samples was performed using dissolution in 0.7 to 1 mL of oxygen peroxide (H_2O_2, 35%, Carlo-Erba analytical quality) and 2 to 3 mL of nitric acid (HNO_3, 65%, Carlo-Erba analytical quality), depending on the dry mass of the sample. After digestion, samples were diluted adding 10 to 15 mL of ultra-pure water (18.2 MΩ/cm^2). Blanks (acid + ultra-pure water) and Certified Reference Materials (CRM, TORT-2 (lobster hepatopancreas) and DOLT-4 (dogfish liver), National Research Council, Canada) were prepared and analysed using the same methods than the samples. Average recoveries of the CRM are presented in Supplementary Material (Tables S1 and S2 for 2010 and 2011 analyses, respectively). When average recoveries for both DOLT and TORT ranged between 75% and 125%, the analyses were considered as acceptable. When they ranged between 50% and 75% or 125% and 150%, they were considered as under- or over-estimating, respectively. Otherwise, they were considered as not acceptable and were not used further in the statistical analyses.

PCBs (14 congeners), organochloride insecticides (OCs, 13 active substances or metabolites), anticholinesterase (AChEs) and organophosphorus (OPs) insecticides (18 active substances or metabolites), the molluscicide metaldehyde, anticoagulant (anti-vitamin K) rodenticides (AvKs, 10 active substances), and herbicides and fungicides (10 active substances and metabolites) were also measured in the faeces. The herbicides/fungicides, metaldehyde, OCs, and PCBs, were analysed by gas chromatography (Agilent technologies 6890N for herbicides/fungicides and metaldehyde, 6850 for OCs and PCBs), the AvKs by UV-high-pressure liquid chromatography (Hitachi – Science and Technology – LaChrom Elite HPLC System, L-2130 HTA pump, L-2450 diode Array detector/L-2480 fluorescence detector, L-2200 autosampler), and AChEs and OPs by both techniques. PCBs were measured in all samples, whereas the other pollutants were measured when the sample mass was sufficient. All metal and organic compound concentrations are expressed as micrograms per grams dry mass (μg/g dm). A complete list of the organic compounds analysed in the present work is provided in Supplementary Material.

2.4. Individual genotyping and molecular sexing

Bat DNA contained in droppings was used both for individual genotyping and molecular sexing. Genetic analyses were used to highlight and quantify a potential population fragmentation due to constraints to dispersal. DNA was extracted from the bat droppings using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions, the modifications recommended by Puechmaille et al. (2007), and the other modifications described below to improve DNA recovery. Each dropping was first placed individually in a 2 mL microtube. Droppings were then squashed with a sterile toothpick in 1.6 mL of lysis buffer (ASL) until the solution was homogenized. Samples were incubated overnight at 56 °C. An Inhibitex tablet was added to each sample, and the mixture was vortexed continuously until the tablet was completely suspended. The suspension was then incubated 1 min at room temperature. The mixture was centrifuged at 13,200 rpm for six min. Immediately after centrifugation, 600 μL of the supernatant was transferred into a new 2 mL microtube containing 25 μL of Proteinase K. A volume of 600 μL of buffer AL (a lysis buffer) was added to the mixture, and then the mixture was vortexed 15 s. The mixture was incubated 15 min at 70 °C. A volume of 600 μL of ethanol (96%–100%) was added to the lysate, which was briefly vortexed. 600 μL of this mixture was then applied in a QIAmp spin column placed in a 2 mL collection tube, and the tube was centrifuged 1 min at 7200 rpm. This step was repeated twice. A volume of 500 μL of AW1 buffer (wash buffer) was added in the QIAmp spin column placed in a new 2 mL collection tube, and the mixture was centrifuged 1 min at 10,000 rpm. A volume of 500 μL of AW2 buffer (wash buffer) was then added in the QIAmp spin column placed in a new 2 mL collection tube, and the mixture was centrifuged 1 min at 14,000 rpm. Finally, the QIAmp spin column was transferred in a 1.5 mL microtube and 80 μL of water at 60 °C was applied in the column. The suspension was then incubated for 5–10 min at room temperature and centrifuged at 14,000 rpm for 2 min. A second elution step was introduced to maximize the amount of DNA collected: 40 μL of water at 60 °C was added to the suspension, which was then incubated for 30 min at room temperature before a final centrifugation of 3 min at 14,000 rpm. Eluates were stored at −20 °C until DNA amplification.

Table 1
Microsatellites used for the genotyping of *R. hipposideros* from dropping samples: loci comprised in the three multiplex PCR with the fluoro-label used, primer concentration for multiplex PCR, and number of alleles.

Multiplex PCR mixture	Locus	Fluoro-label	Concentration in PCR mixture (μM)	Number of alleles
1	RHC108	FAM	0.28	6
	RHD2	NED	0.20	13
	RHD9	VIC	0.11	8
	RHD113	VIC	0.20	7
2	RHD103	NED	0.20	8
	RHD119	PET	0.10	5
3	RHD102	PET	0.40	11
	RHD111	NED	0.40	7

Because samples were collected without identification of the individual that excreted the droppings, each sample was genotyped to assign it to an individual. The eight microsatellite loci recommended by Puechmaille et al. (2005, 2007) were used to genotype the samples. DNA amplification was performed in three different multiplex reactions. Each reaction was performed in a mixture (14 μL) consisting of 3 μL of DNA extract, 1 x Multiplex PCR Master Mix (QIAGEN), and the primer concentrations reported in Table 1. The amplification programme consisted of an activation step of 15 min at 95 °C followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 45 s, and primer extension at 68 °C for 90 s. A final extension was performed for 30 min at 72 °C. Amplification reactions were performed using an Eppendorf Mastercycler DNA Engine. The PCR products were then diluted in 24 μL of RNAse-free water and stored at −18 °C. Allele size was quantified using an Applied Biosystems 3130 Genetic Analyzer in a reaction containing 1 μL of the diluted PCR product, 0.25 μL of an internal lane standard (500-LIZ; Applied Biosystems) and 9.75 μL of deionized formamide. Genetic profiles were acquired using the program Genemapper version 3.7 (Applied Biosystems).

Multilocus genotypes were determined using a comparative multiple-tubes approach, as recommended by Puechmaille and Petit (2007) to reduce genotyping errors. Samples exhibiting inconsistencies between replicates or samples for which consensus genotypes were not complete (i.e., comprising less than eight loci) were discarded from the analysis. Multiple comparisons between genetic profiles were performed using the allelmatch package (Galpern et al., 2012) in the R 2.14.0 software (R Development Core Team. R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2011. http://wwwR-project.org).

To determine the sex structure of the maternity colonies (*i.e.*, essentially females with a possible presence of isolated males), all individuals from the 2011 session were sexed using molecular typing (Bryja and Konecny, 2003). Part of the *Sry* gene was amplified by duplex PCR using two sets of primers. A 447/445-bp region of the *Zfy-Zfx* genes was amplified as a positive control of PCR using XP15EZ/XP23EZ primers (Aasen and Medrano, 1990), and a 202-bp fragment of the SRY-HMG box of the *Sry* gene located on the Y-chromosome was amplified for males using the SRYhmg-F/SRYhmg-R primers (Sánchez et al., 1996). PCR amplifications were conducted in a reaction mixture (12 μL) consisting of 5 μL of DNA extract, 1 x HotStarTaq Master Mix (Qiagen), and 0.3 μM of each primer and PCR-grade water. A negative control (PCR-grade water) was included for every 20 samples. The amplification cycling programme consisted of an activation step of 15 min at 95 °C followed by 32 cycles of denaturation at 93 °C for 60 s, annealing at 50 °C for 60 s, and primer extension at 72 °C for 60 s. A final extension was performed at 72 °C for 5 min. The PCR products were separated and visualized using the QIAxcel device (an automated capillary electrophoresis system produced by Qiagen), and a QIAxcel DNA high-resolution kit (Qiagen).

2.5. Faecal prevalence of E. hessei in lesser horseshoe bats

The faecal prevalence for *E. hessei* was measured by a molecular diagnosis using the DNA extracted from droppings. The amplification of *E. hessei* was performed with the generic PCR primers F1E (5′- TACCCAATGAAAACAGTTT - 3′) and R2B (5′- CAGGAGAAGCCAAGGTAGG - 3′), targeting the 18S rRNA gene (Orlandi et al., 2003). The PCR amplifications were conducted in a reaction mixture (25 μL) consisting of 2 μL of DNA extract, 1 x HotStarTaq Master Mix (Qiagen), 0.2 μM of each primer, and PCR-grade water. A negative control (PCR-grade water) was included for every 20 samples. The amplification cycling programme consisted of an activation step of 15 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and primer extension at 74 °C for 90 s. A final extension was performed at 72 °C for 10 min. The PCR products were separated and visualized using the QIAxcel device and a QIAxcel DNA high-resolution kit (Qiagen). Each sample was amplified in duplicate and ten samples with discordant results between two PCR were re-amplified twice to ensure a reliable diagnosis. In a previous study, PCR products from positive samples were sequenced and *E. hessei* was the only species identified (Afonso et al., 2014).

With the genotyping analyses, each sample was assigned to one individual. Among the 707 samples genotyped, 348 were unique and 359 had a genotype present at least twice in the dataset. Thirty-two of the 101 individuals with at least two tests for *E. hessei* had discordant diagnostics during the sampling period (15 days). Faecal prevalence was then estimated by re-sampling to take into account that individuals could have both positive and negative PCR results. To estimate the prevalence of *E. hessei* in each bat colony, the number of individuals positive for the parasite was divided by the total number of individuals sampled in the maternity roost. One sample was randomly selected for each individual, and the faecal

prevalence was calculated. This procedure was repeated 100 times to estimate a mean faecal prevalence per maternity roost, *i.e.*, the mean number of individuals positive for *E. hessei* among the 100 repetitions divided by the number of individuals sampled in the maternity roost.

2.6. Statistical methods

2.6.1. Colony size

Most often, bat colony size is difficult to readily assess by visual counting within maternity roosts. The number of bats in a roost varies daily and individuals are difficult to count when they are located in inaccessible places or when they are flying within the roost. The size of each colony was thus estimated via statistical methods, using the sequential Bayesian estimator method developed by Petit and Valiere (2006) and implemented in the R software using a script provided by Eric Petit (University Rennes 1).

2.6.2. Genetic diversity within colonies and differentiation among colonies

Genetic diversity within colonies was assessed by estimating the allelic richness per colony using a rarefaction procedure corrected by the number of individuals sampled per colony. Genetic variation was estimated over all loci within each colony by assessing the observed H_O and expected H_E heterozygosity. Deviations from the Hardy–Weinberg equilibrium (HWE) and from linkage equilibrium performed for each locus within each colony were estimated using the exact test based on a Monte Carlo Markov chain method (MCMC). All these calculations were performed using the R package hierfstat (Goudet, 2005). The genetic differentiation between colonies was quantified by computing the Weir and Cockerham (1984) estimator F_{ST}. Because Verity and Nichols (2014) showed that Weir and Cockerham estimator may be insufficient on its own to measure genetic differentiation, we also assessed the differentiation index D (Jost, 2008). F_{ST} was computed with the R package hierfstat, while D was assessed with the R package DEMEtics (Gerlach et al., 2010).

Finally, the software STRUCTURE 2.3 (Pritchard et al., 2000) was used to detect the potential genetic structure and assign individuals to inferred clusters based on multilocus genotypes. Twenty independent runs of $K = 1$–10 were performed at 1,000,000 MCMC repetitions with a 100,000 burn-in period using no prior information and assuming correlated allele frequencies and admixture. K was identified using the maximum values of $\ln P(D)$ (the log probability of the data for a given K) returned by the software.

2.6.3. Statistical tests

The number of individuals sampled in each colony was compared between the two sampling sessions using a Fisher-test. The paired Wilcoxon signed-rank test was used to test for significant differences in metal concentrations (Cd, Pb, and Zn) between the two sampling sessions. The association between metals was assessed using a principal component analysis (PCA). Chi-squared test was used to compare faecal prevalence of *E. hessei* between sampling sessions. The Fisher's exact test for count data was used when assumptions of the Chi-squared tests were not met. Spearman rank correlation test was used to investigate relationships of faecal prevalence for *E. hessei* and pollutant concentrations.

3. Results

3.1. Habitat description and sample characteristics

All 20 sampled maternity roosts were located in buildings surrounded mainly by woodland and shrublands (on average 47.7% of the surface surrounding roosts, min–max: 23.8%–75.5%), grasslands (29.1%, min–max: 10.7%–54.3%) and arable lands (14.7%, min–max: 3.6%–41.7%). Hedgerows, urban and water areas represented a low proportion of the surface surrounding the roosts (3.7%, 3.6% and 1.2% on average, respectively).

A total of 440 and 432 samples were collected in 2010 and 2011, respectively: 234 individuals were sampled in 11 maternity roosts in 2010, and 232 individuals were sampled in 18 maternity roosts in 2011. Among these individuals, 17 were sampled in both sessions. Thus, a total of 449 distinct individuals were sampled in the two combined sessions. On average, 15.5 individuals were sampled per roost in both sessions, and the number of individuals sampled differed significantly between the two sessions (Fisher-test, $F = 45.95$, $df = 1$ and 27, $P < 0.001$): an average of 21.3 individuals were sampled per roost in 2010 (when 40 droppings were collected per roost), while 11.9 individuals were sampled per roost in 2011 (24 droppings were collected per roost). With colonies comprising from 16 to 226 individuals (according to statistical estimations), a variable part of the colony was sampled in each roost (the number of individuals sampled and the estimation of colony size are given Table 2).

The 17 individuals sampled in the two sessions were all re-sampled in the same maternity roost; among them, the 12 individuals for which sex was known were females. Among the 232 individuals sampled in 2011, 143 were females, and 2 were males (87 were undetermined due to uninterpretable results).

3.2. Genetic diversity of colonies

All 8 loci analysed with microsatellite markers were polymorphic with 5–13 alleles per locus and an average of 5.00 alleles per locus per colony (Table 1), and no linkage disequilibrium was detected between locus pairs across populations. Global

Table 2
Genetic characteristics of the samples collected in 20 lesser horseshoe bat maternity roosts during June 2010 and June–July 2011. Maternity roost: name of the village hosting the maternity roost; Samples: number of samples collected in each maternity roost; Genotypes: number of reliable genotypes; Individuals: number of distinct individuals; Effective size: estimated number of individuals in the colony based on a sequential Bayesian estimator; Allelic richness: allelic richness corrected for sample size; H_O: observed heterozygosities; H_E: unbiased estimates of expected heterozygosities; HWE: probabilities associated with the rejection of the Hardy–Weinberg equilibrium.

Maternity roost	Sampling session	Samples	Genotypes	Individuals	Effective size	Allelic richness	H_O	H_E	HWE
Alaise	2010	–	–	–	–	–	–	–	–
	2011	24	22	20	226	5.03	0.80	0.78	0.411
Andelot-Morval	2010	–	–	–	–	–	–	–	–
	2011	24	23	11	16	4.60	0.78	0.73	0.376
Aresches	2010	40	35	18	24	5.74	0.79	0.76	0.361
	2011	24	15	10	28	4.48	0.77	0.75	0.721
Bartherans	2010	–	–	–	–	–	–	–	–
	2011	24	20	12	24	3.96	0.73	0.71	0.781
Belleherbe	2010	40	38	20	28	5.63	0.79	0.75	0.563
	2011	24	19	14	49	4.76	0.78	0.74	0.427
Barretaine	2010	–	–	–	–	–	–	–	–
	2011	24	11	10	209	4.53	0.77	0.73	0.137
Châtillon-le-Duc	2010	–	–	–	–	–	–	–	–
	2011	24	17	9	16	4.59	0.70	0.72	0.728
Charmes-Saint-Valbert	2010	40	38	27	56	5.70	0.76	0.76	0.903
	2011	24	20	12	24	4.85	0.80	0.77	0.625
Cussey-sur-Lison	2010	–	–	–	–	–	–	–	–
	2011	24	22	14	30	4.80	0.76	0.74	0.624
Crouzet-Migette	2010	40	36	32	114	5.68	0.73	0.75	0.565
	2011	24	21	11	18	5.01	0.78	0.78	0.946
Chevigny	2010	40	32	15	18	4.32	0.63	0.67	0.482
	2011	24	15	9	20	3.88	0.68	0.68	0.961
Échenoz-la-Méline	2010	–	–	–	–	–	–	–	–
	2011	24	12	10	118	4.88	0.71	0.76	0.601
Epenouse	2010	–	–	–	–	–	–	–	–
	2011	24	21	13	27	4.68	0.79	0.75	0.352
Fouvent-le-Bas	2010	–	–	–	–	–	–	–	–
	2011	24	14	10	38	4.49	0.71	0.71	0.960
Fourg	2010	40	39	20	28	5.71	0.76	0.73	0.281
	2011	24	17	13	59	5.05	0.73	0.75	0.767
Laval-le-Prieuré	2010	40	32	21	31	5.78	0.72	0.78	0.251
	2011	24	22	15	27	4.89	0.79	0.76	0.642
Offlanges	2010	40	28	22	34	5.26	0.73	0.72	0.954
	2011	24	18	10	18	4.29	0.68	0.73	0.332
Les Planches-près-Arbois	2010	40	34	18	24	5.79	0.69	0.76	0.206
	2011	–	–	–	–	–	–	–	–
Pretin	2010	40	34	18	24	5.69	0.75	0.73	0.721
	2011	–	–	–	–	–	–	–	–
Randevillers	2010	40	36	23	37	5.96	0.82	0.77	0.223
	2011	24	16	12	51	5.04	0.74	0.75	0.853
Total		872	707	449	1416	–	-	–	-
Average (± Standard Error)		30.07 (± 2.88)	24.38 (± 3.25)	15.48 (± 2.11)	48.33 (± 19.28)	5.00 (± 0.21)	–	–	–

tests by locus and colony showed no deviation from the HWE (Table 2). The overall F_{ST} and D values were low for both sessions ($F_{ST} = 0.023$ and $D = 0.076$ in 2010, $F_{ST} = 0.028$ and $D = 0.097$ in 2011), indicating a low genetic differentiation among the sampled colonies. Pairwise F_{ST}values between the colonies were low to moderate, ranging from 0.003 (F_{ST} between Alaise and Crouzet-Migette) to 0.082 (F_{ST} between Châtillon-Le-Duc and Chevigny colonies). The 20 independent runs of the STRUCTURE software showed that ln $P(D)$ was maximized when $K = 1$ (-11142.27) and decreased when $K > 1$. The Bayesian clustering method thus failed to identify genetic clusters among the 20 sampled colonies.

3.3. Pollutant concentrations

Among the organic compounds tested in the guano samples, only organochlorines were detected. Endosulfan alpha was detected in one colony (Châtillon-Le-Duc, 0.035 μg/g dry mass), whereas lindane was detected in seven colonies (Barretaine:

Table 3
Trace metals concentrations (μg/g dry mass) for each lesser horseshoe bat colony sampled. Data for Cd, Pb, and Zn are mean concentrations of the two sampling sessions (except for Les Planches-près-Arbois and Pretin, where sampling occurred only in 2010) for each of these three trace metals, whereas the other trace metals concentration were measured only in 2011. UDL: under detection limit, OVER: above detection limits, NM: not measured.

Maternity roost	Al	As	Ca	Cd	Cr	Cu	Fe	Hg	K	Mg	Mn	Na	Ni	Pb	Se	Zn
Alaise	18.53	3.68	1057.54	0.86	UDL	54.71	160.14	UDL	6627.72	1485.84	111.75	790.60	0.37	0.47	2.86	301.45
Andelot-Morval	25.75	3.07	OVER	1.42	0.13	68.98	214.50	UDL	5683.06	1491.45	72.35	1343.10	0.50	1.12	1.93	381.07
Aresches	28.99	4.11	OVER	1.21	0.12	68.03	230.47	UDL	6805.52	1717.60	71.92	1005.24	0.67	0.58	2.60	392.85
Bartherans	126.83	3.36	OVER	1.71	0.47	51.12	387.12	UDL	6876.20	1335.89	70.97	757.94	0.69	2.76	1.58	269.57
Belleherbe	31.66	3.85	1076.17	0.83	0.14	57.88	203.91	UDL	6447.73	1175.05	45.63	1103.01	0.57	0.59	2.27	199.40
Barretaine	41.83	1.90	OVER	1.80	0.25	64.15	262.75	UDL	6165.64	1435.45	77.62	1026.82	0.68	1.68	3.27	291.72
Châtillon-le-Duc	74.42	12.14	OVER	0.94	0.47	54.40	360.17	UDL	8880.71	2084.83	145.75	1329.53	0.51	2.91	2.35	311.31
Charmes-Saint-Valbert	789.94	4.93	OVER	0.43	2.14	44.38	1491.97	UDL	7820.95	2312.54	131.34	1044.15	1.50	1.97	2.64	306.26
Cussey-sur-Lison	35.02	4.38	OVER	1.17	0.19	53.76	196.07	UDL	10921.72	1530.20	90.04	3429.55	0.56	1.24	2.91	268.99
Crouzet-Migette	9.96	3.65	OVER	2.96	0.05	80.79	215.54	1.09	4965.76	1571.98	57.34	1110.68	0.49	13.27	1.96	667.29
Chevigny	6.74	2.70	OVER	0.38	UDL	42.48	119.90	UDL	6531.61	1279.54	63.31	1109.44	0.40	1.41	1.41	271.76
Échenoz-la-Méline	12.25	3.62	OVER	1.28	0.04	42.96	118.37	UDL	8019.03	1368.47	47.53	672.77	0.37	0.59	2.47	227.60
Epenouse	14.20	1.34	627.76	3.61	UDL	66.21	160.15	UDL	5589.58	1197.28	47.24	724.86	0.45	0.41	2.55	341.05
Fouvent-le-Bas	108.20	9.08	OVER	0.67	0.46	40.93	300.04	UDL	9803.27	1466.01	63.06	1012.48	0.68	2.19	2.51	230.38
Fourg	41.13	4.74	1277.77	1.06	0.11	62.89	243.04	UDL	7364.61	1708.22	81.19	523.64	0.63	12.48	2.46	296.20
Laval-le-Prieuré	65.54	3.32	OVER	2.80	0.06	55.37	240.76	UDL	6550.84	1480.68	64.85	1029.97	0.77	2.24	2.40	437.68
Offlanges	63.85	3.37	OVER	0.35	0.04	91.01	225.63	UDL	7164.52	1577.19	100.42	964.44	0.48	1.03	2.36	316.48
Les Planches-près-Arbois	NM	NM	NM	0.92	NM	NM	NM	NM	NM	NM	NM	NM	NM	1.97	NM	494.34
Pretin	NM	NM	NM	3.89	NM	NM	NM	NM	NM	NM	NM	NM	NM	4.72	NM	499.53
Randevillers	22.57	1.25	939.16	1.76	0.29	59.92	504.22	0.25	6253.87	1272.91	42.37	1117.48	0.79	5.86	2.73	303.73
Median (Min-Max)	33.34 (6.74–789.94)	3.64 (1.25–12.14)	1057.54 (627.76–1277.77)	1.19 (0.35–3.89)	0.14 (0.04–2.14)	56.63 (40.93–91.01)	228.05 (118.37–1491.97)	0.67 (0.25–1.09)	6716.62 (4965.76–10921.72)	1483.26 (1175.05–2312.54)	71.45 (42.37–145.75)	1028.40 (523.64–3429.55)	0.57 (0.37–1.50)	1.83 (0.41–13.27)	2.47 (1.41–3.27)	305.00 (199.40–667.29)

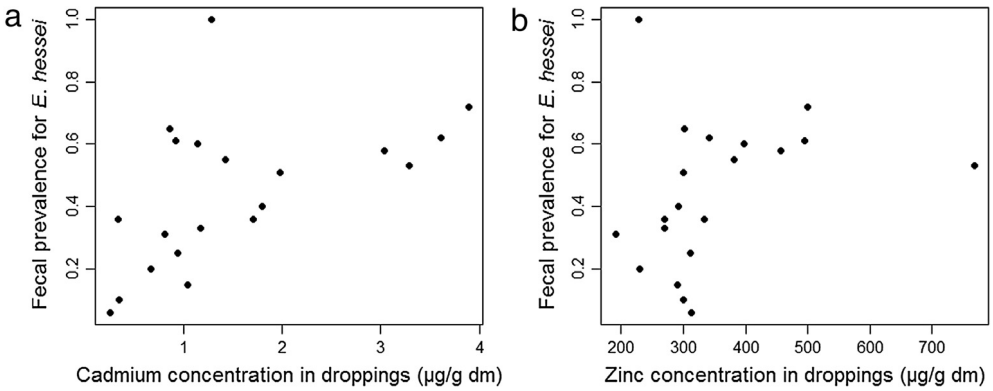

Fig. 2. Prevalence of *Eimeria hessei* in lesser horseshoe bats and Cd (a) and Zn (b) concentrations in each maternity roost.

0.02 μg/g dm; Charmes-Saint-Valbert: 0.013 μg/g dm; Chevigny: 0.03 μg/g dm; Laval-le-Prieuré: 0.05 μg/g dm; Offlanges: 0.112 μg/g dm; Pretin: 0.04 μg/g dm; Randevillers: 0.035 μg/g dm). The metal concentrations measured in the bat guano are presented in Table 3 and in Figure S1, with metrological information given in Tables S1 and S2. Fourteen elements were considered metrologically satisfactory even if average recoveries from Certified Reference Materials indicated that concentrations of Cd, Pb, and Zn in 2010, and Ca, Fe, K, Mg, Mn, Na, Ni, and Zn, in 2011 may have been slightly underestimated (overestimated for Se in 2011). Ca concentrations were almost always above detection limits whereas only one Hg concentration could have been measured, the rest being under detection limits. The concentrations did not differ between the two sampling sessions for the sites sampled both in 2010 and in 2011 for Cd (Wilcoxon signed rank test, 9 matched observations, $z = 0.18$, $P = 0.910$), Pb (Wilcoxon signed rank test, 9 matched observations, $z = 0.53$, $P = 0.652$) and Zn (Wilcoxon signed rank test, 9 matched observations, $z = 0.89$, $P = 0.426$). Therefore, the data from 2010 and 2011 for Cd, Pb, and Zn were pooled for further analyses. Charmes-Saint-Valbert was the colony for which high concentrations of Al, Cr, Fe, Ni, and Mg were found, compared to the other colonies. Concentrations of As and Mg were also high in Châtillon-le-Duc, Pb and Zn in Crouzet-Migette, As in Fouvent-le-Bas, Pb in Fourg, and Na in Cussey-sur-Lison. A PCA (Figure S2) showed that Cr, Fe, Ni, and Mg and Cd, Cu, Pb, and Zn were correlated. Taken together, these results suggest homogeneous exposure to metals among colonies (except for the colony of Charmes-Saint-Valbert that exhibited high concentrations of 5 elements).

3.4. Faecal prevalence of E. hessei and relationships to pollutant concentrations

The overall faecal prevalence of *E. hessei* was 0.42 (185/438) and did not differ between the two sampling sessions ($\chi^2 = 0.47$, $P = 0.494$): 0.42 (98/234) in 2010 and 0.38 (87/227) in 2011. The faecal prevalence did not differ significantly between the two sessions in all 9 maternity roosts collected both in 2010 and in 2011 (Fisher's exact test for count data, $0.164 < P < 1$).

Among the 17 individuals sampled in both sessions, 7 were positive for *E. hessei* in 2010, 5 were positive in 2011, and 2 were positive in both sessions. The faecal prevalence did not differ between these 17 individuals between 2010 (9/17 individuals positive for *E. hessei*) and 2011 (7/17; $\chi^2 = 0.12$, $df = 1$, $P = 0.731$).

The faecal prevalence was highly variable depending on the maternity roost, with values between 0.00 (at Chevigny in 2010) and 0.80 (at Aresches in 2011) (Table 4). The faecal prevalence was not related to the effective size of the colony estimated by a sequential Bayesian estimator, neither in 2010 (Spearman, $r_s = 0.07$, $P = 0.840$) nor in 2011 ($r_s = 0.26$, $P = 0.303$).

The faecal prevalence of *E. hessei* was not related to lindane concentrations in droppings (Spearman, $r_s = -0.39$, $P = 0.114$). Faecal prevalence, however, was related to the Cd and Zn concentrations in droppings but not to other metals (Spearman, $0.082 < P < 0.782$). The prevalence increased with increasing Cd concentration ($r_s = 0.59$, $P = 0.006$) and was always >50% when the Cd concentrations in the droppings were between 2 and 4 μg/g (Fig. 2). The faecal prevalence was also positively related to Zn concentration ($r_s = 0.48$, $P = 0.034$), and was always > 50% when the Zn concentrations in the droppings were between 400 and 800 μg/g (Fig. 2).

4. Discussion

This study shows that several indicators of stress that can affect bat populations, such as potential population fragmentation (indirectly assessed by genetic differentiation), exposure to pollutants, and parasite faecal prevalence, can be assessed based on less disturbing and non-invasive methods (dropping collection, and molecular and chemical analysis tools), without resorting to capturing individuals. This alternative to tissue sampling has been developed to study species

Table 4
Faecal prevalence for *E. hessei* in 20 lesser horseshoe bat maternity roosts sampled in June 2010 and June–July 2011. Number of diagnostics: number of individuals with a reliable test for *E. hessei*; Positive: mean number of individuals positive for *E. hessei* after 100 re-sampling tests; Faecal prevalence: faecal prevalence for *E. hessei* (=Positive/Number of diagnostics); 95%CI: 95% confidence interval of the faecal prevalence for *E. hessei*.

Maternity roost	Sampling session	Number of diagnostics	Positive	Faecal prevalence	95%CI
Alaise	2010	–	–	–	–
	2011	20	13	0.65	[0.43; 0.82]
Andelot-Morval	2010	–	–	–	–
	2011	11	6	0.55	[0.28; 0.79]
Aresches	2010	18	9	0.50	[0.29; 0.71]
	2011	10	8	0.80	[0.49; 0.94]
Bartherans	2010	–	–	–	–
	2011	12	4	0.33	[0.14; 0.61]
Belleherbe	2010	20	6	0.30	[0.18; 0.49]
	2011	11	4	0.36	[0.15; 0.65]
Barretaine	2010	–	–	–	–
	2011	9	4	0.44	[0.19; 0.73]
Châtillon-le-Duc	2010	–	–	–	–
	2011	6	2	0.33	[0.10; 0.70]
Charmes-Saint-Valbert	2010	27	10	0.37	[0.22; 0.56]
	2011	12	4	0.33	[0.14; 0.61]
Cussey-sur-Lison	2010	–	–	–	–
	2011	13	5	0.38	[0.18; 0.64]
Crouzet-Migette	2010	32	18	0.56	[0.39; 0.72]
	2011	11	5	0.45	[0.21; 0.72]
Chevigny	2010	15	0	0.00	[0.00; 0.20]
	2011	9	2	0.22	[0.06; 0.55]
Échenoz-la-Méline	2010	–	–	–	–
	2011	7	5	0.71	[0.36; 0.92]
Epenouse	2010	–	–	–	–
	2011	13	8	0.62	[0.36; 0.82]
Fouvent-le-Bas	2010	–	–	–	–
	2011	10	2	0.20	[0.06; 0.51]
Fourg	2010	20	4	0.20	[0.08; 0.42]
	2011	13	1	0.08	[0.00; 0.33]
Laval-le-Prieuré	2010	21	12	0.57	[0.37; 0.76]
	2011	15	9	0.60	[0.36; 0.80]
Offlanges	2010	22	1	0.05	[0.00; 0.22]
	2011	10	1	0.10	[0.01; 0.40]
Les Planches-près-Arbois	2010	18	11	0.61	[0.39; 0.8]
	2011	–	–	–	–
Pretin	2010	18	13	0.72	[0.49; 0.88]
	2011	–	–	–	–
Randevillers	2010	23	14	0.61	[0.41; 0.78]
	2011	12	4	0.33	[0.14; 0.61]
Total		438	185	0.42	[0.38; 0.47]

sensitive to disturbance and/or difficult to capture (Taberlet et al., 1999; Puechmaille and Petit, 2007) and was particularly useful here because the bats were sometimes inaccessible with respect to capture and handling. Individual genotyping and a Bayesian statistical method were used to estimate bat colony size. The maternity colonies sampled in this study comprised 16–226 individuals, depending on the colony. These values are consistent with sizes reported in previous studies by visual counting (10–245 individuals; Bontadina et al., 2002; Holzhaider et al., 2002; Reiter, 2004) and suggest that non-invasive approaches can be used to estimate colony size with confidence, as already shown by Puechmaille and Petit (2007). This sampling method also has the advantage of facilitating the large scale of the sampling plan, with 20 maternity roosts sampled across the Franche-Comté region (an area of approximately 16,202 km^2), and a total of 449 individuals sampled over two consecutive field sessions.

Landscape composition around maternity roosts was mainly predominated by woodlands and shrublands (47.7% of the surface surrounding roosts, on average), while hedgerows represented 3.7% of the surrounding areas. These results are consistent with previous studies (Schofield, 1996; Bontadina et al., 2002; Reiter, 2004; Boughey et al., 2011). In terms of the ecological requirements of the lesser horseshoe bat, the Franche-Comté region is amenable to the presence of this species. Numerous old farm buildings offer numerous potential roosting sites. The region is mostly covered by forests (forests cover 42% of the study area; Tournant et al., 2013), and its specific karstic geologic context provides abundant caves for winter roosts. When woodland cover is extensive enough, the lesser horseshoe bat is expected to be able to avoid open areas to

realize daily or inter-seasonal movements (Holzhaider et al., 2002; Tournant et al., 2013). This suggests that gene flow can occur at a wide geographical range. By using the F_{ST} fixation index, the differentiation index D and a Bayesian clustering method, this study showed that colonies exhibited a low genetic differentiation and were likely not divided into genetic clusters across the study area. These results reinforce the assumption that no obvious physical barrier acts as a constraint with respect to gene flow. Non-migratory bat species are expected to show genetic partitioning among populations, due to female fidelity to their natal colony and short-distance movements for mating and dispersal (Moussy et al., 2013). However, both a good genetic diversity and a low differentiation among lesser horseshoe bat colonies evoke that unknown mechanisms allow gene flow, like inbreeding avoidance during mating. In other non-migratory bat species, inter-colony gene flow may occur mainly via mating rather than by permanent immigration of individuals between colonies, as observed in the greater horseshoe bat (*Rhinolophus ferrumequinum;* Rossiter et al., 2000). Female bats gather with males at unknown locations for breeding before reaching the winter roost. Mating probably involves females and unrelated males; this swarming behaviour thus promotes genetic diversity and the low differentiation observed between geographically close colonies, as suggested for Natterer's bat (*Myotis nattereri*; Rivers et al., 2006) and Daubenton's bat (*Myotis daubentonii*; Atterby et al., 2009). The low genetic differentiation between maternity colonies and the presence of a winter roost in the centre of the Franche-Comté region that hosts a quarter of the total lesser horseshoe bats of the area (CPEPESC Franche-Comté, unpublished data) supports the assumption that gene flow may occur via mating among unrelated individuals.

Faecal sampling allowed the re-sampling of 17 individuals over the two field sessions, conducted one year apart. All these individuals were re-sampled in the same roost in both years, and all the sexed individuals were females, which may indicate female philopatry. Female philopatry is well known in bats (Lewis, 1995) but was never formally reported in the lesser horseshoe bat, to our knowledge. These results are in accordance with studies conducted on lesser horseshoe bat movements showing that this species appears to exhibit poor natal dispersal ability. As the few numbers of individuals re-sampled likely reflect a low sampling frequency, female dispersal behaviour would benefit from being studied with greater and constant sampling effort, especially in roosts with many individuals. The non-invasive approach developed in this study may provide a good alternative to capture–mark–recapture studies in this way.

Despite the ban of its use in 1998 in France, residues of lindane (a substance used in wood preservation) were detected in the bat guano from seven colonies. Residues of pesticides, even those banned for decades, may persist in the environment, and this substance in particular has been reported regularly in various bat tissues many years after its restriction in other countries (Shore et al., 1990; Bayat et al., 2014). The bats sampled in this study roosted mainly under the roofs of old buildings (churches, barns, and residential houses) and may have been in contact with beams treated with lindane. The lindane concentration values measured in the present study (0.013–0.112 ppm) are comparable to those found in bat guano in previous studies (see Clark and Shore, 2001 for a review). Like other organochlorine pesticides, lindane may be transferred from mother to offspring before birth via movement through the placenta or after birth via breastfeeding (Clark and Shore, 2001). Exposure to residues of persistent organochlorine pesticides can manifest in acute and lethal impacts, as well as sub-lethal and chronic effects, such as immune suppression or increased metabolism (Clark and Shore, 2001). To our knowledge, there are no toxicological reference values for lindane in the guano of bats. Lindane is mostly excreted via urine and very little is excreted via faeces (ATSDR, 2005). Therefore, even a low concentration in guano may correspond to a relatively high exposure, an issue that would need further investigation.

Bats sampled in the present study were also exposed to various metals, with concentrations lower than or of the same order of magnitude as those reported in other studies of bat guano (Clark and Shore, 2001; Zukal et al., 2015), even if one should keep in mind that the concentrations of some elements may have been underestimated in the present work. Metals occur naturally in the environment (in soil, water, and living organisms) at background concentrations, but anthropogenic activities can drastically increase their concentrations (Kabata-Pendias, 2010). Non-essential metals play no (known) physiological role in living organisms and can be toxic at low concentrations although essential elements play a crucial role in many biological functions of the organisms but can be toxic at elevated concentrations. In the absence of baseline metal concentrations in guano with respect to lethal or sub-lethal effects, it is difficult to estimate the risk of adverse effects for maternity colonies based on the concentrations measured in the present study. It is even very difficult to extrapolate metal concentrations in internal tissues from the concentrations in the guano because, to the best of our knowledge, no studies relating concentrations in tissues and the guano are available for bats. However, Zukal et al. (2015) reported that metal concentrations in guano are generally higher than in tissues. Therefore, since the concentrations measured in the present study were always lower than or of the same order of magnitude as those reported in other studies of bat guano, our data suggest chronic exposure to metals rather than acute contamination. This is in agreement with the fact that, among the 20 colonies sampled in the present study, only 2 (Belleherbe and Echenoz-la-Méline) contain polluted sites identified by BASOL, the French national stocklist for polluted sites (http://basol.developpement-durable.gouv.fr/), in a 5-km radius buffer around the centroid of the villages where colonies are located. In these 2 cases, the pollutants identified are hydrocarbons, cyanide, and fungicides (that have not been looked for in the present work). The interpretation of metal concentrations in guano for toxicological purposes would need further research on (i) relationships between concentrations in internal tissues such as liver or kidneys (for which toxicological reference values exist, at least for rodents) and concentrations in the guano (for instance on dead animals, using the droppings present in the intestinal tract), or, even better, on (ii) studies allowing the development of toxicokinetic models as they exist for human or rodents. This would be extremely valuable to improve the usefulness of guano as non-invasive material for toxicological purposes because many chemicals, whatever the exposure route (inhalation, ingestion, dermal contact), are primarily excreted via faeces.

This study is also the first epidemiological study of an endoparasite conducted on the lesser horseshoe bat. We measured the faecal prevalence of the protozoan *E. hessei*, which could reflect a first excretion period of oocysts after primary infection, or a re-excretion period, as suggested by two individuals positive for the parasite in both sampling sessions. The faecal prevalence of *E. hessei* was highly variable amongst the colonies (0%–80%) and did not seem to depend on the size of the colony. This result may indicate that the environmental contamination by *E. hessei* oocysts within the roosts is not the main source of contamination for bats. Duszynski (2002) hypothesized that *Eimeria* species of bats might utilize invertebrate intermediate or transport hosts. That may explain how bats feeding primarily on insects in flight become infected with oocysts that previously were deposited via the faeces of another conspecific. Except a few exceptions, prevalence of *Eimeria* species in bats reported in the literature appear to be moderate, with on average ~10% of infected individuals (see a review in Duszynski, 2002). The high faecal prevalence found in this study shows the need to better understand the life-cycle and pathogenicity of these parasites. Although nothing is known on *E. hessei* pathogenicity, it cannot be excluded that this parasite may induce clinical signs in lesser horseshoe bats.

E. hessei faecal prevalence was positively related to Cd and Zn concentrations, two of the metals detected in the guano. The study of the relationships between chemical pollutants and pathogenic organisms is a recent research topic that has been investigated with respect to different host species, chemical substances, and pathogenic organisms. Although only a few studies have focused on non-aquatic or semi-aquatic animal species, most have concluded that pollutants might increase the parasite burden or parasitic prevalence in hosts. For example, Tersago et al. (2004) found a positive relationship between the nematode burden in the small intestine of wood mice (*Apodemus sylvaticus*) and Cd concentrations in the liver. Similarly, Halbrook et al. (1993) showed that the prevalence of *Taenia taeniaeformis* in muskrats (*Ondatra zibethicus*) was higher in animals inhabiting a site contaminated by a mixture of pollutants than in animals from a non-contaminated site. One of the main hypotheses explaining the relationship between pollutants and parasites is that elevated exposure to contaminants may disrupt certain components of the immune system of individuals, rendering them more susceptible to infection by pathogenic organisms. In the present case, *E. hessei* faecal prevalence was related to the concentrations of both non-essential Cd and essential Zn. Franche-Comté is a region dominated by calcareous soils that are naturally rich in Cd and Zn (Gis Sol, 2011). It is not unlikely that Cd exposure in the present case, even if it can be considered relatively low compared to literature data on metal concentrations in guano, may cause a decrease in some components of the immune system, thereby increasing the susceptibility of the lesser horseshoe bat to *E. hessei*. Zn, whose physico-chemical properties are relatively similar to those of Cd (Kabata-Pendias, 2010), may have been transferred to the bats via the same pathways as Cd and may therefore be coincidentally associated to *E. hessei* prevalence. There is, however, no data in the present work arguing for a causal relationship between Cd or Zn concentrations and the prevalence of *E. hessei*. From a general point of view, the various mechanisms underlying the relationships between chemicals and pathogens in terrestrial wildlife are still unknown. They may involve both environmental processes, e.g., chemical-driven increases in pathogen diversity and density, and immunological disturbances, which render individuals more susceptible to pathogens and parasites (see for instance Rohr et al., 2008). Such a correlation between pollutants and pathogens argues for a more systemic approach of the ecology of bat species, and more generally wildlife species, to better ensure their conservation.

5. Conclusions

The present study, by sampling 20 maternity roosts of the lesser horseshoe bat and 449 individuals over two sampling sessions, brings important insights on the lesser horseshoe bat ecology, and its exposure to stressors thought to be demographic disruptors. Generally considered as one of the main threats to bat populations when woodland is low extensive and/or fragmented, landscape appeared here to be favourable to lesser horseshoe bat populations, with woodlands predominating habitats around roosts and a low genetic differentiation between all maternity colonies over the study area. We thus conclude that individuals sampled belong to a unique population not fragmented by obvious dispersal barriers. Even if exposure of bats to chemical pollutants (assessed by concentrations in guano) is difficult to link to adverse effects due to the lack of toxicological references, residues of lindane, a pesticide banned of its use in France years ago, was detected in 7 over the 20 bat colonies. This result raises questions about the potential effects of this pesticide that persists in the environment or is used illegally, and therefore still constitutes a source of contamination and a potential threat for bats. Concentrations of metals measured in bat colonies suggest chronic exposure rather than an acute contamination. However, the concentrations of two metals (Cd and Zn) were positively related to the faecal prevalence of the protozoan parasite *E. hessei*, which reached very high values. Although the lesser horseshoe bat population is increasing in the Franche-Comté region, our results indicate that attention should focus on pollutant and pathogen interactions, which are still largely unknown in terrestrial ecosystems. It would be relevant to investigate if the exposure to a mixture of chemical pollutants, even at low concentrations, can induce adverse effects on bats, and/or increase their susceptibility to infectious diseases. Pathogenic organisms are still largely unstudied in bats, and if we focussed only on a protozoan parasite, many others probably occur in bat populations with an unknown pathogenicity. Our study stresses out the importance of systemic approaches in the study of ecology of wildlife species.

Acknowledgements

The authors thank Sébastien Puechmaille and Fabrice Poncet for their helpful comments during the molecular analysis processes, Nadia Crini and Caroline Amiot for the analysis of trace metals, Jean-François Cosson and Karine Berthier for their advice about statistical analysis, and Eric Petit for providing a R script for the estimation of bat colony size. The authors sincerely thank Richard Shore and two anonymous referees for their useful comments on the previous versions of the manuscript. The study sites in Franche-Comté belong partly to the Long Term Ecological Research (LTER) Network "Zone Atelier Arc Jurassien" (http://zaaj.univ-fcomte.fr/). This work was supported by the Région Franche-Comté, the Fondation pour la Recherche sur la Biodiversité (FRB), the Observatory of Earth Sciences "Terre Homme Environnement Temps Astronomie" (OSU THETA, Bourgogne-Franche-Comté), and the French Ministry of Ecology, Energy, Sustainable Development, and the Sea (ITTECOP program) as part of the Graphab project managed by the USR 3124 MSHE Ledoux.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.gecco.2016.09.002.

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