Combining noninvasive genetics and a new mammalian sex-linked marker provides new tools to investigate population size, structure and individual behaviour An application to bats

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Combining noninvasive genetics and a new mammalian sex-linked marker provides new tools to investigate population size, structure and individual behaviour: an application to bats

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Abstract:

Monitoring wild populations is crucial for their effective management. Noninvasive genetic methods provide robust data from individual free-ranging animals, which can be used in capture-mark-recapture (CMR) models to estimate demographic parameters without capturing or disturbing them. However, sex- and status-specific behaviour, which may lead to differences in detection probabilities, is rarely considered in monitoring. Here, we investigated population size, sex ratio, sex- and status-related behaviour in 19 *Rhinolophus hipposideros* maternity colonies (Northern France) with a noninvasive genetic CMR approach (using faeces) combined with parentage assignments. The use of the DDX3X/Y-Mam sexual marker designed in this study, which shows inter- and intra-chromosomal length polymorphism across placental mammals, together with 8 polymorphic microsatellite markers, produced high quality genetic data with limited genotyping errors and allowed us to reliably distinguish different categories of individuals (males, reproductive and non-reproductive females) and to estimate population sizes. We showed that visual counts represent well adult female numbers and that population composition in maternity colonies changes dynamically during the summer. Before parturition, colonies mainly harbour pregnant and non-pregnant females with a few visiting males whereas after parturition, colonies are mainly composed of mothers and their offspring with a few visiting non-mothers and males. Our approach gives deeper insight into sex- and status-specific behaviour, a prerequisite for understanding population dynamics and developing effective monitoring and management strategies. Provided sufficient samples can be obtained, this approach can be readily applied to a wide range of species.

Introduction

Monitoring wild populations is crucial for their effective management (McMahon *et al.* 2011; Pereira *et al.* 2013). Yet, developing robust monitoring protocols is challenging (Nichols & Williams 2006), particularly regarding detection probability issues (Vos *et al.* 2000; Yoccoz *et al.* 2001; Pollock *et al.* 2002). While extrinsic factors (related to observer identity, time, climate, habitat, or site) that lead to detection probability variation can be accounted for using covariates, this is not the case when variation results from inter-individual variation in behaviour. A common source of intra-specific variation is sex, with males and females displaying different behaviour that leads to large differences in detection probabilities (e.g. Ogutu *et al.* 2006; Christy *et al.* 2010; Singh *et al.* 2014). This observation lead to the development of statistical frameworks accounting for specific forms of intra-specific variation (e.g. Veech *et al.* 2016), but the most obvious and efficient way of correcting potential bias is to *a priori* identify subsets of populations that differ in detection probabilities.

Numerous bat species are threatened by human induced perturbations and global change, and their loss could dramatically impact ecosystem functions and services (Mickleburgh *et al.* 2002; Jones *et al.* 2009; Rebelo *et al.* 2010). Monitoring programs that are set up to assess bat population trends most often use visual counts or, more recently, passive acoustics. One important caveat of these approaches is that different categories of individuals, which are not identifiable during census, may differ in detection probabilities. For example, sexual segregation varies both spatially and temporally in bats and many other organisms (Wearmouth & Sims 2008; Angell *et al.* 2013). Different behaviour may even occur between individuals of the same sex (Senior *et al.* 2005), potentially leading to differences in detection probabilities (Marescot *et al.* 2011). Moreover, visual counts may cause disturbance to roosting bats, especially during the parturition and hibernation periods

(Kunz *et al.* 2009). Despite these limitations, visual counts are widely used in maternity colonies to study bat population structure and dynamics, either by assuming that males are not a substantial component of maternity colonies (Johnson *et al.* 2011; Olson & Barclay 2013) or by acknowledging that the unknown amount of males within colonies does not permit to correctly estimate essential parameters such as fecundity (Seckerdieck *et al.* 2005). When unverified, the first assumption is particularly likely to lead to incorrect inferences on population structure and dynamics. Furthermore, because females are the most important contributors to population dynamics in bats and many other animals, it is crucial to be able to differentiate both sexes when estimating colony sizes.

Capture-mark-recapture (CMR) techniques deliver individual level data which allow insights into bat life history and ecology, including longevity, physiology, social organization, movement behaviour, reproduction, sex ratio, and, to some degree, survival and population size (O'Shea *et al.* 2004; Ellison 2008). However, CMR methods have generally proven unsuccessful for reliably estimating bat population size due to bias associated with the need to capture individuals multiple times, heterogeneity in individual detection probability, low recapture rates, trap happiness or shyness, or mark loss (Kunz 2003; Schorr *et al.* 2014). Therefore, CMR is difficult to apply to bats, which are elusive and sometimes rare organisms that are usually small-sized, nocturnal, cryptic, highly mobile and capture-sensitive. Superior censusing methods should minimize disturbance and sampling bias (Yoccoz *et al.* 2001; Kunz *et al.* 2009).

Noninvasive genetic methods have become a well-established tool to study free-ranging animals by identifying each individual through its unique DNA fingerprint, without having to capture or disturb them. DNA is extracted from noninvasive samples (hair, faeces, urine) and amplified at genetically informative markers. As a drawback, noninvasive samples often contain only low amounts of degraded target DNA, resulting in genotyping errors (Broquet *et*

al. 2006). Previous work has proven that high quality genotypes can be efficiently obtained from bat feaces (Puechmaille *et al.* 2007). Multilocus genotypes are then treated as individual molecular marks, and identical genotypes are considered as recaptures. This information can be used in CMR models to estimate demographic parameters (Lukacs & Burnham 2005). The data provided can also be analysed using population genetic tools to gather critical information on sex-ratio (using a sex-linked genetic marker), relatedness, pedigrees, population structure or genetic diversity within or between populations (Waits & Paetkau 2005).

Rhinolophus hipposideros is the Palearctic bat species whose population decline is best documented. During the last century, it became rare or extinct at the northern edge of its range in Northern Europe, likely due to pesticides use, food shortage, and habitat loss (Bontadina *et al.* 2000; Weiner & Zahn 2000; Farcy *et al.* 2009). Females are known to return to their natal roost in late spring or early summer to give birth and raise their single young (Gaisler 1966). Males are usually assumed to live alone or to gather in small groups throughout the year but their presence in maternity roost has already been reported (Gaisler & Chytil 2002; Bontadina *et al.* 2002). Pregnant and lactating females have stricter roost requirements than others (Speakman *et al.* 2003). Such possible intra-specific variations in roosting requirements and behaviour depending on sex or reproductive status could result in intrinsic heterogeneity in detection probabilities, and therefore, affect colony size (i.e. total number of adults) and fecundity (i.e. ratio juveniles/adults) measurements when estimated by visual counts (Safi *et al.* 2007; Veech *et al.* 2016). Here, we provide evidence that genetic approaches, including molecular sexing, are of great interest to elucidate the population structure of bat maternity colonies and to investigate possible differences in sex- and/or status-related behaviours.

The main goals of this study were to investigate population size, sex ratio and sex-

related behaviour in *R. hipposideros* maternity colonies with a noninvasive genetic CMR approach combined with parentage assignment. We developed a robust molecular protocol, using 8 polymorphic microsatellites and one sexual marker which we applied on samples from 19 *R. hipposideros* colonies situated in Northern France, assessing the importance of taking into account sex and reproductive status when monitoring populations.

Material and Methods

Study site and sampling protocol

19 colonies located in Northern France (Picardie) were investigated (Fig 1). Bat faeces were collected during two successive sessions conducted before and after parturition in 2013. Newspaper was spread on the ground under the main hanging sites of each colony to gather bat faeces. After approximately 10 days, faeces were collected and stored on containing silica gel fragments to prevent DNA degradation until analysis (Puechmaille & Petit 2007). The number of visible adults was recorded during each visit.

DNA isolation

All pre-PCR procedures were carried out with aerosol barrier tips and single-use non-talc gloves, and pre- and post-PCR procedures were performed in separate rooms to avoid cross-contamination. For each colony, the desired number of faeces (twice the number of bats visually counted during colony sampling; see Table 2) was randomly picked. Faeces were individually homogenized using two glass beads (2 mm diameter) for 2 × 30 s at 30 Hz (Tissuelyser, Retsch). DNA was extracted using the NucleoSpin® 96 Plant II Kit (Macherey-Nagel) with slight modifications from the original protocol (Appendix A).

Development of a new polymorphic sex-linked marker

A primer pair was designed to target a pseudo-autosomal portion of the 8th intron of the sex-

linked DEAD-box helicase 3 gene (DDX3). This gene was previously amplified in bats, with amplicon lengths suitable for degraded DNA analyses (Hellborg & Ellegren 2003). The new primer pair (DDX3X/Y-Mam-F: CAGATCTATGAGGAAGCCAGAAA; DDX3X/Y-Mam-R: TCATACCGCTCTAGAGTTCGC) targets a locus that exhibits different length on the X (154 bp from the DDX3X gene, also called DBX) and Y (131 bp from the DDX3Y gene, also called DBY) chromosomes, respectively.

The suitability of this marker for *R. hipposideros* sex identification was tested using 22 male and 17 female pure DNA samples extracted from postmortem biopsies following a salting out protocol (Petit *et al.* 1999). PCR reactions were conducted in a final volume of 15µL containing 1 µL of template DNA, 10X PCR Buffer (20 mM Tris–HCl ph 8.4, 50 mM KCl; Promega), 0.2mM dNTPs (Invitrogen), 1.5mM MgCl2 (Promega), 0.3µM of forward and reverse primer, 0.05U/µL Taq DNA polymerase (Promega). The cycling conditions included an initial denaturing step of 95°C for 15 min followed by 45 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min and a final extension step of 72°C for 1 h. The PCR products obtained from two female and two male were cloned and purified (Strataclone PCR Cloning Kit, Agilent). Consensus sequences for DDX3 fragments amplified from both X- and Y-chromosomes were obtained by forward/reverse sequencing seven clones from each individual (Sanger technology).

In silico PCR (ecoPCR programme: Ficetola *et al.* 2010) was conducted to assess the theoretical coverage (Bc) of our sex-linked primer pair. To do so, we compiled a customized sequence database by blasting (Blastn) our consensus X-linked amplicon on the entire EMBL database, and by retrieving from GenBank the mRNA sequences of each available mammalian (sub)species (taxid: 40674). We then filtered out those that (i) did not align correctly, (ii) were too short to cover the entire length of the corresponding amplicon (i.e partial sequences) and (iii) contained ambiguous nucleotides (e.g. N). We randomly kept one

sequence per (sub)species to reduce overrepresentation of a few species. Sequences were trimmed to contain only the amplicon (including primer binding sites), and internal insertions were removed. Our customized mRNA sequence database, containing 91 unique mammalian (sub)species (Appendix B), was used with ecotaxstat (Ficetola *et al.* 2010) to compute Bc (allowing 3 mismatches).

We also looked for length polymorphism of our sex-linked marker in Mammalian species. We first compiled a customized sequence database by blasting (Blastn) our consensus X-linked amplicon on all available mammalian reference genomic sequences (refseq_genomic) and by retrieving the sequences (and corresponding chromosome, gene and sex annotations) from each matching (sub)species. We then filtered out those that did not align correctly, as well as partial sequences, and trimmed the sequences to contain only the amplicon (including primer binding sites). Finally, we calculated the theoretically amplified amplicon length for all the 162 sequences, belonging to 85 unique mammalian (sub)species, contained in our customized genomic sequence database (Appendix B).

Multilocus genotyping

Bat DNA was amplified and genotyped using 9 polymorphic molecular markers including a panel of 8 microsatellites selected from the literature, and our new sex-linked maker (Table 1, Appendix C). These 9 markers were optimized into a single 8 µl multiplexed reaction containing 3.5 µl Multiplex PCR buffer mix (Qiagen), 0.0875 µM of each primer (Table 1) and 2 µl DNA templates.

A touchdown thermal cycle program was used to prevent non-specific amplification, which included an initial denaturation at 95°C for 15 min followed by 16 cycles each of denaturation at 94°C for 45 s, 58°C with 0.5°C decrease per cycle for 45 s, and extension at 72°C for 1 min, and completed with 20 additional cycles with an annealing temperature of 50°C. A final extension at 72°C for 1 hour was included at the end of the cycles before

holding at 4°C. Amplifications were replicated three times to allow for genotyping error detection and to build robust consensus genotypes. Alleles were scored using GeneMapper v.5 from electrophoreses run on a 3730xl DNA Analyzer sequencer with GeneScanTM–500LIZ size standard (Applied Biosystems).

Bioinformatic pipeline and genotyping quality

An automatic procedure that scores genotypes and determines consensus genotypes was developed to avoid problems associated with subjective/inconsistent scoring of alleles and human errors and minimize the time necessary for scoring alleles. This bioinformatic pipeline is based on a comparison between alleles obtained from different scoring parameters for each replicate, and a comparison between replicates. A first scoring mode, "stringent", only considered peaks high enough to avoid genotyping errors associated with small peaks, with a threshold depending on the fluorescent dye (200 for PET, 100 for others). A second scoring mode, "non-stringent", employed peak height thresholds of 20, 40, 50, and 60 for FAM, VIC, NED, and PET, respectively. This allowed the recovery of peaks that were not detected with the first thresholds. Thresholds for stringent and non-stringent analyses were chosen after careful observation of randomly selected samples to account for the different markers' propensities to generate higher peaks or smaller artefacts. Peaks detected with the non-stringent analysis were only scored if they were detected in at least two of the three PCR replicates. Automated scoring with GeneMapper tends to not consider peaks obviously high enough when another peak is drastically higher. To overcome this issue, in a third approach, we scored our data as Amplified Fragment Length Polymorphisms (AFLP) instead of microsatellites. This allowed the detection of all the peaks within defined bins independently of the height of other peaks, retaining only those that had already been detected in previous scorings in at least one other replicate. When more than two alleles per locus were scored by the software, only the two highest were kept.

The three replicates obtained from each sample were then used to create a consensus genotype by applying the following rule: one allele of the consensus must appear at least twice over the three replicates. In the rare case of problematic consensus (three alleles seen twice), the smallest peak was discarded. To rule out that distinct genotypes actually corresponded to the same individual because of genotyping errors, we manually checked every consensus that differed from others by one or two loci (mismatch 1 and 2 - Puechmaille & Petit 2007). If there was any evidence for distinct genotypes originating from the same individual (i.e. if a particular allele was present in at least one replicate of the genotype of the individual scored as homozygous for that locus) this allele was retrospectively validated, in order to create a common genotype and merge samples.

Samples with missing data on at least one locus were discarded for subsequent analyses, except the juvenile dataset for parentage assignment (see below). Allelic Drop Out (ADO) and False Allele (FA) rates, as well as the Quality Index (QI), were estimated by comparing consensus genotypes to PCR replicates (Broquet & Petit 2004; Miquel *et al.* 2006). The expected heterozygosity of each locus was computed with unique genotypes (i.e. individuals) using the adegenet package (Jombart 2008) to evaluate the markers' polymorphism.

Deviations from Hardy-Weinberg proportions were quantified and tested with FIS and the corresponding permutation test using the software GENETIX (Belkhir *et al.* 1996). Only individuals encountered before parturition (adults) were used in these two tests.

Parentage assignment

To identify mothers, parentage was assigned using the software Colony 2 with a full-pedigree likelihood method (Jones & Wang 2010). Males were designated as polygamous, and mothers as monogamous with only one possible offspring because samples were taken over a single reproductive event (Gaisler 1966). Individuals sampled before parturition were considered as

potential parents while those only sampled after parturition were considered as potential juveniles. Only individuals successfully genotyped at 9 loci were considered, except potential juveniles for which the selection threshold was lowered to 7 loci since these individuals, only sampled after parturition, were less likely to represent a clean genotype due to the lack of comparison between sampling sessions. We ran analyses ten times to assess the robustness of the results. Only individuals assigned at least five times out of ten runs were considered as parents.

Population size estimates

Population sizes were calculated using a Bayesian estimator based on single session CMR data (Petit & Valière 2006). This method, adapted to noninvasive genetic data, considers every population size between a minimum (the number of genotypes detected) and a maximum size (set to 10 times the visual count) and determines the most probable population size given the distribution of recaptures (i.e. the number of faeces corresponding to the same individual). This method assumes a homogenous detection probability and a closed population. The homogeneity of detection probability was tested with the method developed in Puechmaille & Petit (2007), which consists in simulating the sampling process under an assumption of homogeneous capture probability, and to compare the observed with the expected number of captures per individual. Population closure is highly likely since samples were deposited over a maximum of two weeks. Population size estimates were computed when considering adult (i) females and males and (ii) females only. Individuals assigned as juveniles were removed from population size estimates. To compare visual and genetic estimation methods, we performed a generalized linear mixed model (glmm) with population size as a response variable (following a Poisson distribution) and with the estimation method and sampling session as fixed effects. Colonies were considered a random effect. Fixed effect significances were tested with a Wald chi square test followed by post-hoc pairwise least-

square means test. Glmm and Chi-square tests were performed in R 3.2.2 (R Core Team 2016). For comparison, we also estimated the population size with the Capwire package (Pennell *et al.* 2013), by performing either the "Equal Capture Model" or the "Two-Innate Rates Model" (depending on the likelihood ratio test included in the package) before performing the same glmm and subsequent tests. To validate the suitability of our molecular markers to discriminate among a large number of individuals, population size estimates were compared with the reciprocal of the probabilities of identity computed for unrelated individuals (PID-rand) and full siblings (PID-sibs) using Gimlet (Valière 2002) and for samples collected before parturition.

Sex related behaviour

The overall and offspring sex-ratio of each colony (i.e. the ratio of males to total number of individuals genotyped) was estimated separately before and after parturition. To investigate the capture probability of females and males depending on the sampling session, we performed a generalized linear mixed model (glmm) with the number of recaptures for each individual as a response variable (following a Poisson distribution) and with sex and sampling session as fixed effects. Because the same individual could be sampled before and after parturition, we considered individuals as a random effect. The fixed effect significance was tested with a Wald chi-square test followed by a pairwise least-square means post-hoc test. A Chi-square test was performed to investigate if the inter-session recapture rate (number of individuals captured before and after parturition) differed between sexes and with female reproductive status.

Results

Molecular protocol validation and sex ratio

The results of the amplification tests carried out with the DDX3X/Y-Mam sex-linked marker on 37 known *R. hipposideros* males and females confirmed its reliability for sex identification (100% were correctly sexed). Sequenced amplicons provided one allele from the X-Chromosome (154 bp) and two from the Y-Chromosome (129 and 131 bp). Furthermore, *in silico* PCRs confirmed the reliability of the DDX3X/Y-Mam marker for sex identification, its length polymorphism between and within 85 unique mammalian (sub)species, and the wide taxonomic coverage of the corresponding primer pair that theoretically amplified 88% of the 91 tested (sub)species belonging to all described placental mammal super-orders (detailed results are available in Appendix B).

In this study, 3544 samples out of 5099 (69.5%) were genotyped at all 9 loci. Mean ADO and FA rates over loci were 6.7% and 2.5% respectively, resulting in a QI of 91.6%. The average expected heterozygosity of the eight microsatellite loci was 70% (range 43–83%). Over all loci and colonies, the minimal number of individuals that could be discriminated for unrelated individuals (1/*P*ID-rand) and assuming full siblings (1/*P*ID-sibs) was above 1.46 x 10^6 and 2.77 x 10^4, respectively, and was always clearly greater than the corresponding colony sizes estimated visually or genetically (Table 2).

We distinguished 1337 unique genotypes, inferred to correspond to different individuals, 345 (25.8%) of which were males. The number of unique genotypes identified for each colony and sampling session ranged from 4 to 200 with a mean of 43.7, and was in the range of corresponding visual counts (Table 2). F_{IS} values were variable between colonies, with four significantly positive values (Pic4, Pic5, Pic6, Pic19) and one significantly negative (Pic8). Males were sampled in each colony and at each sampling session, except in the smallest colony (Pic1) before parturition. The mean overall sex-ratio of

the sampled colonies was 0.22 (range 0 - 0.5) and varied between colonies and sampling sessions (Table 2).

Population size estimates

Population sizes estimated from all individuals ranged from 8 to 235, with a mean of 64.16 (SD = 46.16) individuals before parturition, and 60.28 (SD = 34.49) after parturition (Table 2). When only considering females, average population sizes decreased to 47.9 (SD = 28.71) and 44.56 (SD = 24.49) before and after parturition, respectively. However, capture probabilities were not homogenous in half of the colonies (in 9 to 11 colonies depending on male presence and sampling session, see Table 2). Because no recapture occurred at Pic1 after parturition, we were unable to estimate its population size.

Mean population sizes calculated from all individuals, before and after parturition, were greater than those estimated from females only. Mean adult population sizes estimated through visual counts were closer to those calculated from females only (Fig. 2) and no significant difference was observed between them before parturition (Least-square means, p-value = 0.22). Independently of the estimation method, adult population sizes decreased after parturition. Capwire estimates gave similar results, with no significant differences between visual counts and female population size before and after parturition, with a p-value of 0.25 and 0.32, respectively (data not shown).

Sex ratio and sex related behaviour

Among the 662 females sampled before parturition and the 670 potential juveniles, only 174 were assigned as mother-juvenile pairs at least 5 times among 10 Colony runs. Mother-juvenile assignments were possible for all 19 sampled colonies, but with very heterogeneous proportions of mothers, varying from 8% to 80% (31.4% on average). This analysis allowed us to distinguish mothers and unassigned females (hereafter, non-mother females).

The number of samples collected from individuals assigned as mothers was significantly higher than for males for both sampling sessions (Least-square means, p-values < 0.05). The number of recaptures for mothers was particularly high after parturition, and significantly higher than for non-mothers (Fig. 3). There were significantly more inter-session recaptures for mothers (63.8%) than for non-mothers (34.2%), and more for both types of females than for males (20.6%) (Chi-square test; p-values < 0.001).

Discussion

Molecular protocol validation

We developed a widely applicable noninvasive molecular protocol, involving eight microsatellite loci and one sex-linked marker, for population genetic studies conducted on *R. hipposideros*. Our results confirmed the suitability of the DDX3 sex-linked marker for reliable molecular-sexing from DNA extracted from different sources, including noninvasive samples. Indeed, the amplification of different pseudo-autosomal DNA fragment sizes (i.e. length polymorphism) from both X and Y chromosomes in a single PCR reaction allowed the unequivocal distinction between sexes by avoiding misidentification due to PCR failure (i.e. false negative results) when using chromosome-specific markers. We showed that this inter-chromosomal length polymorphism, which is linked to an indel located in the 8th intron of the DEAD-box helicase 3 gene, is observed in 85 mammalian (sub)species, making it a potentially widely applicable molecular sexing tool (Appendix B.2).

Despite the challenging low concentration and degraded nature of noninvasive DNA samples, our protocol allowed the complete genotyping of 70% of analysed faeces and provided high quality genetic data (QI 91.6%) with low genotyping error rates (ADO <7% and FA <3%). When including the samples with missing data, the Quality Index dropped to 73.3% but ADO and FA rates remained relatively stable (6.6% and 3.2%, respectively). These

values are among the best recorded when compared to other studies directly amplifying nuclear DNA from faeces (Broquet *et al.* 2006), but are lower than those obtained in Puechmaille *et al.* (2007), where microsatellites markers were only genotyped if mtDNA was previously amplified in a given sample. This suggests that directly performing three PCR replicates per sample can represent a good compromise between sufficiently reducing genotyping error rates while keeping the cost of large scale studies as low as possible.

Here, 1337 unique genotypes were obtained from 19 colonies, which, in combination with the low PIDs calculated for our data set (even assuming full siblings), demonstrates that our molecular marker set is variable enough to discriminate a large number of individuals.

F_{IS} spanned a wide range of values around zero, with the most extreme ones being significantly different from zero (Table 2). No particular locus was involved in these extreme values (data not shown), excluding potential experimental artefacts. These F_{IS} values may rather be related to different underlying structures (e.g. Wahlund effect or family structures, see Parreira & Chikhi 2015) in the different colonies, a topic that will require further investigation.

Extrinsic factors affecting detection probability and population size estimates

Mean bat colony sizes estimated visually were always closer to the genetic estimates of female population sizes than to those of the total population (Fig. 2). Hence, visual counts, which do not allow sex differentiation, underestimate the total number of individuals that occupy colonies. Indeed, individuals which are not present when the visual count occurs are missed. Individuals with the highest philopatry, i.e. females, are more likely to be detected. Beyond sex, individual detection probability may be heterogeneous due to a multitude of time- or site-dependent extrinsic factors affecting the observation process itself (observers' skills, timing and duration of censusing) and/or conditions (environmental factors restraining visibility or observers' proximity to the animals and/or factors related to species biology and

ecology) (Kunz *et al.* 2009; Veech *et al.* 2016). Our results confirm that visual counts represent a reliable and cost effective method for censusing the more settled individuals within colonies (i.e. adult females, see below) when only interested in bat demographic trends. However, when more detailed demographic data are required (e.g. population structure, reproduction or vital rates), sampling noninvasively over several consecutive days combined with CMR approaches represents a powerful alternative. Genotypes were obtained at a cost of 7.25€ per sample (consumables only) and approximately two months of lab work and data analysis were required to obtain clean consensus genotypes for all 19 colonies. This investment provided high quality data at the individual level and while minimizing both disturbance of colonies and the effects of extrinsic factors on individual detection probability and population size estimates (Petit & Valière 2006; Puechmaille & Petit 2007; Kunz *et al.* 2009).

Half of the population size estimates did not meet the assumption of homogeneous detection probability, and this heterogeneity can alter population size estimates (Link 2003). Therefore, population size estimates taken at the colony level should be treated carefully. Indeed, in most wild animal populations, standardizing sampling effort is helpful to reduce biases due to extrinsic factors, but will not totally eliminate heterogeneity, because other intrinsic variables, like inter-individual behavioural variations, will influence their detection probability through space and time (Yoccoz *et al.* 2001; Veech *et al.* 2016). It is thus crucial to define subsets of populations that differ in detection probability for a more accurate evaluation of key parameters such as population sizes or vital rates.

Intrinsic factors affecting detection probability and demography

Within a population, individuals may exhibit distinct types of behaviour that will make them more or less detectable. This heterogeneity in detection probability can be related to one or several intrinsic factors including age (e.g. Crespin *et al.* 2006), sex (e.g. Tavecchia *et al.* 2002), breeding or social status (e.g. Ogutu *et al.* 2006).

Although intrinsic heterogeneity remains difficult to measure and to control for statistically (Veech *et al.* 2016), its underlying factors are important to identify and consider whenever possible, because behaviour is often the primary force responsible for changes in density, genetic diversity, and sex or age structure.

Sex related behaviour

The set of molecular markers used allowed us to assess variation of bat maternity colony structure over time. Our results show that these colonies are indeed mainly composed of females but also that the number of males is substantial (25.8%). This is in agreement with sex-ratios recorded in other European maternity colonies (Bontadina *et al.* 2002), though our results demonstrate that sex-ratio is not homogenous between colonies and through time within the same colony.

Unsurprisingly, a greater number of samples was analysed per female than per male before and after parturition (Fig 3). This number significantly increased in females while it decreased in males after parturition, suggesting that most males leave or spend less time in the roost and are not recaptured (Table 3). This is supported by the fact that our female population size estimates were slightly lower (13.7% on average) than visual counts, which only encompass the most settled individuals independently of their sex. Such behaviour has been observed in many other temperate bats where females segregate from males in spring and summer to form maternity colonies and raise their young (McCracken & Wilkinson 2000; Senior *et al.* 2005; Safi *et al.* 2007; Ibáñez *et al.* 2009). Indeed, during this period of low mating willingness, the lower fidelity of males to maternity colonies may reduce local resource competition with pregnant and lactating females that display more stringent foraging and roosting requirements (Senior *et al.* 2005; Safi *et al.* 2007).

These results highlight the potential of molecular approaches to investigate sex-related

behaviour, in relation to species biology and ecology, and to evaluate how it could affect individual detection probabilities during population surveys. Such sex-specific information can inform conservation programs, especially in social and/or colonial species in which females, which are key individuals for population growth, form isolated groups to give birth and raise their young, a period in which they are particularly vulnerable.

Female reproductive status

The combination of our noninvasive CMR approach with parentage analysis allowed deeper insights into the structure of maternity colonies, revealing that female roost fidelity can change over time according to reproductive status, and providing fecundity estimates.

Only 26.3 % of the females sampled before parturition were assigned a mother status. This is surprisingly low considering previously reported values of this species' fecundity (e.g. 0.38 and 0.5 in Schofield 1996 and Petit *et al.* 2014, respectively). A possible explanation would be that we failed to assign a significant number of mothers due to insufficient sampling, or because of genotyping failure of some mothers before parturition and/or some juveniles after. The latter is particularly likely because sex-ratios of assigned juveniles, which are supposed to be balanced (Gaisler 1966), were biased towards females in our study (21.8% males, data not shown). This could be explained by an unexpected difficulty to sample juveniles (e.g. particular composition and/or frequency of deposition of juvenile faeces, coprophagy behaviour of the mother), or by a significant part of the assigned juveniles actually corresponding to philopatric adult or subadult females sampled only after parturition. Nevertheless, variation in offspring sex-ratio has been reported in numerous mammal species including bats (Barclay 2012), and different adaptive explanations have been proposed (Clutton-Brock & Iason 1986).

We cannot currently accept or reject one of those two hypothetical explanations for the low number of assigned mothers observed, the reality being probably a mix of both. If low juvenile sampling prevails in shaping the observed patterns, sampling twice in a single year might not be optimal for correctly identifying all mothers of that year, or to estimate fecundity. Sampling colonies twice a year over multiple years would enhance both adult and offspring sample sizes and could help elucidating the causes of the observed low parentage assignment and female-biased offspring sex ratios.

Differences between the fecundity observed in bibliography, estimated from visual counts, and our molecular approach could also be related to intrinsic heterogeneity in individual detection probabilities due to behavioural differences in roost occupancy between individuals of different reproductive status. Indeed, the mean number of samples analysed per individual after parturition was higher for females assigned as mothers than for any non-mother in both sampling sessions (Fig. 3). For mothers, the number of samples analysed per individual increased after parturition, suggesting a smaller mobility of females that gave birth. This number did not vary for non-mother females between sampling sessions, suggesting a stable behaviour over this period. Furthermore, the proportion of females resampled after parturition was higher for females assigned as mothers (Tab. 3). These results clearly show that all females seem to frequent the maternity colony before the permanent settlement of mothers. Because the costs and benefits of site fidelity for females may change with reproductive conditions and associated energetic requirements, we hypothesize that non-mothers are less faithful to their colony because of intra-specific competition for local resources with mothers (see Anthony *et al.* 1981; Kunz *et al.* 2009; Ngamprasertwong *et al.* 2014).

Finally, our results highlight the suitability of our approach (involving a well conserved polymorphic pseudo-autosomal sexual marker) to (i) gather high quality data at the

individual level, (ii) decipher complex inter-individual behavioural variations that may influence individual detection probability, and (iii) consider these factors for more precise population size and reproduction rate estimates, which are fundamental for efficient management of wild populations.

Conclusion

With noninvasive genetic sampling and an analysis method adapted to large datasets, we obtained high quality genetic data with minimal disturbance of the colonies. We showed that distinguishing males from females, using a sex-linked marker, offers unique opportunities to more reliably determine different categories of individuals (males, females, potential breeders, reproductive individuals), to identify extrinsic and intrinsic factors of heterogeneity in individual detection probabilities, and to estimate population sizes, which is critical for understanding population dynamics and optimise wildlife management. By combining parentage with CMR analyses, we showed that the population structure of *R. hipposideros* in maternity colonies changes dynamically during the summer. Parentage assignment provided valuable insights into female behaviour revealing that, after parturition, maternity colonies are mainly composed of mothers and their offspring while males and non-mother females continue to frequent them without residing there permanently. Because reproductive females crucially contribute to the establishment and long-term persistence of social animal colonies, disentangling behaviour specific for these different categories of individuals is essential for an effective monitoring and conservation of wild populations. Finally, our study highlights that noninvasive genetic samples, when analysed both as individual marks (CMR) and genetic fingerprints (parentage) instead of as allelic frequencies only, allow to investigate both individual (behaviour, mating, etc.) and population (genetics and dynamics) level processes. When combined with information on other biotic or abiotic factors, such data offer unprecedented information to better understand population dynamics and the underlying responsible

processes (Jan *et al.* 2017). This knowledge is urgently needed for conservationists, managers and policy makers to understand how species are responding to global environmental change (e.g. climate, habitat) and to make long-term predictions.

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Data Accessibility:

DNA sequences: Genbank accessions KY793696-KY793699

R scripts and genotypes used in this study were deposited at DRYAD entry doi:10.5061/dryad.8fh54

Author contributions:

This project was designed by EJP with input from SJP and fundraising was conducted by EJP. Lab experiments were performed by DZL, PLJ, ALB and LL. Data were analysed by DZL, PLJ, TG, ALB, LL, SJP and EJP. The paper was written by DZL, PLJ, SJP and EJP. Both co-first authors contributed equally to the study. All authors were involved with data interpretation and draft corrections.

Supporting Information:

Appendix A: DNA isolation using the NucleoSpin® 96 Plant II Kit (Macherey-Nagel).
Appendix B: In silico validation of our sex-linked primer pair.
Appendix C: Example of genotype profiles obtained from one male (A) and one female (B) (three replicates each).

Table 1: Description, sources and amplification conditions of markers used this study and their respective measures of allelic drop-out rate (ADO), false allelic rate (FA), quality index (QI) and expected heterozygosity (Hs).

	Locus	Size range	Fluoro-label	Annealing Temperature (°C)	Concentration of each primer (µM)	Source	ADO	FA	QI	Hs
Microsatellites	**RHA101**	130-150	NED	*56*	*0.0875*	Struebig et al. 2011	0.07	0.01	0.94	0.43
	RHA7	221-249	VIC	*56*	*0.0875*	Struebig et al. 2011	0.07	0.03	0.91	0.83
	RHA109	168-198	PET	*56*	*0.0875*	Dool et al. 2013	0.05	0.02	0.93	0.76
	RHA4	264-298	FAM	*56*	*0.0875*	Rossiter et al. 2012	0.07	0.03	0.91	0.77
	RHC108	150-174	FAM	*56*	*0.0875*	Puechmaille et al. 2005	0.09	0.02	0.89	0.73
	RHC3	186-194	FAM	*56*	*0.0875*	Puechmaille et al. 2005	0.10	0.05	0.87	0.52
	RHD102	224-272	PET	*56*	*0.0875*	Puechmaille et al. 2005	0.06	0.02	0.91	0.80
	RHD103	200-240	NED	*56*	*0.0875*	Puechmaille et al. 2005	0.05	0.03	0.93	0.73
Sexing Marker	**DDX3X/Y-Mam**	128-157	VIC	*56*	*0.0875*	This study	0.06	0.02	0.95	0.23

Table 2: Colonies' demographic and genetic characteristics. The number of adults visually counted, the number of unique genotypes (and corresponding proportion of males) and population sizes estimated genetically before and after parturition are presented separately. * indicates that population size estimates did not meet the homogeneous sampling assumption. Genetic characteristics were calculated using microsatellites data from the first sampling session only. F_{IS} values in bold are significantly different from 0 (10 000 permutations), and the ratio 1/probability of identity considering unrelated (PID-rand) or siblings (PID-sibs) individuals.

Colony	Before parturition				After parturition				Genetic characteristics		
	Count	Number of genotypes (sex-ratio)	Population size estimate (95% CI)		Count	Number of genotypes (sex-ratio)	Population size estimate (95% CI)		FIS	1/PIDunb	1/PIDsib
			Females + males	Females only			Females + males	Females only			
Pic1	5	8 (0.0%)	24 (12-49)	24 (12-49)	5	4 (25.0%)	NA	NA	0.05085	7.03×10^{10}	6.75×10^{2}
Pic2	16	21 (9.5%)	24* (21-30)	22* (19-27)	26	20 (35.0%)	32 (24-60)	20 (15-43)	-0.0254	2.02×10^{7}	4.13×10^{2}
Pic3	60	53 (20.8%)	60* (55-69)	47* (43-53)	60	45 (15.6%)	54* (48-64)	44* (39-52)	-0.00802	3.36×10^{8}	1.24×10^{3}
Pic4	45	43 (20.9%)	90 (67-155)	67 (49-121)	21	17 (23.5%)	21* (18-29)	15 (13-21)	**0.05155**	2.12×10^{8}	1.02×10^{3}
Pic5	40	34 (14.7%)	60* (46-99)	50* (38-87)	6	27 (18.5%)	40 (32-62)	30 (24-45)	**0.06916**	1.09×10^{8}	7.86×10^{2}
Pic6	70	50 (12.0%)	87 (69-128)	73 (58-108)	101	62 (12.9%)	65* (62-70)	56* (54-60)	**0.09212**	7.25×10^{7}	7.87×10^{2}
Pic7	30	36 (36.1%)	58 (46-89)	33 (27-53)	20	24 (33.3%)	28* (25-35)	18* (16-22)	0.01676	1.06×10^{8}	8.00×10^{2}
Pic8	35	35 (17.1%)	38* (35-44)	31* (29-36)	50	30 (16.7%)	40 (33-55)	32* (27-45)	**-0.069**	1.59×10^{8}	8.20×10^{2}
Pic9	55	36 (8.3%)	54* (44-79)	49* (40-72)	25	33 (39.4%)	45* (37-62)	26 (21-38)	-0.00381	2.36×10^{8}	1.20×10^{3}
Pic10	35	37 (32.4%)	44 (39-55)	30 (26-38)	40	28 (32.1%)	41 (33-63)	26 (21-40)	-0.01185	8.93×10^{7}	8.20×10^{2}
Pic11	37	33 (18.2%)	44 (37-59)	34 (29-45)	7	17 (17.6%)	18* (17-21)	15* (14-17)	-0.01587	4.93×10^{7}	7.59×10^{2}
Pic12	91	60 (20.0%)	65* (61-72)	53* (49-59)	50	62 (16.1%)	68* (63-74)	56* (52-61)	-0.03741	1.27×10^{8}	9.58×10^{2}
Pic13	50	51 (15.7%)	58* (53-66)	48* (44-55)	25	45 (13.3%)	60 (52-78)	50 (43-63)	0.00619	1.79×10^{7}	5.95×10^{2}
Pic14	80	63 (22.2%)	108 (88-151)	86 (68-129)	45	25 (20.0%)	58 (40-135)	41 (28-98)	-0.031	2.94×10^{7}	8.05×10^{2}
Pic15	5	10 (50.0%)	35 (17-59)	19 (7-29)	10	16 (31.3%)	85 (36-84)	60 (21-59)	0.01548	5.15×10^{8}	4.52×10^{2}
Pic16	222	200 (39.0%)	235* (222-253)	140* (131-152)	215	143 (32.2%)	152* (146-159)	101* (97-105)	-0.00448	7.90×10^{7}	1.09×10^{3}
Pic17	41	39 (25.6%)	51* (44-67)	41* (33-59)	3	24 (16.7%)	36* (28-57)	29* (23-48)	-0.01043	1.46×10^{6}	2.77×10^{2}
Pic18	40	55 (16.4%)	67 (60-80)	54 (49-64)	60	33 (3.0%)	45* (37-62)	43* (36-59)	0.04052	9.37×10^{7}	9.94×10^{2}
Pic 19	10	12 (41.7%)	17 (13-34)	9 (7-20)	10	4 (25.0%)	8 (5-29)	6 (3-23)	**0.12659**	1.09×10^{8}	3.04×10^{2}

Table 3: Number of adults sampled only before parturition *vs* in both sessions, depending on their sex and, for females, their assignment as a mother. *: significant Chi square-test (p-values < 0.001)

	Sampled only before parturition	Sampled before and after parturition	
Males	170	44	*
Unassigned females	321	167	*
Mothers	63	111	

Figure 1: Location of the 19 monitored colonies (Picardie, France)

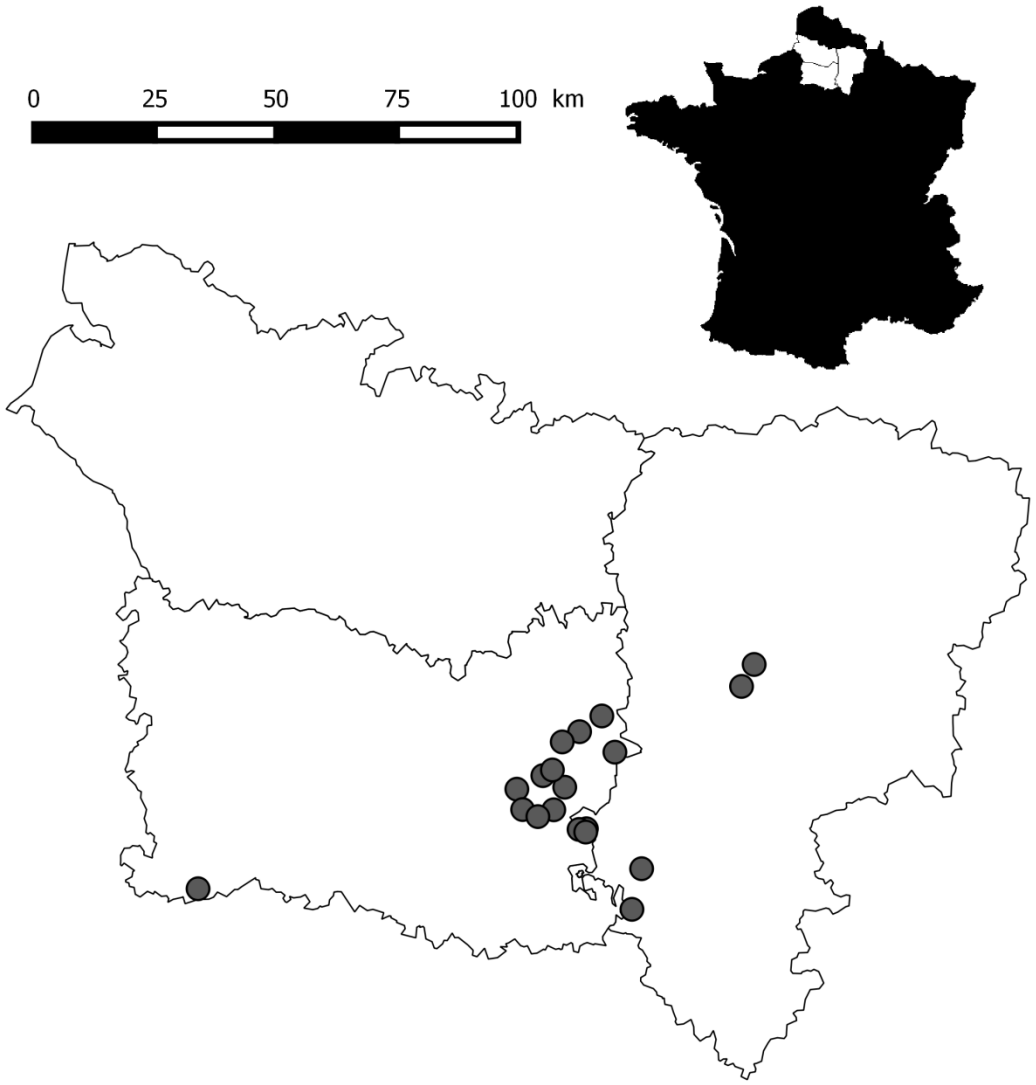

Figure 2: Colony sizes estimated by visual counts or CMR approach for both sampling sessions. CMR models were applied to all sampled individuals and to adult females only. Error bars represent standard error. Letters represent significant differences (Least-square means, p-values < 0.05)

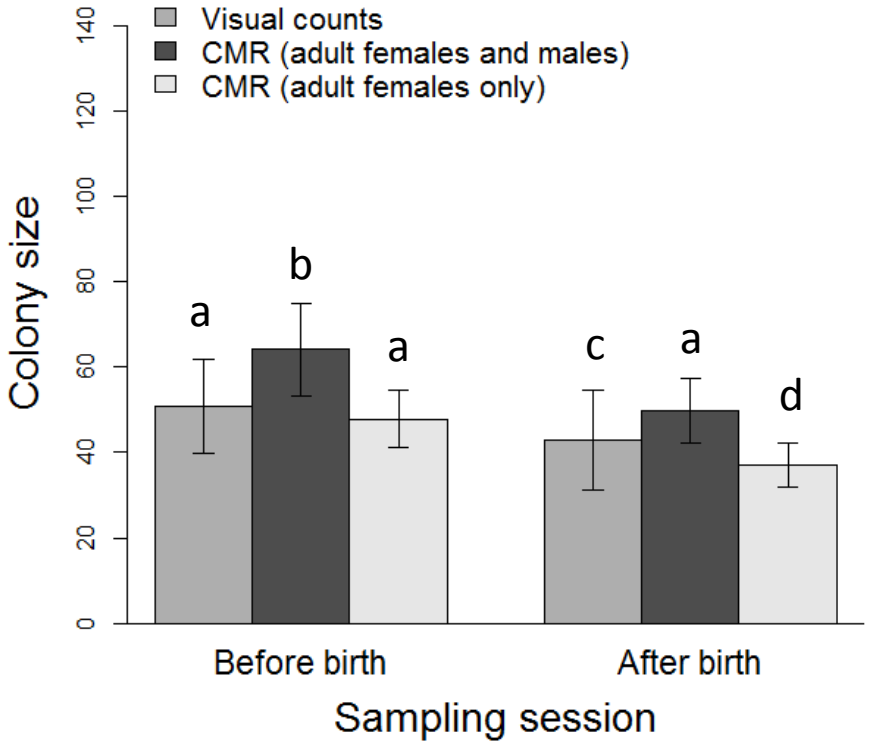

Figure 3: Number of captures for males, non-mother females, and females assigned as mothers for both sampling sessions. Error bars correspond to the standard error. Different letters correspond to a significant difference (Least-square means, p-values < 0.05)