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Article

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Bat ecological traits behind *Leptospira* distribution and genetics in the north-eastern Palaearctic

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Abstract

Leptospirosis is a globally distributed zoonosis caused by spirochaetes of the genus *Leptospira*. A wide variety of vertebrates are susceptible to the infection, including humans, domestic animals, rodents, and bats. However, the biological and ecological drivers of bacterial transmission in Chiroptera remain poorly understood. To bridge this gap, we divided the studied bat taxa by roosting and foraging preferences as well as migration behaviour, and compared these traits with PCR-positivity of *Leptospira* in bat urine. In total, 2,455 individuals across 37 species were analysed in the north-eastern Palaearctic, and 27 species were found positive for *Leptospira* infection. Species and age strongly influenced *Leptospira* prevalence, whilst sex effects varied across species. Large maternity colony size, void-roosting behaviour, the presence of sex-segregated colonies, and foraging over water appeared to be the most significant factors associated with PCR-positivity. Sequencing of the secY, pntA, LipL32, and LipL41 genes identified *Leptospira kirschneri, L. interrogans, L. borgpetersenii*, and a number of unique haplotypes belonging to related genetic lineages.

Keywords Leptospirosis, Chiroptera, Reservoirs, Pathogen ecology, Bat ecological categories, Zoonoses

Leptospirosis is a globally distributed infection, considered a neglected and re-emerging disease. Some authors suggest that in Europe this is the only zoonosis which tends to increase alongside climate change [1]. The causative agents are Gram-negative spirochaetes of the genus *Leptospira*, which can be identified by either genetic structure or antigenic agglutination reactions [2]. The genetic classification recognises three groups: pathogenic (P1), intermediate (P2), and saprophytic (S1 and S2). In contrast, the serological classification is based on 29 serogroups comprising more than 300 serotypes. These two schemes do not correspond directly, as a single serotype may occur across multiple species — both pathogenic and saprophytic forms [3].

Leptospira infection affects a wide range of vertebrates — mammals, birds, reptiles, amphibians and fish — with humans among the susceptible hosts [4]. Chiroptera are no exception and possess several significant ecological features that may predispose these mammals to serve as a spirochaete reservoir. Firstly, during pregnancy and nursery periods, bats commonly form large colonies, occasionally numbering several thousand individuals [5].

Secondly, some bat species prefer roosting in crevices [5], where animals are tightly packed and the possibility of infection spillover is high. Noteworthily, homing — the ability to locate the same roost annually — is common among Palaearctic bat species and could lead to repeated contamination of a given shelter over many years. For example, pond bats can use one main roost for more than 50 years [6]. The situation is further compounded by some bat taxa following the same foraging routes annually [7]. For rodents, it has been shown that year-by-year excretion of *Leptospira* leads to its accumulation in water and soil, thereby forming natural foci of infection [8]. As bats typically urinate upon arousal from torpor, chiropteran roosts are likely to be heavily contaminated with urine [9]. Thus, even if bats do not infect other mammals directly, their contribution to environmental contamination might be underestimated.

Thirdly, seasonal flux is common among Chiroptera in the north-eastern Palaearctic. Species undertaking either short- or long-range seasonal movements demonstrate a high degree of roost fidelity during both hibernation and summer activity. For *Eptesicus (Cnephaeus) nilssonii* (Keyserling & Blasius, 1839), the distance between seasonal shelters is estimated at approximately 10–30 km [10]; for *Myotis dasycneme* (Boie, 1825) distances of up to 300 km have been documented [7]. Some migratory species are able to fly much further — up to 2,500 km [11]

— potentially spreading the bacteria over considerable distances. In addition, some synanthropic species may be accidentally transported to new territories by car, public transport or cargo [12, 13].

Most hibernating species in the north-eastern Palaearctic find their winter shelters in natural or artificial caves, abandoned mines, or fortification structures that are regularly visited by tourists. During the warmer part of the year, depending on the locality and species, bats may inhabit urban or rural areas, roosting in tree holes in parks and churchyards or directly under eaves in towns [5, 7, 14]. Owing to the ability of *Leptospira* to survive in water and soil for several months [15], the bacteria persisting in summer or winter roosts might remain infectious even if bats have abandoned their shelter.

Numerous studies on bat-borne leptospirosis have been conducted globally [16, 17]. Although, the majority of these papers have concentrated on molecular and genetic aspects, while relatively few — such as those undertaken in Brazil and across tropical Africa [18, 19, 20, 21] — have explored certain ecological traits of bats in the context of *Leptospira* infection. However, identification of ecological drivers of pathogen transmission is considered a key element in predicting spillover events [21, 22]. Moreover, data on chiropteran leptospirosis across the Palaearctic are strikingly limited [17].

To bridge this gap and to understand which ecological inter- and intraspecific features facilitate *Leptospira* nucleic acid detection, we established an ecological classification of studied Chiroptera (based on species roosting and foraging preferences and migration behaviour) and compared its traits against the PCR results of bat urine and sequences of secY, pntA, LipL32, and LipL41 of *Leptospira* collected from 37 species of Vespertilionidae, Miniopteridae, and Rhinolophidae.

Results

Bat sampling

In total, 2,455 bats of 37 species belonging to the families Vespertilionidae, Miniopteridae, and Rhinolophidae were examined across the north-eastern Palaearctic (Supplementary Table S1). Chiroptera were collected during hibernation (938 specimens), autumn and spring migratory flux (397), and summer active stage (1,120). Females predominated among adult bats captured during active periods (72.9%), as the majority of mist nets were deliberately set near maternity roosts to increase sample size. Approximately half of the individuals collected in summer were sexually mature (52.4%). In hibernacula, no distinction was made with respect to age, and the sex ratio was close to 1:1.

Sampling was carried out in 33 regions of the north-eastern Palaearctic (Fig. 1) and encompassed both synanthropic and natural sites.

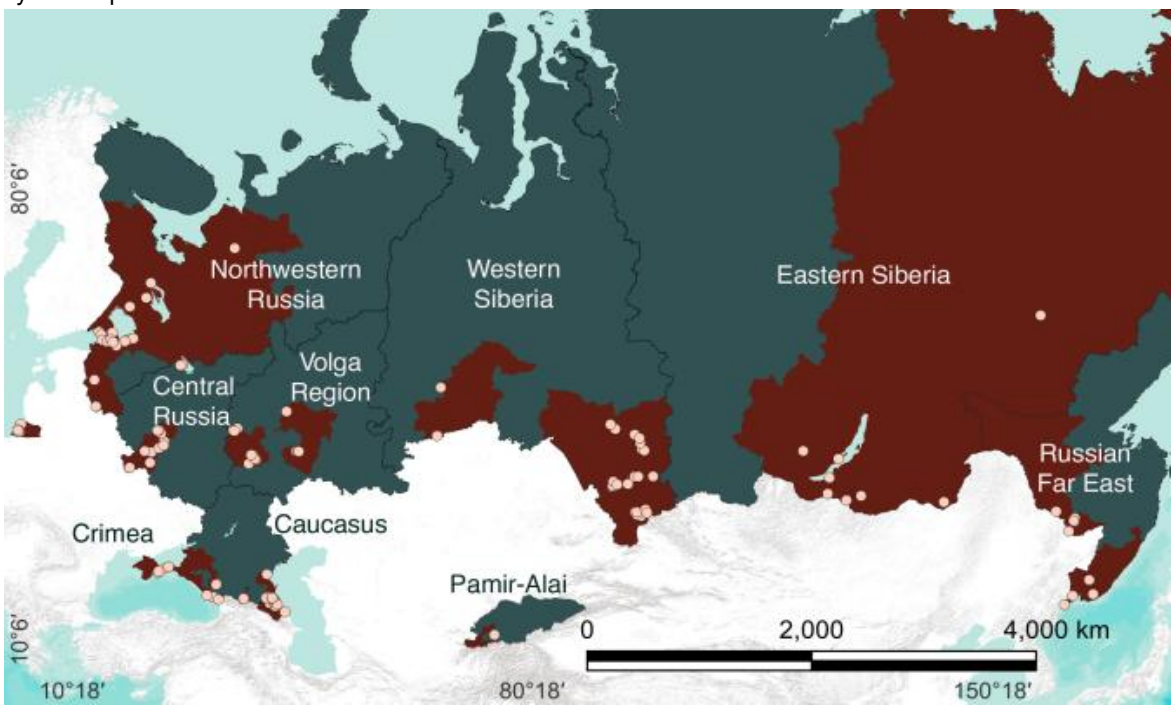


Fig. 1. Map of the study regions of the north-eastern Palaearctic, with dots indicating the exact bat-trapping sites. Regions where collection took place are shown in red. The map was created using QGIS [23].

Species composition varied markedly among regions (Fig. 2), reflecting the uneven distribution of bat taxa across the survey area.

Nursery colonies were found for *Myotis blythii* (Crimea, Altai Krai, Republic of Dagestan), *M. tschuliensis* (Republic of Dagestan), *M. dasycneme* (Kurgan Region), *Eptesicus nilssonii* (Pskov Region), *Pipistrellus nathusii* (Pskov, Kaluga, and Penza regions), *P. pygmaeus* (Penza Region) and *Vespertilio murinus* (Pskov and Leningrad regions). A male colony was directly observed only for *Myotis dasycneme* (Pskov Region) (Supplementary Table S1).

Leptospira detection

Of the 2,455 bats examined, 600 (24.44%; 95% CI 22.74–26.14%) were PCR-positive for *Leptospira* (Fig. 2, Supplementary Table S1). With the exception of *Barbastella barbastellus*, all species represented by no fewer than 20 examined individuals yielded at least one PCR-positive sample (Fig. 2).

PCR positivity varied substantially by bat species (Fig. 2, Fig. 3, Supplementary Data S2). Compared with *Plecotus auritus* — a species with roughly 3% prevalence of shedding *Leptospira* nucleic acids — 10 of the 16 species for which at least 31 individuals were collected showed significantly elevated odds of testing PCR-positive for *Leptospira* (Supplementary Data S2: model IS-1). About 8- to 30-fold higher odds ratios (OR) were found in *Myotis blythii*, *M. petax*, *M. daubentonii*, *M. dasycneme, Nyctalus leisleri*, and *N. noctula*. These species demonstrated strong statistical support (p_{BH}=4.58×10^{-5}–1.61×10^{-3}); their PCR positivity varied from 30.3% to 55.9% in adult bats. Markedly lower, but still significant odds ratios were observed for *Murina hilgendorfi, Plecotus ognevi, Vespertilio murinus,* and *Myotis bombinus* (point OR=4–5, p_{BH}=0.04–0.09), species with adult PCR positivity at 10.5-16.7%. *Eptesicus nilssonii*, *Myotis brandtii*, *M. mystacinus*, *M. nattereri*, and *M. sibiricus* did not differ significantly from *Plecotus auritus* in the prevalence of *Leptospira* shedding (p_{BH}=0.25–0.997) and their PCR positivity ranged from 0% to 10% across these five species.

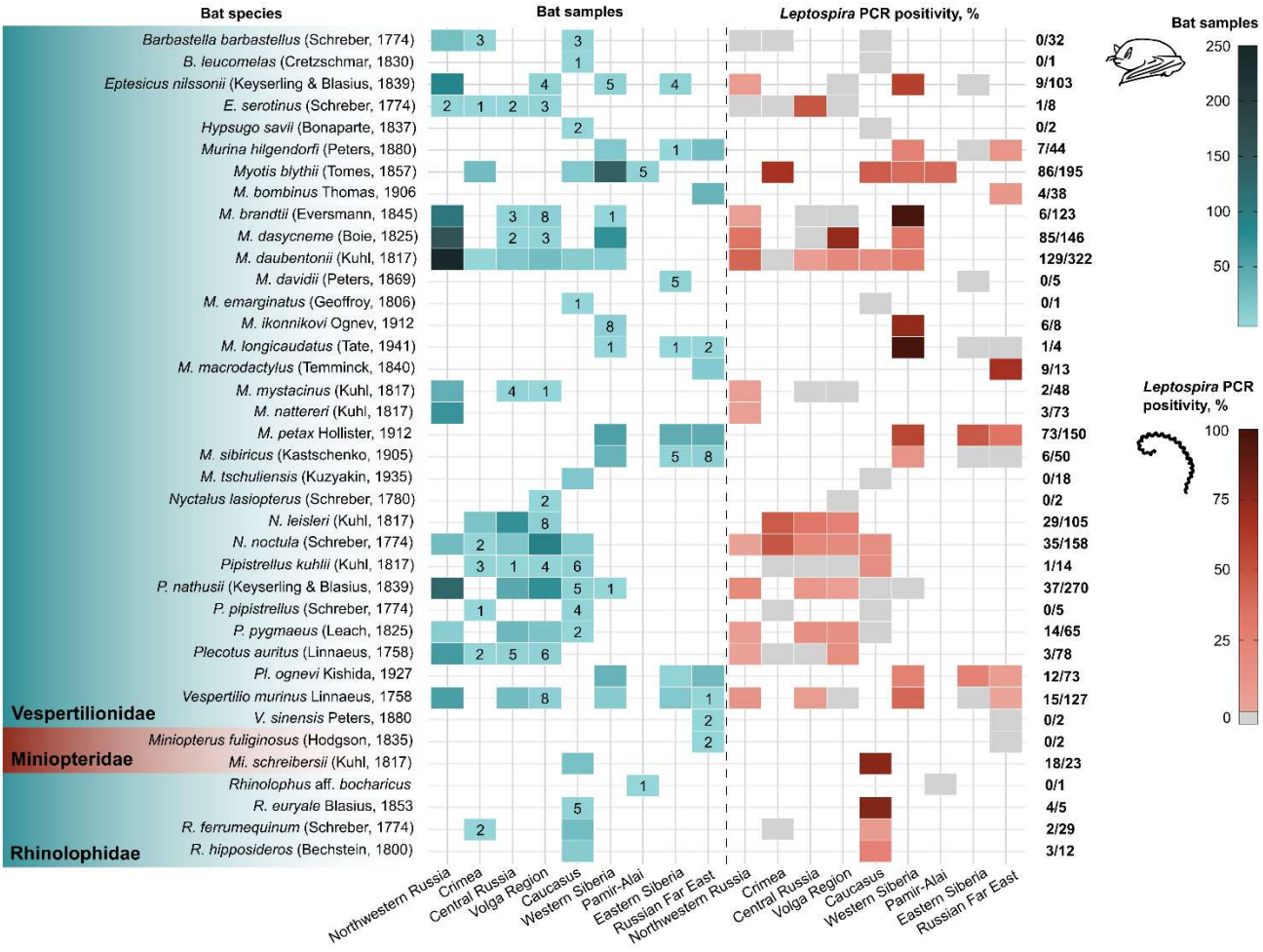


Fig. 2. Bat species trapped in the various regions (teal; sample sizes of fewer than 10 individuals are indicated by numbers) and *Leptospira* PCR positivity in tested urine samples (red). Numbers in the right-hand column represent the sample size for each species, given as the number of PCR-positive bats/number of bats collected.

Subadults had significantly lower PCR positivity rates than adults, both across the whole sample (model IS-2: OR=3.5, CI: 2.6-4.7; $p=3.63\times10^{-16}$) and within particular species (Fig. 3). In terms of predicted probability, this corresponded to an increase from approximately 5.8% positivity in younger animals to 17.8% in adults. When the sexes were considered separately, adult females of well-sampled species had significantly higher odds of PCR positivity than subadult females (model IS-2f: OR=7.4, CI: 4.6-11.9; $p=1.7\times10^{-16}$), whereas adult and subadult males of the same species did not differ significantly (model IS-2m: OR=1.1, CI: 0.7-1.7; $p=0.657$).

In *Myotis daubentonii, M. petax*, and *M. dasycneme*, the odds ratios of *Leptospira* nucleic acid shedding were positively associated with age. First-year adults had twice the odds of subadults (model IS-2myo: OR=2.0, CI: 1.0-3.9; $p=0.052$), second-year adults had the odds roughly four times higher compared to subadults (model IS-2myo: OR=3.9, CI: 1.7-9.0; $p=0.002$), and bats older than two years were significantly more frequently PCR-positive than the younger age group (model IS-2myo: OR=3.8, CI: 2.0-7.2; $p=3.4\times10^{-5}$).

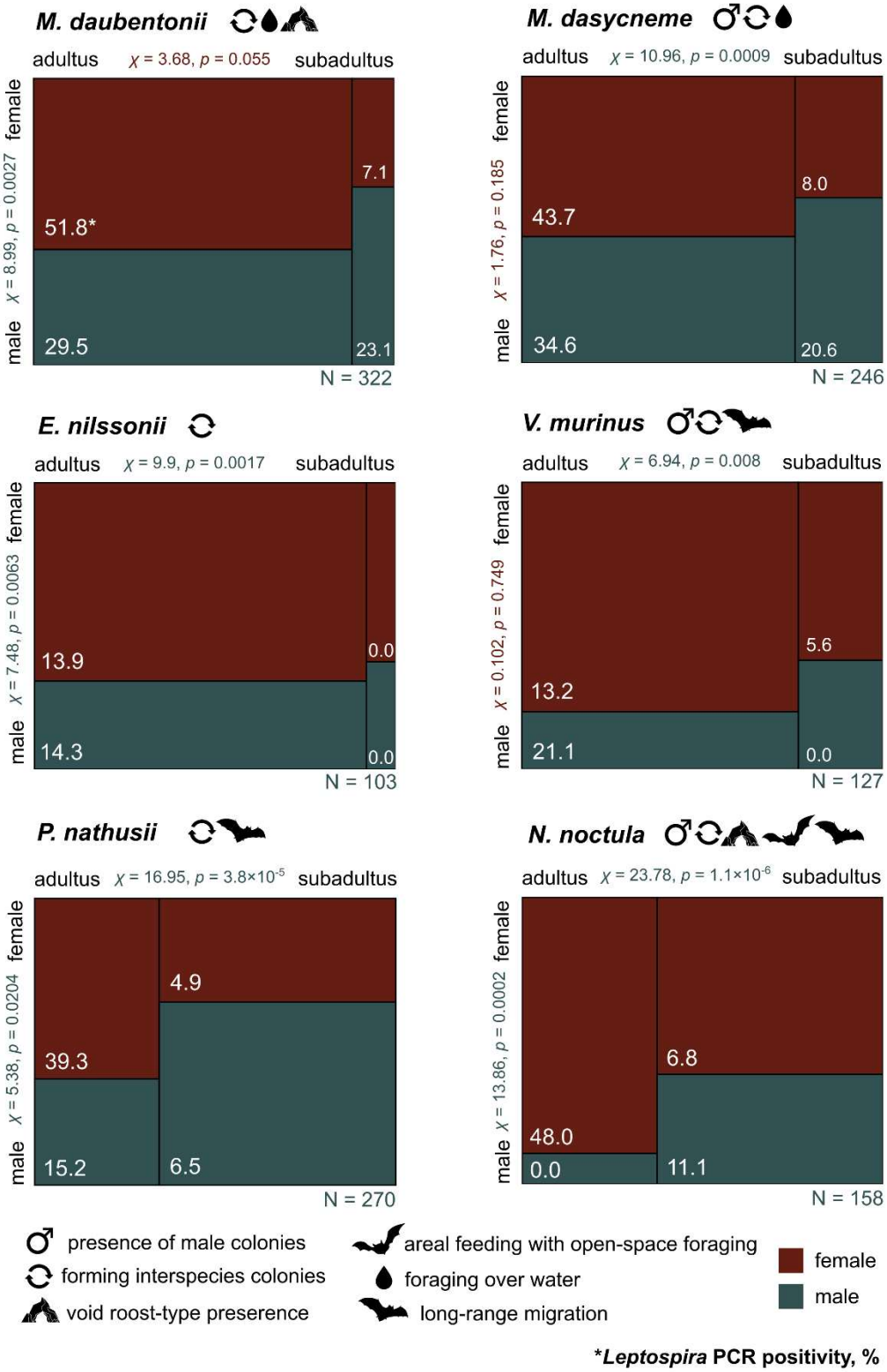


Fig. 3. *Leptospira* PCR positivity (%) in each sex and age groups in six bat species. The proportions within each square represent age (adults on the left, subadults on the right) and sex (males at the bottom, females at the top), and the numbers indicate *Leptospira* prevalence within each group. Results of Pearson's chi-squared (χ^2) goodness-of-fit test for differences in *Leptospira*-positivity between the sex and age groups are shown in green where differences are significant and in red where they are not. The pictograms correspond to the ecological features examined in the present study.

The influence of the annual cycle stage on *Leptospira* positivity was assessed for the same three *Myotis* species, as they were sampled throughout the year and widely distributed across the survey area. Positivity during the summer differed slightly from that during hibernation (model IS-4myo: OR=1.5, CI: 1.0-2.1; p=0.066).

For ringed bats, the individual dynamics of *Leptospira* nucleic acid shedding were inferred. Thirty-seven bats of ten species were recaptured and tested between two and five times (Fig. 4).

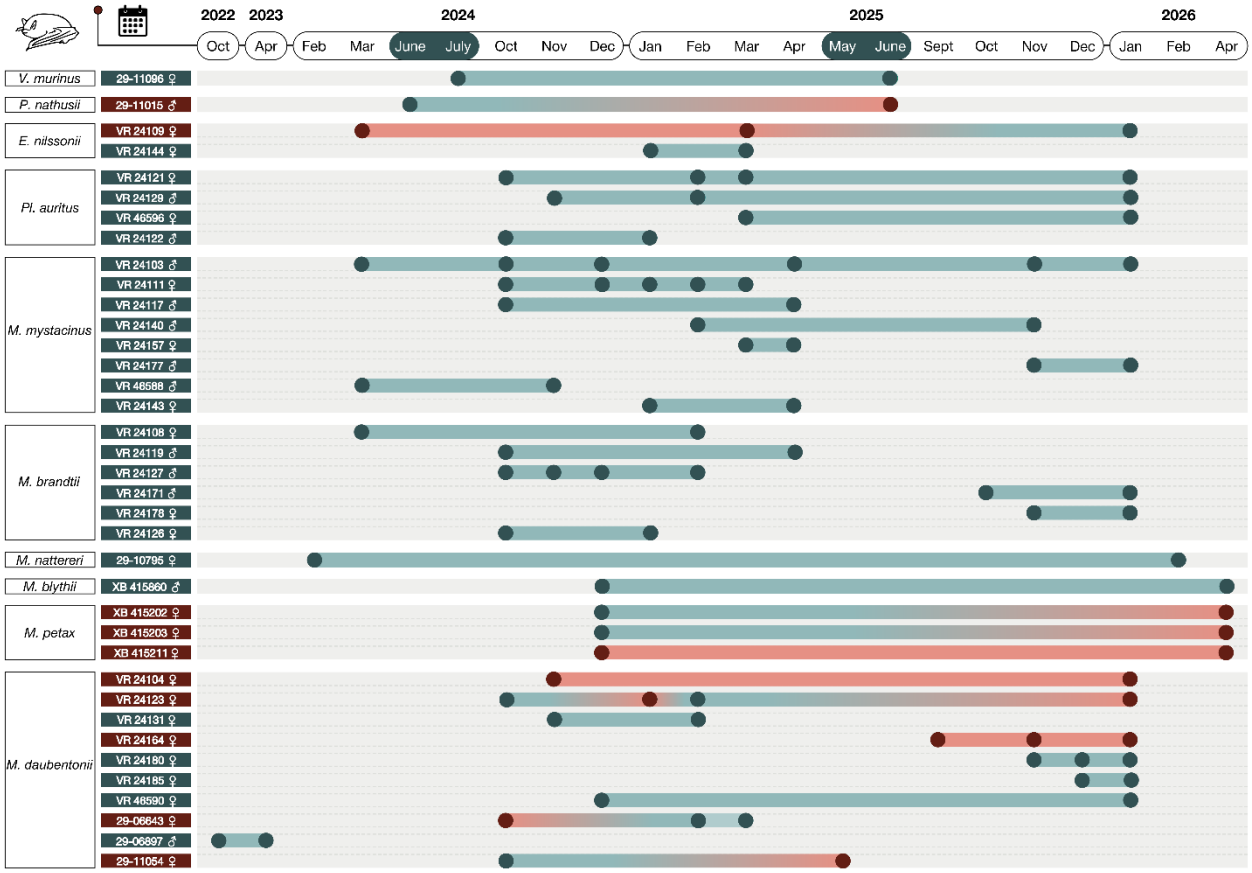


Fig. 4. *Leptospira* PCR results for recaptured bats, by species, ring number, and sex. Rings whose numbers begin with letters are bird rings with smoothed edges, marked with the index 'Moskva'; the remainder are bat rings bearing the index 'Russia'. Each dot represents a capture date and the connecting lines indicate the interval between recaptures for each individual. PCR-negative results are shown in green, PCR-positive results in red. Months coloured in white represent hibernation, and those in green indicate bat summer activity (no ringed bats were trapped during flux periods).

Individuals of bat species that typically exhibit low *Leptospira* PCR positivity — *Myotis mystacinus* (eight bats), *M. brandtii* (six), *Myotis nattereri* (one), and *Plecotus auritus* (four) — were consistently PCR-negative during the following recaptures. One *Vespertilio murinus* and one *Myotis blythii* remained PCR-negative over a period of approximately one year. One specimen of *Pipistrellus nathusii* changed from negative to positive after a year, whilst one (out of two tested) *Eptesicus nilssonii* was no longer shedding *Leptospira* nucleic acids in February 2026, although it had been PCR-positive in the 2024−2025 season. Among highly PCR-positive Chiroptera species, *Myotis petax* (three) and *M. daubentonii* (ten), the following pattern was observed: changed from PCR-negative to positive five times, maintained negative five times, persisted positive results twice, and changed from positive to negative once (Fig. 4).

Among ecological covariates, forming large maternity colonies had approximately six-fold higher odds of PCR positivity than those in the small roost-size category (Supplementary Data S2, models EA-1: OR=5.7, CI: 1.5-21.4; p_{BH}=0.025; ES-1: OR=6.5, CI: 1.9-21.9; p_{BH}=0.010). Bats forming moderately sized roosts demonstrated marginal significance and approximately 2-fold increase in the odds ratios in the whole sample, but given the CI almost reaching one it could not be regarded as robustly significant (model ES-1: OR=2.3, CI: 1.0-5.7; p_{BH}=0.086). The adult-based model instead showed more robust significance (model EA-1: OR=3.3, CI: 1.3-8.9; p_{BH}=0.025). Highly gregarious species — *Miniopterus schreibersii, Myotis blythii*, and *M. petax* — exhibited PCR positivity of up to 46% (Fig. 5). In contrast, small colonies are not always associated with low prevalence: *M. daubentonii* and

Nyctalus leisleri, both PCR-positive above 43%, form comparatively modest roosts. Bats with PCR positivity below 10% typically roost in small clusters.

PCR-positivity varied between different *Myotis blythii* aggregations: in Western Siberia maternity colony numbering about 1,500 individuals exhibited 39% adult PCR prevalence, while in Crimea nursery colony of approximately 2,000 bats showed 73% adult prevalence.

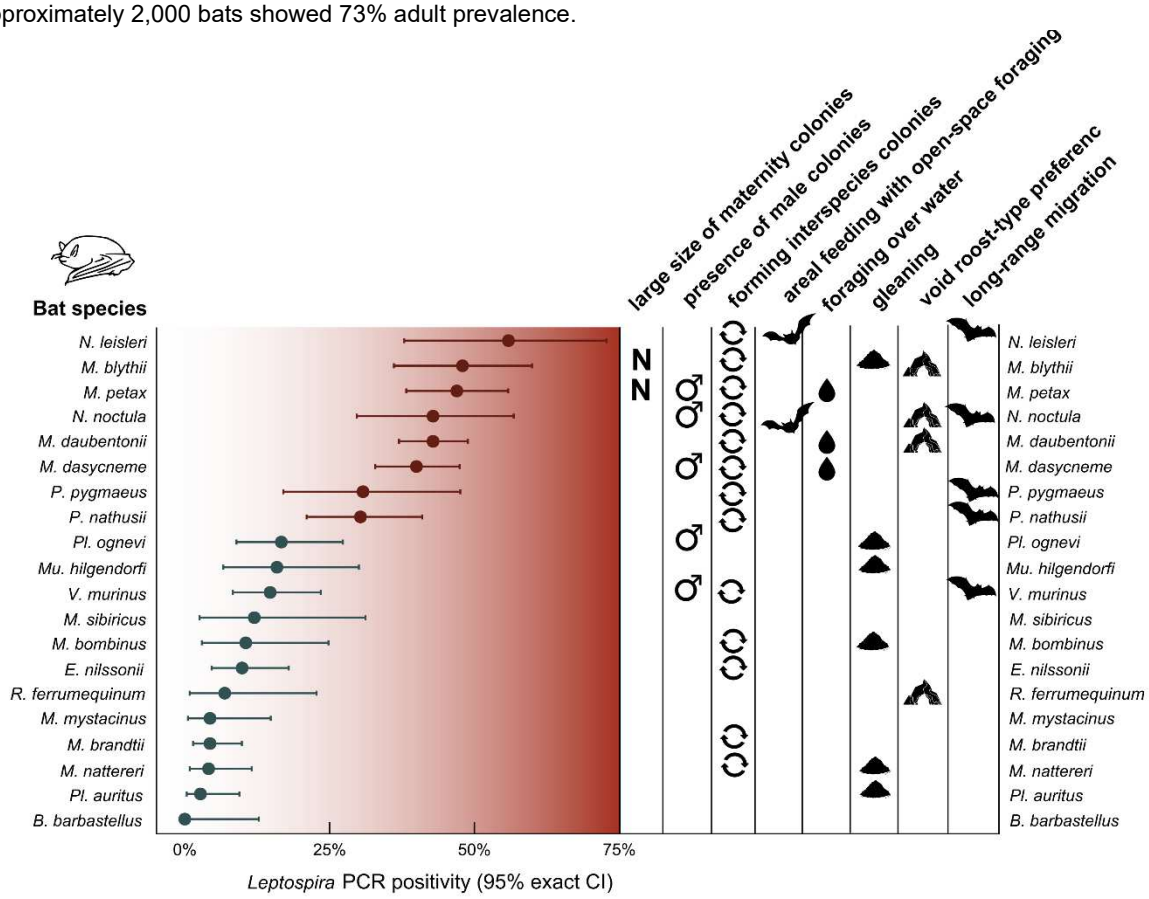


Fig. 5. Bat ecological traits and *Leptospira* PCR positivity, with 95% Clopper–Pearson confidence intervals. Green dots indicate species with *Leptospira* PCR positivity below the global average for insectivorous bats (<22%) [17]. The pictograms correspond to the ecological features examined in the present research. Only data from adult bats were included in the figure; species with fewer than 25 adults were excluded.

The presence of male colonies was significantly associated with higher odds ratios of PCR positivity (models EA-2: OR=4.1, CI: 1.4-12.5; p_{BH}=0.025; ES-2: OR=4.1, CI: 1.5-11.2; p_{BH}=0.018). At the species level, *Myotis dasycneme, Myotis petax, Vespertilio murinus, Plecotus ognevi, Nyctalus noctula,* and *Miniopterus schreibersii* forming male colonies were characterised by *Leptospira* PCR positivity above 15%. Fitting models for species with and without male colonies suggested that the presence of male aggregations raised the overall positivity of species by equalising positivity between sexes. In species where male colonies were recorded, the probability of shedding spirochaete nucleic acids did not differ appreciably between the sexes (model IS-3-4: OR=1.3, CI: 0.9-2.0; p=0.136). However, females were significantly more frequently PCR-positive, if male colonies were not registered for these taxa (model IS-3-5: OR=2.3, CI: 1.6-3.2; p=2.54×10^{-6}).

Forming interspecies colonies was associated with three- to four-fold higher odds ratios of PCR positivity (models EA-3: OR=4.0, CI: 1.3-12.1; p_{BH}=0.025 for adults; ES-3: OR=3.0, CI: 1.0-8.8; p_{BH}=0.072 for the whole sample), however in the model that included subadults the confidence interval approached 1 and this effect could not be considered significant (Fig. 6). Mixed-species colonies are usually recorded both in rarely positive *Myotis brandtii* (*Leptospira* PCR prevalence at 4%) and in the species with the highest positivity, *Nyctalus leisleri* (56%).

Roost-type preference (void or crevice) exhibited a strong association with *Leptospira* nucleic acid shedding (Fig. 6). Void-roosting species had approximately six-fold higher odds of testing positive relative to the crevice-roosting taxa, an effect consistent in both the adult and whole-sample models (models EA-4: OR=6.2, CI: 2.0-19.0; p_{BH}=0.005; ES-4: OR=5.7, CI: 2.0-16.0; p_{BH}=0.005). Bats using a comprehensive roosting strategy did not differ significantly from the reference group (models EA-4: OR=1.7, CI: 0.6-5.4; p_{BH}=0.368; ES-4: OR=1.7, CI: 0.6-4.6; p_{BH}=0.415). Void-roosting bats, namely *Nyctalus noctula, Myotis daubentonii, Myotis blythii,* and *Miniopterus*

schreibersii, exhibited adult PCR positivity above 43%, whilst crevice- and comprehensive-roosting strategies occurred across the entire spectrum of positivity levels (Fig. 5).

Species foraging over water had almost seven to eight-fold higher odds of PCR positivity than bats foraging in cluttered areas, consistently in adults and the whole sample (models EA-5: OR=7.5, CI: 2.3-25.0; p_{BH}=0.005; ES-5: OR=8.4, CI: 2.6-26.9; p_{BH}=0.004). Among the remaining feeding strategies, gleaning did not differ from the reference group (models EA-5: OR=1.2, CI: 0.5-3.2; p_{BH}=0.688; ES-5: OR=1.4, CI: 0.6-3.6; p_{BH}=0.502). In the model based on adults only, open-space foraging showed a significant effect on PCR positivity (model EA-5: OR=8.8, CI: 2.4-32.1; p_{BH}=0.005). In contrast, the whole sample demonstrated lower odds ratios with CI approaching 1, rendering the association inconsiderable (model ES-5: OR=4.5, CI: 1.3-15.1; p_{BH}=0.033, Fig. 6). Species foraging in open space and over water displayed *Leptospira* PCR positivity above 40%, whereas gleaning and cluttered-area foraging species spanned the full range of observed PCR positivity rates, from near zero to about 50% (Fig. 5).

Migratory behaviour was not robustly associated with PCR positivity. Medium-range migrants exhibited nearly five-fold higher odds of testing positive relative to the short-range migrants independent variable and reached nominal significance (models EA-6: OR=5.1, CI: 1.1-24.4; p_{BH}=0.059; ES-6: OR=4.8, CI: 1.1-21.3; p_{BH}=0.070). However, this variable is perfectly collinear with the water-foraging strategy factor (Supplementary Data S2). Long-range migrants did not differ significantly from the short-range group (model EA-6: OR=2.8, CI: 0.8-9.9; p_{BH}=0.130; model ES-6: OR=1.4; CI: 0.5-4.2; p_{BH}=0.564).

Species with low *Leptospira* PCR positivity shared several ecological features: small numbers of individuals in summer colonies, no recorded male colonies, short-range migration, and gleaning or cluttered-area foraging strategies (Fig. 5).

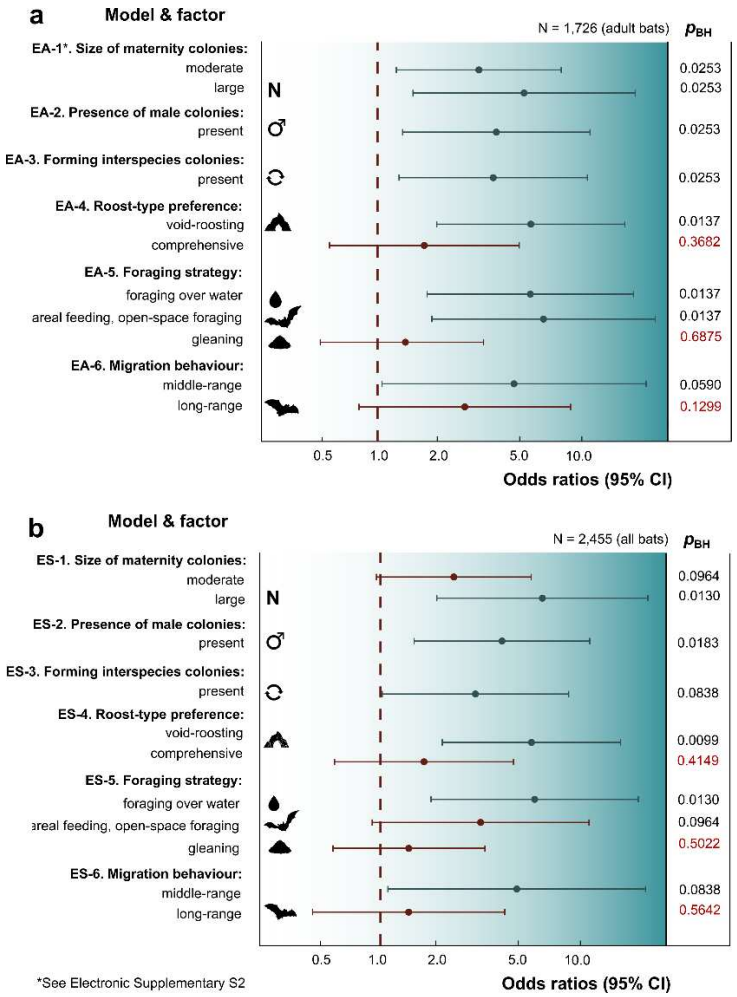


Fig. 6. Odds ratios (OR) with 95% confidence intervals for the ecological models for adult bats (A) and the whole sample (B). An OR greater than 1 indicates higher odds of testing PCR-positive relative to the reference category of the variable, whereas an OR below 1 indicates lower odds; a confidence interval (CI) that does not cross 1 indicates that the effect is statistically supported (green dots). Numbers in the right-hand column are the *P* values adjusted for multiple comparisons using the Benjamini–Hochberg procedure.

Genetic diversity of *Leptospira*

Most of the PCR-positive samples with Ct values from 15.20 to 25.91 that were selected for sequencing (51 out of 72) did not yield PCR amplification of at least one of the four chosen MLST loci, even when newly designed primers were used (Supplementary Methods). Amplification of all four loci was unsuccessful in 12 urine samples obtained from *Miniopterus schreibersii*, two from *Nyctalus noctula*, and one each from *N. leisleri, Myotis blythii, M. petax, Rhinolophus euryale, R. ferrumequinum,* and *R. hipposideros*. As a result, 32 sequences were obtained for secY, 27 for pntA, 30 for LipL32 and 30 for LipL41 (Supplementary Table S3).

In a few chromatograms, multiple peaks were detected, suggesting that a single bat may harbour more than one *Leptospira* species. Moreover, the obtained sequences showed that bats of one species collected from the same site may carry different *Leptospira* species (for example, *Myotis dasycneme* from Tyumen Region excreted nucleic acids belonging to the *L. interrogans*-related group and *L. borgpetersenii*-related cluster).

The majority of bat sequences, obtained in the present study and downloaded from GenBank, based on each of the four genes, were related to *L. borgpetersenii* (peach in Fig. 7, 8, Supplementary Table S3). This lineage comprises all non-bat sequences of this species, as well as most of the sequences from Madagascar, Comoros, South Africa, Vietnam, and, to a lesser extent, China and Japan. Among samples analysed in the present study, leptospires obtained from some *Myotis dasycneme, M. daubentonii*, and *Miniopterus schreibersii* individuals clearly belong to *L. borgpetersenii*, whereas predominantly Asian samples (Japan, China, Kazakhstan, and Russian Western Siberia) with a single sample from Northwestern Russia (Leningrad Region) appear to form a sister clade designated the '*borgpetersenii*-like group' (orange in Fig. 7, 8). All of these samples were obtained from *Myotis* bats. In addition, several sequences from Chinese and Japanese bats separate into a second '*borgpetersenii*-like' group (yellow in Fig. 7).

Sequences clearly belonging to *Leptospira kirschneri* were found in only a few bat specimens: *L. kirschneri* was detected in *Miniopterus natalensi*s (A. Smith, 1833) and *Miniopterus griffithsi* Goodman, Maminirina, Bradman, Christidis & Appleton, 2010 from South Africa, as well as in *Plecotus ognevi* from the Russian Far East (dark green in Fig. 7, 8). The following *Myotis* samples could be attributed to the *'kirschneri*-like' group (light green in Fig. 7, 8): *M. dasycneme* from Northwestern Russia and from Western Siberia, *M. blythii* from Crimea, and *M. petax* from Western Siberia.

Leptospira interrogans was not identified in our samples, but it was found in most bats from Brazil and the Czech Republic, and in *Vespertilio sinensis* and *Rhinolophus cornutus* Temminck, 1834 from Japan (deep indigo in Fig. 7, 8).

The remaining sequences can be classified as independent genetic lineages. One lineage probably represents an unknown *Leptospira* species most closely related to *L. kirschneri* and *L. interrogans*, and includes samples obtained from *Eptesicus nilssonii* and certain *Myotis* species (Fig. 7, 8, the '*nilssonii*' group coloured in aquamarine). Although this group is sister to *L. kirschneri* and *L. interrogans*, it forms a distinct cluster in all networks and comprises only samples from bats, supporting its recognition as a valid lineage.

Another distinct group is formed by sequences obtained from *Nyctalus* spp. (Fig. 7, 8, the '*noctula*' group, shown in red), with similar samples found in *Vespertilio murinus, Eptesicus serotinus,* and *Pipistrellus pygmaeus*. Haplotypes of a single *Pipistrellus pygmaeus* from Central Russia, *Vespertilio sinensis* from Japan, and *Triaenops afer* Peters, 1876 from Mozambique form a sister group. Notably, according to secY and LipL41, samples from the '*noctula*' group are most closely related to *Leptospira borgpetersenii*. However, based on pntA and LipL32, this group is closely related to *L. interrogans* and *L. kirschneri*.

Samples from Brazil may represent two distinct unknown *Leptospira* species (shown grey in Fig. 7), including one closely related to the '*nilssonii*' group.

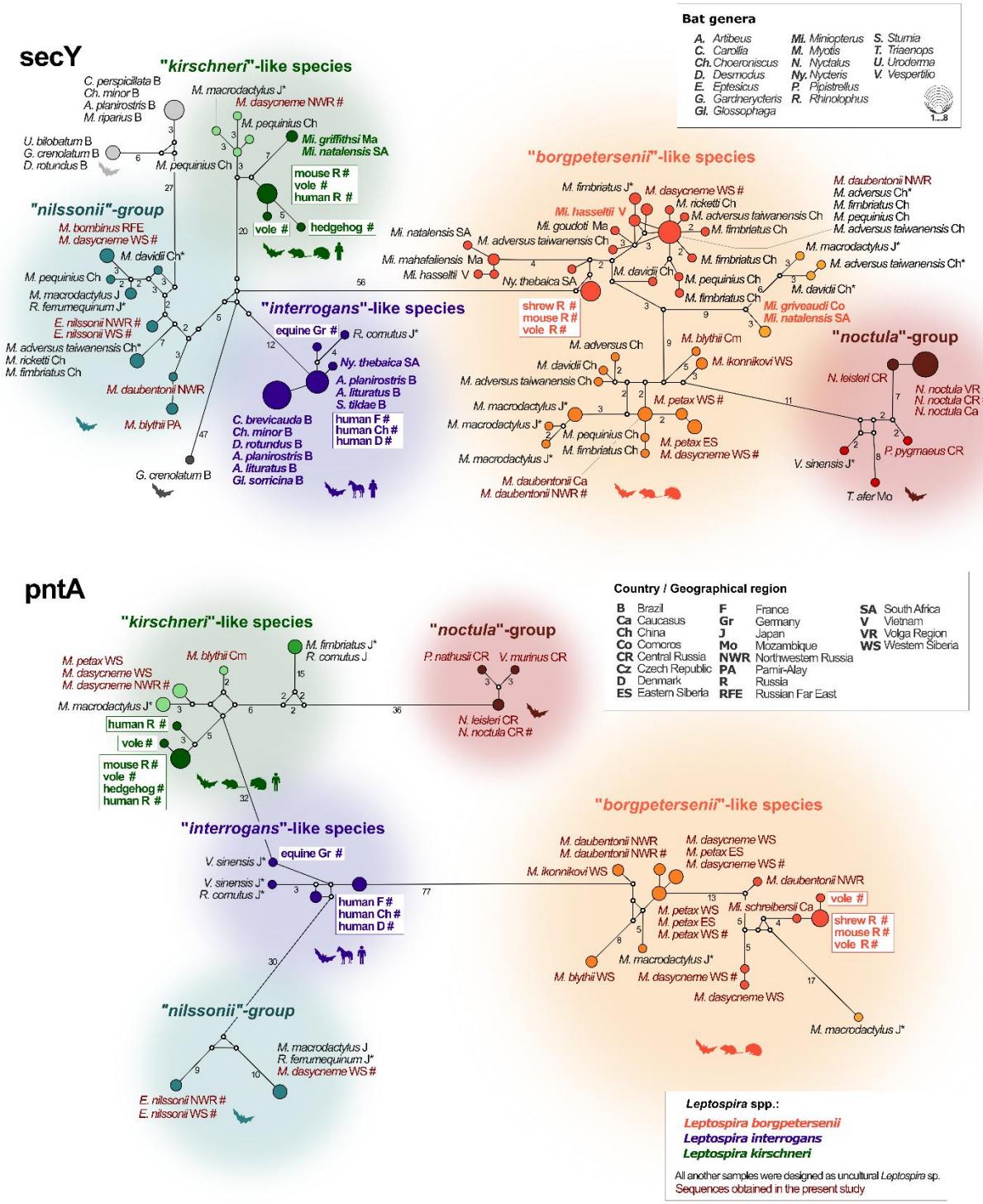


Fig. 7. Haplotype network of *Leptospira* spp. based on secY and pntA sequences. Each circle represents a unique haplotype, and its size is proportional to the number of sequences. Colours within circles correspond to different genetic lineages; length of the lines between circles represents the number of mutational steps separating the haplotypes (the grey numerals above the line indicate the exact number of nucleotide substitutions). White frames were used for non-bat samples. Samples marked with hash (#) were analysed in all four genes each; with asterisk (*) are GenBank samples for which two genes are known. Sequences obtained in the present study are coloured in red. GenBank accession numbers for all samples are given in Supplementary Table S3.

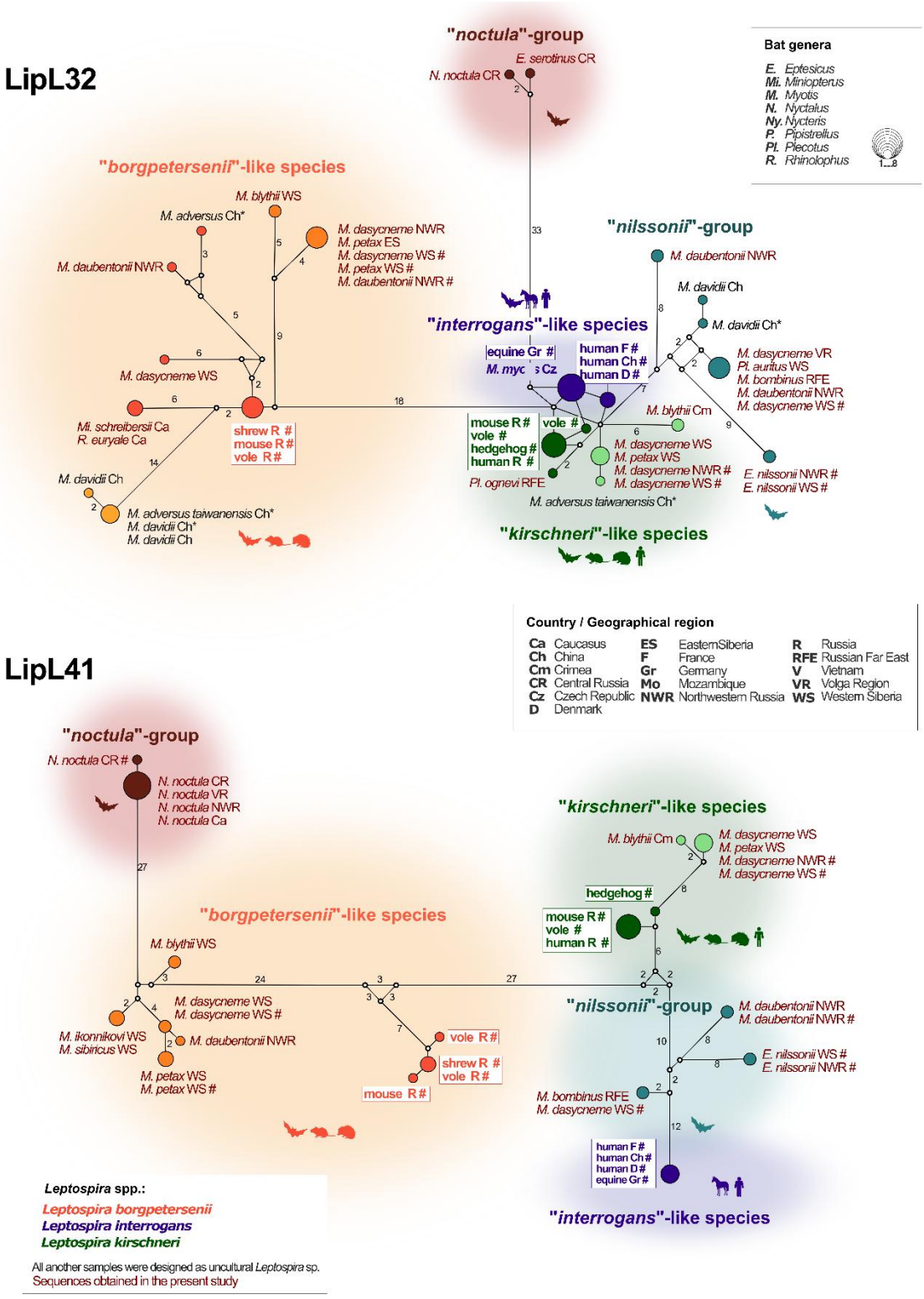


Fig. 8. Haplotype network of *Leptospira* spp. based on LipL32 and LipL41 sequences. Each circle represents a unique haplotype, and its size is proportional to the number of sequences. Colours within circles correspond to different genetic lineages and match those used in Fig. 7; length of the lines between circles represents the number of mutational steps separating the haplotypes (the grey numerals above the line indicate the exact number of nucleotide substitutions). White frames were used for non-bat samples. Samples marked with hash (#) were analysed in all four genes each; with asterisk (*) are GenBank samples for which two genes are known. Sequences obtained in the present study are coloured in red. GenBank accession numbers for all samples are given in Supplementary Table S3.

For samples with two or more loci attributed to a specific genetic lineage, the within-group and between-group p-genetic distances were calculated (Supplementary Table S4). Within-group distances did not exceed 3% for any locus, whilst between-group divergences varied depending on the gene. For the secY and pntA, the range of values was from 4% to 19%, and for the LipL32 and LipL41, from 2% to 15%. Nonetheless, the '*noctula'* group yielded the highest between-group divergences across all loci, ranging from 6% to 19%, while within-group values were below 0.3%.

For the majority of sequences, no association was observed between genetic lineage and place of collection. For example, *Leptospira* samples obtained from *Eptesicus nilssonii* in Northwestern Russia and Western Siberia form a single haplotype. The only notable exception comprises identical samples obtained from *Miniopterus* and *Rhinolophus* captured in a single cave in the Caucasus.

Nevertheless, in most cases the same *Leptospira* species collected from bats of different species are reliably distinguishable by Fst, with a couple exceptions (Supplementary Table S4). Thus, the *'borgpetersenii*-like' sequences obtained from *Myotis daubentonii* (Northwestern Russia) and *M. petax* (Western Siberia) did not differ in LipL32, nor did the second '*borgpetersenii*-like*'* group sequences obtained from *M. adversus* and *M. davidii* (China). Only minor differences in the secY gene were found among *L. borgpetersenii* sequences obtained from the Chinese *Myotis adversus*, *M. fimbriatus*, and *M. pequinius.*

Nucleotide diversity (π) was high for all loci studied: 8.57% for secY, 10.82% for pntA, 5.82% for LipL32, and 9.16% for LipL41. The principal contribution to genetic diversity is due to intergroup differences between *Leptospira* species.

Discussion

Our research identifies 27 north-eastern Palaearctic bat taxa as PCR-positive for *Leptospira* (Fig. 2). We report the shedding of *Leptospira* nucleic acids in eight bat species that had not been previously investigated — *Nyctalus leisleri, Plecotus ognevi, Pipistrellus kuhlii, Myotis sibiricus, M. longicaudatus, M. ikonnikovi, M. petax,* and *M. bombinus* — and in nine species that had been reported only with negative results: *Eptesicus nilssonii, Plecotus auritus, Pipistrellus pygmaeus, P. nathusii, Vespertilio murinus, Myotis mystacinus, Murina hilgendorfi, Rhinolophus euryale*, and *R. hipposideros* [17, 24, 25, 26, 27, 28]. We have conducted the first analysis of *Barbastella leucomelas, Nyctalus lasiopterus, Myotis tschuliensis,* and *Hypsugo savii;* however, the small number of trapped individuals of these species does not allow any conclusions to be drawn. The overall percentage of *Leptospira* shedding detected in the present study (24.44%) corresponds to that recorded in insectivorous bats worldwide (21.93%) [17].

For Palaearctic Chiroptera, several species showed *Leptospira* PCR positivity exceeding 20% and thus could be considered important *Leptospira* reservoirs: *Myotis daubentonii, M. petax, M. macrodactylus, M. dasycneme, M. blythii, Nyctalus noctula, N. leisleri, Pipistrellus pygmaeus, P. nathusii,* and *Miniopterus schreibersii*. According to published data, we could also add to this list *Myotis myotis* (Borkhausen, 1797), *M. fimbriatus* (Peters, 1871), *M. pequinius* Thomas, 1908, *M. davidii,* and *Miniopterus fuliginosus* [29, 30, 31, 32, 33, 34]. In contrast, *Myotis myotis* and *Miniopterus schreibersii* — species positive for *Leptospira* across Europe [our data, 25, 31, 32] — while trapped in Italy were entirely PCR-negative [28]. It can be best explained given that the urogenital swabs used in the Italian study appear to be unsuitable for *Leptospira* detection. It should also be noted that Kiuno et al. [34] claim *Miniopterus schreibersii* to be a highly infected bat in Japan. However, this European species does not occur in Japan. We therefore consider that these records may be attributable to misidentified *Mi. fuliginosus*, which in 2004 proved to be a distinct species on the basis of morphology, distribution, and phylogeny [35]. The same confusion happened with records of *Miniopterus schreibersii* gathered in China [30].

Some bat taxa showed relatively low spirochaete shedding percentages (11–16%) but appeared to excrete *Leptospira* nucleic acids consistently across different sites. These are, in order of increasing PCR-positivity: *Myotis bombinus, Vespertilio murinus, Murina hilgendorfi, and Plecotus ognevi.* The remaining species — *Barbastella barbastellus, Eptesicus nilssonii, Plecotus auritus, Myotis nattereri, M. mystacinus,* and *M. brandtii* — exhibited lower PCR rates (0–10%). In light of the above, we expect bats to have different species-dependent susceptibility to this bacterium and agree with Matthias et al. [18] and Dietrich et al. [16, 36] regarding specificity in the bat-*Leptospira* relationship. Similarly, differences in susceptibility to this spirochaete are documented among small terrestrial mammals [8, 37, 38]. Lei & Olival suggested that differences in ecological traits between bats and rodents may be the key point stipulated host specificity [39].

Our results established a significant association with bat age: subadults of all studied species exhibited lower infection rates than adults. Matthias et al. [18] and Dietrich et al. [16] reported PCR-negative results for first-year bats. Aguillon et al. [21] hypothesised that juveniles are protected against *Leptospira* by maternal antibodies. Nonetheless, in the present study a substantial number of bats aged approximately one to two months were already shedding spirochaetes, indicating that if such antibody-moderated protection occurs, it is brief or incomplete. An alternative explanation is that juveniles infected with *Leptospira* may exhibit a severe form of infection and therefore

not be captured by researchers, as has been shown for young rodents [37]. Moreover, our study and the published data [21] revealed lower infection rates in first-two-year individuals, suggesting the lifetime accumulation of pathogen exposure.

This idea is further supported by observations on ringed bats that changed their PCR results from negative to positive after several months. However, when trapping animals in the wild, it is always challenging to determine how long an individual has been carrying *Leptospira* and whether the PCR result reflects cycles of clearance and reinfection or consistent bacteria shedding. For example, in rats inoculated with strains non-virulent for rodents — derived from tenrecs and bats — leptospires failed to establish renal colonisation or cause disease, though urinary shedding was nonetheless detected within one to two weeks [40]. Moreover, by forming a kidney biofilm, the spirochaetes may not be excreted in urine [41], which might be the reason for switching from positive to negative for one of the ringed *Myotis daubentonii*.

Both our data and those presented by Aguillon et al. [21] show a low probability of cessation of spirochaete shedding in bats that tested positive at the first capture (Fig. 4). In small terrestrial mammals, *Leptospira* renal colonisation can persist throughout the animal's lifetime [37]; in bats, continuously urinary shedding of this bacterium have been observed for at least five months [24].

Neither the present research nor the study of Grenada bats [42] detected seasonal differences in *Leptospira* prevalence. Only a slight increase in positivity was observed in summer, but this effect is not as evident as annual variation in *Leptospira* shedding recorded in continental and insular African bats [16, 19, 21, 38], which increase their positivity during the reproductive season.

Besides species and age differences, Hayman et al. [43] proposed that *Leptospira* infection may depend on ecological functions, colony density, feeding and migration behaviour. During the present research, statistical analysis revealed four ecological traits to be significantly associated with increased odds ratios of *Leptospira* PCR positivity: forming large maternity colonies, having male colonies, void-roosting behaviour, and foraging over water.

All the species known to form large summer colonies demonstrated high levels of infection, whilst those roosting in small aggregations exhibited low infection prevalence. Nonetheless, among highly infected taxa some exceptions occurred: *Myotis daubentonii* and *Nyctalus leisleri* do not usually form numerous clusters during summer. In the New World, studies of *Carollia perspicillata* and *C. brevicauda* showed that bats living in large colonies can be characterised by low infection rates [18]. Notably, observations of the Réunion Island bat *Mormopterus francoismoutoui* Goodman, van Vuuren, Ratrimomanarivo, Probst & Bowie, 2008 [21] indicate that, among gregarious Chiroptera, *Leptospira* prevalence does not increase with colony size, in contrast to our findings for *Myotis blythii,* where adult positivity was higher in a larger colony.

However, the statistical significance of male colony presence supports the idea of coloniality influence. Among Palaearctic Chiroptera, females of all species form breeding colonies, while males of most taxa usually roost solitarily or in small, unstable clusters [7]. Although for species where both sexes are gregarious, generally high infection rates and, more importantly, no distinguishable difference in PCR positivity between sexes were observed.

Once shed in the roost, *Leptospira*, owing to its corkscrew-like motility, can penetrate host mucosa relatively easily [2]. Bats switch day roosts regularly [44], but that does not necessarily affect *Leptospira* persistence, as the bacteria can survive for weeks in wet soil and more than half a year in suitable pH, temperature, and moisture conditions [15]. Facilitating *Leptospira* persistence, temperatures in the nursery roosts are consistently elevated to compensate for the limited thermoregulatory capacity of juveniles. High and stable humidity is also a critical factor, as maintaining water balance is crucial for the survival of juveniles. Moreover, some chiropteran shelters are not merely wet, but completely dripping [authors' unpublished data, 5].

Nevertheless, conditions in certain roosts do not always correspond directly to *Leptospira* prevalence. We found *Myotis blythii* with high PCR positivity roosting in abandoned quarries in Crimea, where there was a lack of water and soil on the concrete floor. Under such conditions, *Leptospira* is unlikely to persist for a prolonged period. In light of this fact, roost-switching behaviour may even facilitate spirochaete infection, as in other shelters conditions might be more suitable for the bacteria.

According to our data, void-roosting, namely roosting in caves, tree holes or lofts, appeared to be significantly associated with high *Leptospira* positivity. Igreja has reported caves as a suitable environment for *Leptospira* transmission [45]. Indeed, *Myotis blythii, M. myotis,* and *Miniopterus* spp., all cave-dwelling bats, demonstrate high infection rates. Yet, an alternative explanation for these particular species might be their ability to form large summer colonies. To prove or refute the association with cave roosting, a more in-depth study of *Rhinolophus* bats — which are also troglophilic — is required.

Foraging behaviour demonstrates an ambiguous association with *Leptospira* infection. Despite the view that soil is the primary reservoir habitat for *Leptospira*, gleaning — actually foraging with landing on the ground during hunting — was not significantly associated with *Leptospira* positivity. Conversely, the open-space foraging group, namely species flying high above any substrate where the spirochaete can be stored, comprises only taxa that exhibit high *Leptospira* PCR-prevalence. The assumption of higher *Leptospira* positivity among species associated

with foraging over water has been discussed previously [27, 29]. This connection is strongly supported by our findings. At the same time, bats that hunt over water may simply prefer roosts with a wetter microclimate that happens to favour *Leptospira* persistence. Also, according to a meta-analysis by Esteves et al. [17], roosting habitat plays a more important role than feeding habits.

Migration activity seems to have limited influence on *Leptospira* prevalence. Our study found no significant differences in PCR positivity between species undergoing long- or short-distance migration. The group of medium-range migrants — shown to be nominally more heavily infected — coincided entirely with the foraging-over-water group (*Myotis dasycneme, M. petax,* and *M. daubentonii*), which makes it difficult to disentangle the two effects. Becker et al. [46] suggested that longer migrations between wintering and maternity grounds may be associated with weaker immune responses due to the redistribution of resources away from immune systems toward increased body fat and metabolic demands during long-distance flight. In fact, some long-range migrating taxa, *Pipistrellus* spp. and *Nyctalus* spp., demonstrated high *Leptospira* PCR prevalence. Another long-range migrant, *Vespertilio murinus*, had intermediate positivity rates. Nonetheless, it shows a tendency to remain hibernating at the breeding site even in a relatively harsh climate [47].

Ultimately, bat aggregation habits appear to be the most intriguing part of pathogen-associated ecology. Gomard et al. [20] also proposed that inequality in *Leptospira* prevalence among bat families is the result of diverse roosting ecology. Moreover, ecological traits might shape *Leptospira* susceptibility not independently, but in combination with one another.

In the present study, *Leptospira* shed by Chiroptera belonged to the P1 clade. The species *Leptospira kirschneri*, *L. interrogans*, and *L. borgpetersenii* — dominated by the latter — together with genotypes closely related to these species were shown to persist in bats worldwide [17, 19]. Most GenBank bat *Leptospira* sequences corresponded to the secY gene (Fig. 7); consequently, its haplotype network is the most complete, encompassing nine genetic lineages.

A large number of studies, with ours being no exception, report *Leptospira* that could not be confidently assigned to any known species [20, 29, 38, 48, 49]. In the present research, the '*nilssonii*' and '*noctula'* groups are of particular interest. *Leptospira* of the latter group is most closely related to *L. borgpetersenii* according to secY and LipL41, but to *L. interrogans* or *L. kirschneri* based on pntA and LipL32 — these species belonging to different genetic clades within the P1 pathogenic clade of *Leptospira*. This may indicate either horizontal gene transfer — a phenomenon common in bacterial evolution [50] — or earlier divergence from an ancestral group. Moreover, we were unable to obtain sequences of pntA and LipL32 from most *N. noctula* samples, suggesting that mutations may occur within the regions of the genes used for primer placement. It appears that traditional primer sets from MLST schemes cannot be applied to bat-borne *Leptospira*. Obtaining complete MLST data, or at least sequencing of two genes, from every PCR-positive bat failed not only in the present study, but also in several others [20, 29, 34, 48, 49]. We fully agree with Cordonin et al. [49], who emphasise the need for full genome sequencing of bat-borne *Leptospira*.

Possible co-infection with different *Leptospira* species in bats was found both in the present study and in Papua New Guinea [51]. We observed potential inter-family *Leptospira* transmission in samples from *Miniopterus* and *Rhinolophus* collected in a single cave (Fig. 8). However, because of the low species-specificity of LipL32, we could not confirm the identities of these samples.

As bats collected in the present study have not been found to be infected with rodent-associated *Leptospira* genotypes and likely form their own genetic subgroups, *in vitro* studies using isolated bat's strains and serotype identification could help to understand their pathogenicity. Experiments may also shed light on whether species with consistently low infection rates have a transient *Leptospira* infection or demonstrate limited bat-to-bat transmission. To infer the connection between roosting behaviour and *Leptospira* positivity it is important to investigate the culture characteristics of these spirochaetes — comparing them with conditions of bat roosting sites. Some authors attribute variation in *Leptospira* host specificity to different mechanisms for escaping the host immune system across host taxa [52]; this knowledge might also help address some of the gaps in our understanding of ecological and genetic traits of this bacterium.

Methods

Ethical approval

Field procedures were carried out in accordance with local and international wildlife research guidelines. All authors complied with the ARRIVE guidelines. Bats were released safe and sound following measurements and samples collection. The Ethics Committee of the Zoological Institute of the Russian Academy of Sciences approved the conduct of this study for the period January 2021–December 2029 (Permission No. 1-11, 21 March 2024). Approval was also granted by the Commission for the Regulation of Experimental Research (Bioethics Commission) of the Federal Scientific Center of the East Asia Terrestrial Biodiversity (Permission No. 7, 16 March 2026). In Kazakhstan, bat trapping was allowed under the Permission No. 27-02-19/4999-KIKhZhM (18 July 2025).

Animal collection and studied area

Bats were collected across various parts of the north-eastern Palaearctic between 2021 and 2026 (Fig. 1; Supplementary Table S1). Throughout hibernation, animals were caught by hand in hibernacula, whilst over the warm season — when Chiroptera were actively flying — were trapped using mist nets or mobile traps. All captured animals were processed immediately after collection or kept individually in cotton bags to prevent contamination during non-sterile sampling.

Bats were identified morphologically following special keys [53, 54]. In ambiguous cases or for cryptic species, molecular identification based on the partial sequences of mitochondrial cytochrome B (CytB) was applied [55]. Primers and amplification programmes for bat species identification are presented in Supplementary Methods. Sex, age, and physiological condition (where possible) were recorded for each individual. For most bat species, age was estimated only during the summer period, when the phalanx epiphyseal gaps of younger specimens are visible. For *Myotis daubentonii, M. dasycneme,* and *M. petax,* age was estimated based on the presence and colour of the chin spot during the first two years of life [56]. According to the development of the epiphyseal joints, animals were divided into two age groups: subadults (independently flying juveniles) and adultus (sexually mature individuals over half a year old); for the aforementioned *Myotis*, age was recorded more precisely as subadult, first-year adult, second-year adult, or older than two years.

Some bats were banded with bat rings or bird rings with smoothed edges in order to infer *Leptospira* DNA/RNA shedding during subsequent recaptures at the same site.

The bat annual life cycle was defined as comprising hibernation, migratory flux, and summer activity. For each individual, the life stage was determined not only by the date of capture, but also by the circumstances of trapping such as collecting in a cave or on a flyway, and physiological state.

Trapping occurred within a large territory in the north-eastern Palaearctic (Fig. 1), encompassing nine studied regions: Northwestern Russia (Kaliningrad, Arkhangelsk, Leningrad, Vologda, Pskov regions, the city of Saint Petersburg, and the Republic of Karelia), central Russia (Bryansk and Kaluga regions), the Volga Region (Penza and Samara regions, the Republic of Mordovia, the Republic of Tatarstan), Crimea, the Caucasus (Krasnodar Krai [Territory], the Republic of North Ossetia–Alania, the Republic of Dagestan, and the Republic of Abkhazia), Western Siberia (Tyumen, Kurgan, Kemerovo, Novosibirsk, East Kazakhstan regions, Altai Krai [Territory], and the Altai Republic), the Pamir-Alai (Sughd Region), Eastern Siberia (Zabaykalsky Krai [Territory], Irkutsk Region, the Republic of Buryatia, the Republic of Sakha), as well as the Russian Far East (Jewish Autonomous Region and Primorsky Krai [Territory]). European regions were divided as smaller areas due to the bigger trapping size within the territory. For a more detailed description of the study area, see Supplementary Table S1.

Urine collection

Approximately 10–100 µl of urine was obtained non-invasively from each individual bat. As demonstrated by Szentiványi et al. [57] no significant difference was observed between urine and tissue samples during *Leptospira* testing. In most cases, urine was collected with a glass capillary, placed in tubes, and frozen at −70 to −20 °C until testing. Once thawed, samples were processed immediately. In areas where electricity was unavailable, FTA cards were used (Fast Technology for Analysis of nucleic acids) and stored at room temperature until analysis. Prior to DNA/RNA extraction, card disks were minced with sterile scissors.

***Leptospira* detection**

DNA/RNA extraction was performed using a Magno-sorb M kit (Central Research Institute of Epidemiology, Moscow, Russia) for urine samples, and the RIBO-prep (Central Research Institute of Epidemiology, Moscow, Russia) for FTA cards. Leptospira detection was performed targeting the 16S rRNA gene fragment of pathogenic *Leptospira* species using the qRT-PCR Amplisens *Leptospira* sp.-FI Kit (Central Research Institute of Epidemiology, Moscow, Russia) according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

Amplification and sequencing of PCR-positive samples were performed using primers targeting the secY, pntA, LipL32, and LipL41 genes [58, 59]. Primer sequences are provided in Supplementary Methods. PCR products were visualised by electrophoresis with 1.7% agarose gel. Purified PCR products were sequenced bidirectionally using a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Austin, TX, USA) on an Applied Biosystems 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting nucleotide sequences were aligned with reference sequences downloaded from PubMLST [60] using MEGA 11 software [61]. The *Leptospira* secY, pntA, LipL32, LipL41, and cytB bat sequences obtained in this study have been deposited in GenBank (Supplementary Table S3).

519 A median-joining haplotype network was constructed using Fluxus Technology Ltd. NETWORK (version
520 10.2.) [61]. After alignment, the length of sequences used to construct the network was 362 bp to secY (125
521 samples), 522 bp to pntA (56 samples), 426 bp to LipL32 (61 samples), and 463 bp to LipL41 (46 samples). For
522 comparison, we used sequences of *Leptospira* interrogans (Stimson, 1907), *L. kirschneri* Ramadass et al., 1992,
523 and *L. borgpetersenii* Yasuda et al., 1987 obtained from humans, livestock, and small terrestrial mammals in
524 Eurasia [60], bat-borne *Leptospira* sequences from various countries [16, 19, 25, 26, 29, 30, 34, 49, 63, 64, 65]
525 and our own samples (Supplementary Table S3; Fig. 7,8).
526 The inter- and intragroup genetic p-distances were calculated in MEGA 11 for different *Leptospira* species
527 and genetic lineages only on samples that were assigned to the same group at two or more loci. The haplotype
528 (Hd) and nucleotide diversity (π), average number of differences (K), and genetic differentiation indices (Fst) were
529 calculated using DnaSP6 [66] (Supplementary Table S4).
530
531 **Bat ecological categories**
532 Average colony size, duration of each stage in the bats' annual cycle, foraging strategy, migration activity, and
533 other ecological features of the studied species were assessed using both literature [5, 7, 54, 67, 68, 69] and the
534 authors' unpublished data (see Supplementary Table S1). Several species are known to change their ecological
535 features in particular areas, and thus the categories were considered individually for all regions.
536 The estimated roosting features included average maternity roost density, presence of colonies with other bat
537 species (interspecies colonies), ability to form male colonies, and preferred roost type. Summer roost size was
538 estimated as small for bats forming nursery colonies for up to 10–30 individuals, moderate for up to 100, and large
539 from 100 up to thousands of bats. In assigning these categories, we used average colony size rather than the
540 maximum recorded bat number (for example, *Myotis dasycneme* and *Vespertilio murinus* were considered species
541 with moderate maternity colony size, notwithstanding colonies up to 500 individuals were occasionally recorded).
542 Gregariousness in winter was not taken into account. Preferred summer roosting types were classified according
543 to *Bat Roosts in Trees: A Guide to Identification and Assessment for Tree-Care and Ecology Professionals* by
544 Rotherham [5]. This classification comprises void-roosting and crevice-roosting types, as well as a comprehensive
545 type for species that follow both roosting strategies. For seven bat species, their preferred roost type was unknown
546 (Supplementary Table S1), so these bats (34 individuals) were excluded from roosting model sample. Presence of
547 male or interspecies colonies was considered not according to published data only, but also using authors' field
548 experience.
549 Foraging strategies were classified based on a synthesis of findings by Kruskop [67] and Fenton &
550 Bogdanowicz [70]. Kruskop, on the basis of wing morphology, highlighted three foraging types — gleaning,
551 cluttered-area foraging, and open-space foraging — ranking each category by the remoteness from substrate (soil
552 or water). Fenton & Bogdanowicz [70] based on literature data and authors' bat measuring suggested four hunting
553 groups: gleaning, trawling, foraging over water, and aerial feeding. Given that *Leptospira* can persist in both water
554 and damp soil [15], we reasoned that bats' attachment to both substrate types during hunting can play a substantial
555 role in pathogen transmission. Therefore, we divided our sample as: aerial feeders with cluttered-area foraging
556 (*Barbastella, Eptesicus, Hypsugo,* most *Myotis, Pipistrellus, and Vespertilio*), foraging over water (*Myotis*
557 *dasycneme, M. daubentonii, M. macrodactylus, M. petax*), aerial feeders with open-space foraging (*Miniopterus*
558 and *Nyctalus*), and gleaning (*Murina, Plecotus, Rhinolophus, Myotis blythii, M. bombinus, M. emarginatus, M.*
559 *nattereri,* and *M. tschuliensis*). *Vespertilio murinus* according to Kruskop [67], was considered a cluttered-area
560 forager; however, as he himself notes, and as we also observed during the author's field research, it can equally
561 be treated as an open-space forager.
562 Migration activity types were divided into three categories according to average flight distances. Short-range
563 migrants — bats that do not typically undertake long flights, wintering and breeding within 30–50 km (up to a
564 maximum of 150 km) — comprise the genera *Plecotus*, *Eptesicus*, *Barbastella*, *Rhinolophus*, *Murina*, *Hypsugo*,
565 *Miniopterus*, and most *Myotis*; medium-range migrants, which move between summer and winter roosts within one
566 to three regions (usually 100–500 km) belong to species *Myotis daubentonii*, *M. petax*, and *M. dasycneme*; and
567 long-range migrants, whose seasonal flux may cover 1,000–2,500 km or more, comprise the genera *Vespertilio*,
568 *Nyctalus*, and *Pipistrellus*. Within the migration types two subtypes were identified: partial, where part of the
569 population migrates and the remainder stays within the territory of summer roosts; and differential, where only bats
570 of a particular sex, usually females, undertake migration.
571 We assume that for each of the studied bats certain parameters may be more or less likely according to the
572 bat's age, physiological status, features of the landscape or prey abundance. We anticipated that the large number
573 of individuals collected would smooth out these differences.

Statistical analysis and visualisation

To infer any associations with ecological features of various bat species we did not use exact Ct values, instead calculating PCR results as positive or negative. This was consciously conducted owing to the fact that concentration of *Leptospira* in urine could vary significantly depending on whether spirochaetes in the kidneys are in biofilm or planktonic form [41].

Leptospira PCR positivity was analysed using generalised linear mixed-effects models (GLMMs) with a binomial distribution and logit link function, implemented in the lme4 package (version 2.0-6 [71]) in R (version 4.6.1 [72]). Chiroptera species identity was included as a random intercept to account for clustering of observations within species. Models were fitted by maximum likelihood using the Laplace approximation.

The selection of covariates was informed by a directed acyclic graph. Species was considered an important source of confounding because species identity is associated with ecological characteristics as well as immune and evolutionary traits that may influence susceptibility to *Leptospira* infection [46]. Accordingly, species identity was included as a random effect when evaluating ecological predictors.

Analyses were conducted in two stages. First, associations between PCR positivity and basic bat characteristics were examined, including species (n=2,455), sex (n=2,409), age (n=2,294), and stage of the annual cycle (n=2,455) (Supplementary Data S2). Second, species-level ecological characteristics were evaluated, including migratory behaviour, foraging strategy, summer roost size and type, and the ability to form male and interspecies colonies. These models included species as a random intercept.

Because infection prevalence and immune responses may differ between age groups [21], ecological analyses were performed both in the full dataset (n=2,455) and after exclusion of subadult individuals (n=1,726) (Supplementary Table S1).

Sample size varied among analyses because some characteristics were unavailable for individual bats. Analyses were therefore carried out using complete observations for the variables included in each model; no missing values were imputed.

Associations from the GLMMs were evaluated using Wald z-tests. Effect sizes for categorical predictors were reported as odds ratios (ORs) with 95% confidence intervals (CIs) (Supplementary Data S2). For analyses of species differences and ecological characteristics, *P* values were additionally adjusted for multiple comparisons using the Benjamini–Hochberg procedure. Because these analyses were exploratory, the resulting *P* values < 0.10 were considered indicative of evidence for an association [73].

Pearson's chi-square (χ^2) tests were additionally used to compare PCR-positive proportions between sex and age groups. Observed proportions are presented with exact 95% CIs calculated using the Clopper–Pearson method.

Results were visualised using the vcd (version 1.4-14 [74]), ggplot2 (version 4.0.3 [75]), and viridisLite (version 0.4.3 [76]) R packages. For map visualisation, QGIS Geographic Information System (version 3.34.3-Prizren [23]) was used.

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Author contribution

A. L. V. and E. S. M. conceived and designed the study. Field samples were collected by A. L. V., M. S. M., D. G. S., V. A. K., E. A. K., I. G. B., U. V. G., A. A. M., D. V. K., and O. A. S.. The laboratory survey was conducted by A. L. V. and E. S. M.. Chiropteran ecological features were investigated by D. G. S., U. V. G., A. L. V., M. S. M., and I. G. B. and their statistical analysis was conducted by M. S. M., A. L. V., and D. G. S.. Sequence analysis was performed by U. V. G., A. L. V., and E. S. M.. Figures were prepared by M. S. M., A. L. V., U. V. G., V. A. K., and I. G. B.. A. L. V. wrote the main manuscript text with revisions provided by E. S. M., D. G. S., I. G. B., and V. G. A.. All the authors reviewed the manuscript and gave their approval for publication.

Data availability

The collected bat data are available in the Supplementary Table S1. All sequences obtained in this study have been deposited in GenBank under accession numbers: PZ899970-PZ900001 (secY *Leptospira*), PZ900002-PZ900028 (pntA *Leptospira*), PZ900029-PZ900058 (LipL32 *Leptospira*), PZ900059-PZ900088 (LipL41 *Leptospira*), and PZ900093-PZ900099 (cytB bats).

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary Table S1: Database of captured bats and their ecology
Supplementary Data S2: Statistical analysis
Supplementary Table S3: Leptospira sequences used in the present study
Supplementary Table S4: Calculation of *Leptospira* samples genetic distances
Supplementary Methods: Primers and amplification conditions

Correspondence and requests for materials should be addressed to A.L.V.

Figure legends

Fig. 1. Map of the study regions of the north-eastern Palaearctic, with dots indicating the exact bat-trapping sites. Regions where collection took place are shown in red. The map was created using QGIS [23].

Fig. 2. Bat species trapped in the various regions (teal; sample sizes of fewer than 10 individuals are indicated by numbers) and *Leptospira* PCR positivity in tested urine samples (red). Numbers in the right-hand column represent the sample size for each species, given as the number of PCR-positive bats/number of bats collected.

Fig. 3. *Leptospira* PCR positivity (%) in each sex and age groups in six bat species. The proportions within each square represent age (adults on the left, subadults on the right) and sex (males at the bottom, females at the top), and the numbers indicate *Leptospira* prevalence within each group. Results of Pearson's chi-squared (χ^2) goodness-of-fit test for differences in *Leptospira*-positivity between the sex and age groups are shown in green where differences are significant and in red where they are not. The pictograms correspond to the ecological features examined in the present study.

Fig. 4. *Leptospira* PCR results for recaptured bats, by species, ring number, and sex. Rings whose numbers begin with letters are bird rings with smoothed edges, marked with the index 'Moskva'; the remainder are bat rings bearing the index 'Russia'. Each dot represents a capture date and the connecting lines indicate the interval between recaptures for each individual. PCR-negative results are shown in green, PCR-positive results in red. Months coloured in white represent hibernation, and those in green indicate bat summer activity (no ringed bats were trapped during flux periods).

Fig. 5. Bat ecological traits and *Leptospira* PCR positivity, with 95% Clopper–Pearson confidence intervals. Green dots indicate species with *Leptospira* PCR positivity below the global average for insectivorous bats (<22%) [17]. The pictograms correspond to the ecological features examined in the present research. Only data from adult bats were included in the figure; species with fewer than 25 adults were excluded.

Fig. 6. Odds ratios (OR) with 95% confidence intervals for the ecological models for adult bats (A) and the whole sample (B). An OR greater than 1 indicates higher odds of testing PCR-positive relative to the reference category of the variable, whereas an OR below 1 indicates lower odds; a confidence interval (CI) that does not cross 1 indicates that the effect is statistically supported (green dots). Numbers in the right-hand column are the *P* values adjusted for multiple comparisons using the Benjamini–Hochberg procedure.

Fig. 7. Haplotype network of *Leptospira* spp. based on secY and pntA sequences. Each circle represents a unique haplotype, and its size is proportional to the number of sequences. Colours within circles correspond to different genetic lineages; length of the lines between circles represents the number of mutational steps separating the haplotypes (the grey numerals above the line indicate the exact number of nucleotide substitutions). White frames were used for non-bat samples. Samples marked with hash (#) were analysed in all four genes each; with asterisk (*) are GenBank samples for which two genes are known. Sequences obtained in the present study are coloured in red. GenBank accession numbers for all samples are given in Supplementary Table S3.

Fig. 8. Haplotype network of *Leptospira* spp. based on LipL32 and LipL41 sequences. Each circle represents a unique haplotype, and its size is proportional to the number of sequences. Colours within circles correspond to different genetic lineages and match those used in Fig. 7; length of the lines between circles represents the number of mutational steps separating the haplotypes (the grey numerals above the line indicate the exact number of nucleotide substitutions). White frames were used for non-bat samples. Samples marked with hash (#) were analysed in all four genes each; with asterisk (*) are GenBank samples for which two genes are known. Sequences obtained in the present study are coloured in red. GenBank accession numbers for all samples are given in Supplementary Table S3.

Supplementary Files

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- S5Methods.Primersandprogramms.docx
- S4CalculationofLeptospirasamplesgeneticdistances.xlsx
- S1TableDatabaseofcapturedbatsandtheirecology.xlsx
- S2Statisticalanalysis.html
- S3Sequencesusedinthepresentstudy.xlsx