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TARGETED SURVEILLANCE FOR EUROPEAN BAT LYSSAVIRUSES IN ENGLISH BATS (2003–06)

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ABSTRACT: In 2003–06, targeted (active) surveillance for European bat lyssaviruses (EBLVs) was undertaken throughout England, focusing on two species most likely to host these viruses, *Myotis daubentonii* and *Eptesicus serotinus*. Blood was sampled for the detection of EBLV-specific neutralizing antibodies and oropharyngeal swabs were taken for the detection of viral RNA or infectious virus in saliva. Between 2003 and 2006, 273 *E. serotinus* and 363 *M. daubentonii* blood samples were tested by the EBLV-1 or EBLV-2 specific modified fluorescent antibody neutralization test. The EBLV-2 antibody prevalence estimate was 1.0–4.1% (95% confidence interval [CI]; mean=2.2%) for *M. daubentonii*. European bat lyssavirus type 1–specific antibodies were detected only in a single *E. serotinus*. Other nontarget species ($n=5$) were sampled in small numbers ($n=24$), with no EBLV-specific antibody detected. No viral RNA or live virus was detected in any of the oropharyngeal swabs analyzed. Host RNA was detected from 83% of the oropharyngeal swabs analyzed (total swabs 2003–06: $n=766$). These data show that EBLV-2 is present in *M. daubentonii* in England. In contrast, there is insufficient evidence to suggest that EBLV-1 is present in *E. serotinus* in England, although further research is warranted.

Key words: Bats, lyssaviruses, passive surveillance, targeted surveillance, rabies, United Kingdom.

INTRODUCTION

European bat lyssaviruses (EBLVs) were first identified in Europe in 1954 (Kappelar, 1989; Fooks et al., 2003), and over 850 cases have subsequently been recorded between 1977 and 2008 (Rabies Bulletin Europe, 2008). Most reports have involved EBLV-1 (Bourhy et al., 1992; Müller et al., 2007), and 95% of these were linked with serotines, especially *Eptesicus serotinus* (King et al., 2004). By contrast, EBLV-2 in bats has been reported only 20 times to date, in Daubenton's bats (*Myotis daubentonii*) and pond bats (*Myotis dasycneme*). Eight of these records are in *M. daubentonii* in the UK (Fig. 1) (Whitby et al., 2000; Johnson et al., 2003; Fooks et al., 2004a, b, 2006; Harris et al., 2007; Pajamo et al., 2008).

There have been three confirmed cases of EBLV in humans: one EBLV-1 case in Russia in 1985 (Selimov et al., 1989), and EBLV-2 cases in Finland in 1985 (Lumio et al., 1986) and Scotland in 2002 (Fooks et al., 2003). European bat lyssavirus type 1 is believed to have been responsible for an earlier case of bat-associated rabies in Ukraine in 1977, although the virus was not characterized. In all four cases, there was no record of prophylactic immunization against rabies. Spillover of EBLV-1 into sheep has occurred on two separate occasions in Denmark, in 1998 and 2002 (Ronsholt, 2002), and into a stone marten in Germany (Müller et al., 2004); EBLV-1 neutralizing antibodies also were detected in a domestic cat in Denmark (Tjornehoj et al., 2004). In 2007, the first case of rabies to infect a domestic animal in France (Fontenay-le-Comte) since the country was declared free of the disease in 2001 was diagnosed in a cat, and was believed to be an EBLV-1 of bat origin (Dacheux et al., 2009).

In recent years, confirmed (virus-positive) EBLV bat cases in Europe have increased in direct association with in-

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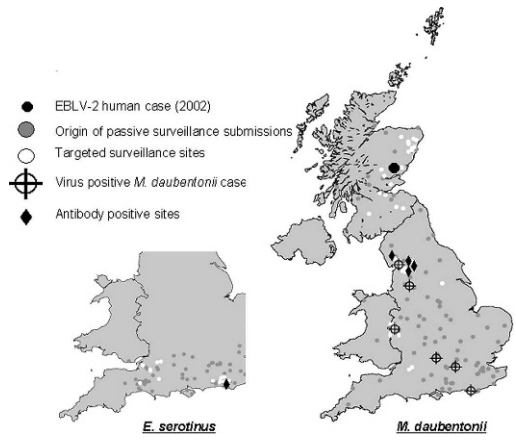


FIGURE 1. Origin of European bat lyssavirus type 2 (EBLV-2) virus-positive Daubenton's bats, origin of passive surveillance submissions, targeted surveillance sites (including antibody-positive sites in England), and location of the fatal human EBLV-2 case in 2002.

creased scanning (passive) and targeted surveillance, giving rise to renewed concern regarding the possibility of bats translocating the virus (Constantine, 2003), perhaps even across the English Channel to the UK. Scanning (passive) surveillance (submission of dead bats) was initiated in the UK in 1987, with tested submissions of over 7,800 UK resident bats, and 12 bats (of six species) of European origin (Harris et al., 2006a). However, passive surveillance is potentially compromised by biases, including geographic, species-specific variation in submission rates (Fig. 1) (Harris et al., 2006a).

Targeted surveillance was initiated in Britain in 2003, with a study in Scotland focusing on seroprevalence in *M. daubentonii*, where prevalence of 0.05% to 3.8% (95% confidence interval [CI]) was detected (Brookes et al., 2005). The Scottish research was augmented by this long-term study of EBLV seroprevalence in England during 2003–06.

The principal target species (*M. daubentonii*) was underrepresented by passive surveillance ($n=175$; ~3% of total submissions), and also showed a restricted geographic distribution of submissions (Harris et al., 2006a). Targeted surveillance of *M. daubentonii* in more than two EBLV-2 a priori areas and in random areas were seen as important steps in beginning to determine prevalence across Britain. The a priori sites were chosen because of their proximity to confirmed EBLV-2–infected *M. daubentonii* and therefore represented regions in which we anticipated finding seropositive bats. The random sites were in regions for which we had no prior data concerning EBLV infection. The serotine *E. serotinus* also was sampled because it is the main reservoir species for EBLV-1 in mainland Europe (Harris et al., 2006b). The other host of EBLV-2—*M. dasycneme*—only has occurred as a vagrant once in Britain (Harris et al., 2006a). In this paper, we report on the results of seroprevalence tests on blood from 273 *E. serotinus* and 363 *M. daubentonii* sampled in England during 2003–06. We also report rabies tissue-culture inoculation test (RTCIT) and real-time (RT) polymerase chain reaction (PCR) assay results for virus and viral RNA in saliva swabs taken from the majority of these bats.

MATERIALS AND METHODS

Sample collection

Between May 2003 and August 2006, 781 bats were caught and sampled at 31 sites across England (Fig. 1 and Table 1). Roosts, commuting routes, swarming sites, and foraging locations were visited, and bats were captured by harp trapping, static hand netting, and mist netting. Bats were identified to species, and their sex, reproductive status, age, body mass, forearm length, condition of fur, behavior, parasite load, and any preexisting injuries were recorded (Anthony, 1988; Hutson and Racey, 2004). A numbered identification ring (Mammal Society, London, UK) was fitted to avoid resampling individuals within 3 mo and to identify repeat captures across years. In this study, bats were identified by an expert based on morphologic findings; however, because genetic typing is a reliable alternative for differentiating between closely related species we also collected wing biopsies

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TABLE 1. Sampling sites, the numbers of bats (*Eptesicus serotinus* and *Myotis daubentonii*) tested by polymerase chain reaction and modified fluorescent antibody virus neutralization, and the number of antibody-positive samples.

County	Site number	Species	Number of bats tested by PCR/mFAVN[a]	Antibody-positive by mFAVN
Sussex	1	*E. serotinus*	18/18	
Sussex	2	*E. serotinus*	14/12	
Sussex	3	*E. serotinus*	52/51	2004: 1 single
Sussex	4	*E. serotinus*	3/0	
Sussex	5	*E. serotinus*	8/8	
Sussex	6	*E. serotinus*	1/0	
Sussex	7	*E. serotinus*	10/9	
Sussex	8	*E. serotinus*	1/0	
Somerset	9	*E. serotinus*	36/35	
Somerset	10	*E. serotinus*	21/21	
Somerset	11	*E. serotinus*	3/1	
Somerset	12	*E. serotinus*	15/15	
Somerset	13	*E. serotinus*	73/72	
Dorset	14	*E. serotinus*	27/27	
Wiltshire	15	*E. serotinus*	45/30	
		M. daubentonii	6/5	
Wiltshire	16	*M. daubentonii*	1/0	
Lancashire	17	*M. daubentonii*	83/52	
Lancashire	18	*M. daubentonii*	6/4	2003: 1 pool of two
Lancashire	19	*M. daubentonii*	75/61	
Lancashire	20	*M. daubentonii*	104/70	2003: 1 pool of two 1 pool of three 2005: 1 single
Lancashire	21	*M. daubentonii*	4/4	
Lancashire	22	*M. daubentonii*	21/21	
Lancashire	23	*M. daubentonii*	4/4	
Lancashire	24	*M. daubentonii*	3/3	
Lancashire	25	*M. daubentonii*	12/12	2003: 1 pool of three
Surrey	26	*M. daubentonii*	30/30	
Cumbria	27	*M. daubentonii*	30/29	2006: 2 singles
Yorkshire	28	*M. daubentonii*	34/33	
Yorkshire	29	*M. daubentonii*	1/0	
Yorkshire	30	*M. daubentonii*	1/1	
Yorkshire	31	*M. daubentonii*	1/0	

[a] PCR = polymerase chain reaction; mFAVN = modified fluorescent antibody virus neutralization.

and stored them for future analysis. A wing tissue punch (3 mm) (Stiefel Laboratories, distributed by Schuco International, London Ltd., London, UK) was taken from both wings of each bat (Worthington Wilmer and Barratt, 1996) for species identification by subsequent DNA analysis if necessary, and for population genetic studies. This approach has been recommended by a network of European scientists involved in active and passive bat lyssavirus surveillance (the European Union–funded Med-Vet-Net), and has been agreed under EUROBATS (Med-Vet-Net Working group, 2005).

Two mouth swabs were usually taken to collect saliva in order to determine the presence of EBLV: one swab stored at 4 C in 'RNA later' (Ambion Europe Ltd., Huntingdon, UK) for RT-PCR and the second in transport medium for virus isolation (L15 medium containing 2 mmol L-glutamine, 50 μg/ml of penicillin, 2 μg/m. of streptomycin, 2 μg/ml of nystatin, and 2% fetal calf serum [Sigma, St. Louis, Missouri, USA]). The swabs remained in these buffers until processed for laboratory analysis, at which time the liquid was aspirated and used directly in the relevant assay.

Finally, a blood sample was taken by puncture of the brachial or uropatageal vein, with target volumes of blood of 100 μl for *M. daubentonii*, and 140 μl for *E. serotinus*. After

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sampling, a rehydration fluid was offered to each bat (Lectade™, Beecham Animal Health, Brentford, Greater London, UK). Bats were released back into the roost (during the day) or at the area of capture (during night). Blood samples were stored at 4 C in the field. Serum was removed within 24–48 hr, with serum samples tested 1–28 days after sampling (mean=12.5; median=9.5). Serum was stored at 4 C if testing was to be completed within 2–3 days or below −20 C if testing was delayed (up to 28 days).

Capture, handling, ringing, and sampling all were undertaken under license from both the UK Home Office (UK Project Licenses PPL 30/1948 and PPL 60/3122, PIL 70/18233) and the relevant statutory conservation organization (Natural England).

Sample analysis

Isolation of host RNA and viral RNA: All saliva samples were analyzed by routine PCR methods to detect the presence of host RNA (Smith et al., 2000), viral RNA (Heaton et al., 1997), and live virus, as described in Brookes et al. (2005). In addition, a RTCIT described by Webster and Casey (1996) was used, including 100 µl of saliva/transport medium per well in duplicate wells on 96-well plates (single passage, 72 hr incubation).

When a blood sample was found to be seropositive, the corresponding saliva sample from that bat was additionally tested by mouse inoculation test (MIT), as described by Koprowski (1996). For the MIT, two 4-wk-old outbred CD1 mice (Charles River, Margate, UK) were injected intercerebrally with 40 µl of swab material (in transport medium) from each bat that was antibody positive. The MIT was performed according to Home Office guidelines (UK Project License PPL 70/4867 or PPL 70/6527), and mice were monitored for a minimum of 28 days before being killed humanely.

Modified fluorescent antibody virus neutralization (mFAVN) test: An mFAVN test was performed on individual or pooled serum samples (*E. serotinus* tested for EBLV-1, *M. daubentonii* and other *Myotis* species tested for EBLV-2) to detect levels of neutralizing antibodies in blood samples, with all samples tested in duplicate. Occasionally, it was necessary to pool serum samples to gain the minimum serum volume required for testing (40 µl), with pooling limited to samples of similar volume, from the same species, site, and date. A threshold was chosen to separate positive and negative results in this quantitative test. Previous studies used a reciprocal titer of 27 (Serra-Cobo et al., 2002; Brookes et al., 2005) or 9 (Perez-Jorda et al., 1995) as a cut-off point for either EBLV-1 or EBLV-2 to eliminate false-positive results. In this study we used a reciprocal titer of ≥27 as the positive cut-off, and negative samples were defined as those with lower titer levels. Raw neutralization data are available upon request. Two polyclonal antisera, raised against EBLV-1 and EBLV-2 in rabbits, were used as positive controls in the respective mFAVN assays. These positive controls provided validation data on the robustness and repeatability of the mFAVNs in lieu of an internationally recognized standard. In addition to the in-house EBLV-1 and -2 control sera, the performance of Office International des Épizooties positive-control dog sera (Agence Française de Sećurité Sanitaire des Aliments, Nancy, France) also was monitored to ensure consistency of testing.

Prevalence estimates and CIs for individual roosts and for all bats sampled were generated using a generalized linear modeling approach to calculate maximum-likelihood estimates of prevalence and confidence limits where multiple different pool sizes were used (Brooks et al., 2005). The method assumes 100% test sensitivity and specificity (Williams and Moffat, 2001).

RESULTS

Summary of bats sampled

Between 2003 and 2006, 710 bats were caught and sampled. However, only 660 of those tested by mFAVN yielded readable results, including 273 *E. serotinus*, 363 *M. daubentonii*, and 24 individuals from five nontarget species (*Plecotus auritus*, *Myotis brandtii*, *Myotis mystacinus*, *Pipistrellus pipistrellus*, *Myotis nattereri*); 50 samples had insufficient serum volume either to pool or to perform repeat testing upon mFAVN test failure. Of those tested, 383 were tested as single samples (214 *E. serotinus*, 165 *M. daubentonii*, and four individuals from nontarget species). The remaining 277 samples were tested in pools (min=2, max=6), 210 of which were in pools of two.

Of the *E. serotinus* and *M. daubentonii* tested by mFAVN, 472 were female (249 *E. serotinus*, 223 *M. daubentonii*) and 164 were male (140 *E. serotinus*, 24 *M.*

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TABLE 2. Breakdown of age and sex classes for *Eptesicus serotinus* and *Myotis daubentonii* tested by modified fluorescent antibody virus neutralization.

Species	Males		Females		Species total
	Adult	Juvenile	Adult	Juvenile	
E. serotinus	5	19	218	31	273
M. daubentonii	100	40	181	42	363
Sex/age totals	105	59	399	73	636

daubentonii), with an age distribution including 504 adults (399 female, 105 male) and 132 juveniles (73 female and 59 male) (Table 2). Samples from *E. serotinus* were collected from 15 sites (all in southern England), and from *M. daubentonii* at 17 sites (the majority of which were in northwest England) (Fig. 1 and Table 1). The number of sites by county, samples tested from each site by PCR and mFAVN, and positives detected are provided in Table 1.

A low mortality rate occurred during handling (less than 1%), mostly at the beginning of the project after which the techniques were refined to ensure the best possible outcomes for the bats. The causes of death were unclear and no pattern in age, sex, condition of the bat, or season was observed.

Myotis daubentonii: When all sites sampled during 2003–06 were included ($n=17$) in the analysis, (including seropositive and seronegative bats; total samples=363), a 95% CI seroprevalence of 1.0–4.1% (mean=2.2%) was estimated. Of the 17 sites sampled, EBLV-2 neutralizing antibody titers ≥27 were detected at four sites in 2003, 2005, or 2006 (Fig. 1 and Table 1).

The 95% CI for overall estimated prevalence from the antibody-positive sites only ($n=4$) from 2003–06 was 2.5–11% (95% CI; mean=5.8%). Of these sites, a single site in Lancashire (Site 20) contained antibody-positive individuals in 2003 and 2005 (Table 1). Antibody-positive samples also were detected from two additional sites in Lancashire in 2003, Site 18 and Site 25. In 2006, two antibody-positive bats were identified from a single site in Cumbria (Site 27). From these results, and allowing for the effects of pooling, a minimum of seven bats, and a maximum of 13 showed a neutralizing antibody level of ≥27.

Individual site seroprevalence estimates for the four sites where EBLV neutralizing antibodies were detected between 2003–2006 are as follows: Site 18 (1 pool of 2 sera positive; 95% CI seroprevalence of 2–80%; mean=29%; $n=4$); Site 20 (one pool of two sera, one pool of three sera, and one single serum seropositive; 95% CI seroprevalence of 1–9%; mean=4%; $n=70$); Site 25 (one pool of three sera seropositive; 95% CI seroprevalence of 1–35%; mean=9%; $n=12$), and Site 27 (two single sera seropositive; 95% CI seroprevalence of 1–20%; mean=7%; $n=29$).

Eptesicus serotinus: When all sites sampled during 2003–06 were included ($n=15$) in the analysis (total samples=273), a 95% CI seroprevalence of 0.001–1.6% (mean=0.37%) was estimated. European bat lyssavirus-1 neutralizing antibodies were detected in a single sample at one site in 2004 only (Site 3, Sussex), with a neutralizing antibody reciprocal titer of 81 (Table 1). Samples obtained and tested from Site 3 in 2004, 2005, and 2006 generated an overall roost seroprevalence 95% CI of 0.1–9% (mean=2%; $n=50$). Unfortunately, insufficient serum was obtained from the seropositive serotine bat to test against other lyssaviruses.

Nontarget species: Forty-two individuals of five other species were caught, from

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which 24 samples were tested by EBLV-2 mFAVN. All blood samples tested were seronegative, with reciprocal titers <27.

Presence of host or lyssavirus RNA and viral RNA from mouth swabs

Swabs were obtained from a total of 766 individual bats during 2003–06 (287 from *E. serotinus*, 439 from *M. daubentonii*, and 40 from nontarget species). All swabs collected (two from each bat) were tested by PCR for the presence of host and lyssavirus RNA, and by RTCIT for the detection of live virus. Host rRNA was detected by PCR in 83% (638 swabs) of swabs indicating that host ribosomal RNA was present and successfully amplified. In the remaining 17% ($n=128$), host rRNA was absent or below the limit of detection. By species, host rRNA was detected in 81% of *E. serotinus* swabs and 84% of those from *M. daubentonii*.

None of the samples yielded lyssavirus RNA, suggesting that none of the bats (for which host rRNA was detected) were excreting virus in saliva at the time of sampling.

Virus isolation tests were conducted using RTCIT (all second oropharyngeal swabs were tested) and MIT (seropositive bats only). All RTCIT samples were found to be negative, and at up to day 41 after infection clinical signs of infection had not developed in any of the inoculated mice. These results indicated that live virus was not detectable in any of the oropharyngeal swab samples.

Potential diagnostic characters

We examined bats for the presence of a field-based prognostic characteristic that could be used to indicate the presence of EBLV-seropositive individuals, such as behavior, condition of fur, and body mass. Such physiologic or behavioral characteristics in seropositive bats may be indicative of a past or current lyssavirus infection. No qualitative records showed any pattern in identifying useful characters, with the majority of bats being completely "normal" in most respects.

The body mass data were explored to determine if they could be used as a diagnostic guide (e.g., if "exposed" bats were underweight). The mean ratio (and its variance) of *M. daubentonii* body mass to forearm length was calculated for all bats excluding those to be found seropositive for each of the four age and sex classes. There was evidence of statistically significant differences between the sexes; females were heavier for their body size ($F=60.77$; df=1; $P<0.001$), and adults were heavier than juveniles ($F=279.82$; df=1; $P<0.001$), with interaction between ages and sex also being significant ($F=6.64$; df=1; $P<0.01$). Location of capture was investigated as another potential factor (accumulated analysis of variance using an unbalanced design), but was found to be nonsignificant in the absence of interactions with other factors ($F=0.48$; df=1; $P=0.49$). The probability that individual bats were either over- or underweight was then calculated as the z-statistic for the 13 potentially seropositive bats, compared to their age and sex grouping (Tables 2 and 3). Nine of these bats were overweight, and four showed evidence of being underweight; only one seropositive bat was significantly distinct (i.e., outside 95% CI), a juvenile male that was significantly overweight ($P=0.047$). Hence, there was no clear association between body mass and seroconversion to EBLV-2 in *M. daubentonii*.

Rates of recapture

Myotis daubentonii: During the 4 yr of sampling, 43 individual *M. daubentonii* were recaptured (11.8% of sampled *M. daubentonii*). Of these, 11 were caught within the same year and within a time span that did not allow for resampling. The remaining 34 *M. daubentonii* (9.4% of sampled *M. daubentonii*) were recaptured between one and five times. Of the 34 resampled recaptures, 33 were caught at the same location each time. From the *M. daubentonii* recaptures, only one individual with a previously antibody-positive

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TABLE 3. Statistical analysis of *Myotis daubentonii* body mass/forearm length ratio for bats seropositive and seronegative for exposure to European bat lyssaviruses.

	Mean ratio of forearm length to body mass (variance)		Number of bats in each age/sex class (seronegative/seropositive)	
	Adult	Juvenile	Adult	Juvenile
Male	4.69 (0.84)	5.49 (0.54)	148/3	47/1
Female	4.43 (0.48)	5.39 (0.57)	240/9	59/0

result was recaptured (Site 20, caught in 2005 and recaptured in 2006). This individual had an initial reciprocal titer of 81 in 2005, which when tested again in 2006, had dropped to 3.

Eptesicus serotinus: Between 2004 and 2006, 15 *E. serotinus* were recaptured (5.5% of *E. serotinus* sampled), all on only one occasion, and at the site of initial capture. All *E. serotinus* individuals recaptured were found to have a reciprocal titer of 9 or less.

DISCUSSION

In the UK, *E. serotinus* often roosts in residential properties. This species appears to be at the edge of its geographic range in the UK; it is limited to southern England and is considered one of the less common UK species. The population size is estimated at less than 15,000 (Harris et al., 1995); the population density considerably lower than in mainland Europe. This could be a reason for the lack of apparent transmission and occurrence of EBLV-1 in UK *E. serotinus*, because virus transmission likely relies on the availability of new vectors.

In contrast, *M. daubentonii* are widespread throughout the UK and are considered to be much more common (estimated to number at least 150,000 by Harris et al. [1995]). Populations are believed to be increasing Europe-wide, including by approximately 4% in the UK in the last 10 yr (Bat Conservation Trust, 2005). This species roosts mainly in unoccupied structures such as bridges and trees. Although roosts are sometimes found in houses, *M. daubentonii* is considered less likely to come into contact with humans than a more synanthropic species such as *E. serotinus*. This difference in behavioral ecology may prove to be a factor in the detection and therefore estimated prevalence of EBLVs within the UK.

Targeted surveillance for rabies exposure in bats is uncommon in Europe. In Finland, a Swiss bat biologist died in 1986 from rabies shown to be an EBLV-2 bat variant. During the summer of 1986, a surveillance study was undertaken in Finland involving 183 bats of seven different species, from both targeted sampling ($n=124$) and passive surveillance ($n=59$) (Hagner et al., 1987). None of the bats analyzed in that study was identified as virus-positive, or found to have lyssavirus antibodies present.

In Spain, targeted surveillance found three of four colonies of *E. serotinus* to be EBLV-1 virus-positive (Perez-Jorda et al., 1995). Between 1992 and 2000, antibodies to EBLV-1 were found in *Myotis myotis*, *Miniopterus schreibersii*, *Tadarida teniotis*, and *Rhinolophus ferrumequinum*. European bat lyssavirus-1 virus-positive results (RNA confirmed by nucleotide sequencing) also were obtained by nested RT-PCR on brain, blood pellet, lung, heart, tongue, and esophagus/larynx/pharynx of *M. myotis*, *M. nattereri*, *R. ferrumequinum*, and *M. schreibersii* (Echevarria et al., 2001). Prevalence of EBLV-1 antibodies in *E. serotinus* increased over a 12-mo period (from 3% to 59%), followed by a decrease to 10%

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at the end of the 6-yr study (Echevarria et al., 2001), suggesting that EBLV-1 infection may not be fatal, with an antibody response possibly mediating host immunity (Serra-Cobo et al., 2002).

Germany reported one of the highest numbers ($n=187$) of EBLV-positive cases in bats in Europe between 1954 and 2005: 57 cases were identified to species, with 92.5% ($n=53$) of these in *E. serotinus* (Müller et al., 2007). In The Netherlands, passive EBLV sampling has detected a prevalence of 21% in tested samples ($n=1,178$) by fluorescent antibody testing (FAT) and PCR in *E. serotinus*, with 19.5% ($n=706$) of males, 23.6% ($n=473$) of females, and 18% of juveniles tested ($n=57$) and 21.3% of adults tested ($n=1,122$) positive by FAT and PCR for EBLV-1 (Van der Poel et al., 2005).

The detection of neutralizing antibodies in blood samples reflects past exposure to EBLVs only, and does not demonstrate active infection (with possible excretion of virus in saliva) at the time of sampling. The antibody prevalence data collected during 2003–06, combined with the oropharyngeal swab results, suggests that exposed bats may have mounted a sufficient immune response to suppress the virus and might therefore remain seropositive without excreting virus in saliva. Detection of antibody in blood (Shanker et al., 2004) and virus (or viral RNA) in saliva may fluctuate over time (Bourhy et al., 1999; Echevarria et al., 2001; Serra-Cobo et al., 2002; Brookes et al., 2005; Amengual et al., 2007). The technique of detecting virus from bat saliva was used successfully for surveillance of an *E. serotinus* colony that had been involved in a human exposure incident in Seville, Spain, in 1999 (Echevarria et al., 2001), where 15 of 71 oropharyngeal swabs were positive for EBLV-1 RNA by nested RT-PCR (Echevarria et al., 2001).

Estimating an overall seroprevalence of titers to EBLV-2 in the English Daubenton's bats sampled within this study was directly affected by the inclusion or exclusion of a priori sites in the analysis. Most measures of prevalence are based on random sampling. However, this study represents both random and selective sampling. As such, it is important to distinguish between the levels of seroprevalence observed. In addition, the necessity to pool several samples resulted in maximum likelihood estimations of seroprevalence at each site. The seroprevalence estimates from random sites (i.e., non–a priori sites) were substantially lower than at sites where infected bats were thought likely to occur. In an initial analysis of the 2003 English data collected, the overall seroprevalence for three random *M. daubentonii* sites was between 1.2% and 11.6% (95% CI), whereas a seroprevalence of 4.9–31.6% (95% CI) was identified for the single a priori site visited that year (Smith et al., 2006), compared to estimated seroprevalence of 2.5–11% (95% CI; mean=5.8%) at all antibody-positive sites ($n=4$) during the entire surveillance period (2003–06). The overall estimated seroprevalence (including both seropositive and seronegative sites) of 1.0–4.1% (95% CI; mean=2.2%) for *M. daubentonii* in our study is comparable with data from sampling in Scotland during 2003, when prevalence of 0.05–3.8% (95% CI) in *M. daubentonii* was estimated (Brookes et al., 2005), and during 2007, when 5.4% of sampled *M. daubentonii* ($n=240$) were reported as EBLV-2 antibody-positive (Scottish Natural Heritage, 2009).

The application of the positive threshold of ≥ 27 to pooled samples may have underestimated the true number of EBLV-1 or EBLV-2 positive samples present, because weakly positive sera may not have been identified. When the positive threshold was reduced from ≥ 27 to ≥ 9 in a secondary analysis of the 2004 and 2005 data, the percentage of serum samples called "positive" increased from 0% to between 5% and 6.7% in *M. daubentonii* in 2004, and 0.9% to 2.8–3.8% in 2005. Using the same method for

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E. serotinus, seroprevalence in 2004 increased from 2.1% to 18.8%, with nine bats seropositive at the ≥9 threshold, rather than just the single individual at ≥27 (Veterinary Laboratory Agency, unpubl. data). The pooling of some samples in this study led to an inability to account for the exact number of positive and negative bats in any given pooled set if a positive result was found, resulting in broader confidence intervals than if pooling had not occurred. However, particularly for the *M. daubentonii* sampled, pooling was reduced over the 3 yr of sampling as blood sampling proficiency in the field increased.

All of the bats that were sampled appeared to be healthy, and none exhibited obvious clinical signs of rabies. The majority of the *M. daubentonii* sampled were caught on the wing, another indication of relative good health considering active rabies infection in bats may lead to paralysis or lack of coordination and an inability to fly (Johnson et al., 2003; Fooks et al., 2004a, 2006). The *E. serotinus* sampled were, in some cases, taken by hand from their day-roost locations, and therefore their ability to fly was not always observed. Throughout the sampling of both *E. serotinus* and *M. daubentonii*, there has been a substantial bias in the number of male and female bats sampled. To enable sampling of both adults and juveniles, maternity colonies were used for the majority of the sampling. Adult male *E. serotinus* are not known to commonly inhabit maternity colonies, which are made up with predominantly breeding and nonbreeding adult females and, later in the season, their offspring (Hutson, 1991). A small number of adult males were sampled from Site 15, a swarming location, and some juvenile males were caught at maternity colonies. The seropositive *E. serotinus* identified in 2004 was a juvenile male at a maternity colony, and he may not have returned to that site after fledging. In the UK, passive surveillance has tested 110 *E. serotinus* (from 1987 to winter 2007), and all have been EBLV negative by FAT, RTCIT, MIT, and PCR.

This study amplifies and extends the findings of previous work that aims to understand the distribution and prevalence of infection by and exposure to EBLVs in Britain. Our data show that despite an apparently constant and low rate of lyssavirus antibody seroconversion in English *M. daubentonii* bats there was no evidence of noticeably sick bats around exposure foci or of virus excretion in any of the wild bats sampled. This, together with the low rate of recovery of morbid *M. daubentonii* bats, may suggest that the pathology, or at least the progression of diagnostic signs in this species, does not follow the path of lyssaviruses in nonflying mammalian species and that the virus is being maintained within this wild host population. Although EBLV-2 does cause rabies in Daubenton's bats, the low number of confirmed cases in the UK and the detection of healthy seropositive bats (one bat was healthy 1 yr after testing positive for EBLV-2 antibodies) is suggestive of recovery. This hypothesis is supported by recapture data for EBLV-1 in *M. myotis* species in Spain, which are suggestive of cycles of infection and a persistent immunity within the community (Amengual et al., 2007). Amengual et al. (2007) proposed that the disparity observed for disease progression of EBLV-1 in *M. myotis* compared to that observed for rabies-associated bat viruses in nonchiropteran mammals is due to a long-established virus/host coevolution.

Further sampling is required on both principal target species (*E. serotinus* and *M. daubentonii*), at both a priori sites (particularly for *M. daubentonii*, in light of the recent virus-positive cases at a new location in England; Harris et al., 2007), and at other sites where there is no prior reason to expect to find antibody-positive bats. Testing a larger sample set of *E. serotinus* over a wider geographic range also would help in determining whether

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detection of the single antibody-positive bat in this study was indeed an isolated case. Testing of other resident bat species in the UK also should be undertaken, particularly focusing on other species that have been found to be EBLV-positive elsewhere in Europe.

In addition, further work is needed to formulate the statistical basis for larger-scale surveillance, which may be necessary for future policy formation or public health risk assessment. Recent work has been focused on sampling at a restricted selection of roost types (for example, maternity roosts) and within a relatively narrow seasonal window. There are both theoretical and statistical concerns that the unbalanced sex and age profile of most of the bats sampled (i.e., 58% are adult females), and their physiologic (most were pregnant or lactating) or immunologic status may bias results, and that immunologically naïve juveniles were poorly represented (19% across both sexes). Some targeted work on an unbiased sample of the population may be needed to ensure that a statistically and scientifically robust distribution of bat ages and sexes are sampled at the appropriate season to ensure a more appropriate interpretation of results.

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