

SHORT NOTE

First records of *Myotis alcaethoe* von Helversen & Heller, 2001 in Belgium

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KEYWORDS: Vespertilionidae, *Alcaethoe* whiskered bat, Wallonia, faecal DNA extraction.

Molecular techniques have led to the discovery of several cryptic bat taxa in Europe (1, 2). One of these recently-discovered species is *Myotis alcaethoe* von Helversen & Heller, 2001, a species in the 'whiskered bat'-complex (3). *M. alcaethoe* is morphologically very similar to the whiskered bat *M. mystacinus* (Kuhl, 1817) and to the Brandt's bat *M. brandtii* (Eversmann, 1845), even though it is not a sister-taxon to either of these species (4).

Although molecular techniques remain the most useful and reliable identification method (5), the species can also be identified based on a number of morphological characteristics, most notably its very small size (forearm < 33.5 mm) and the well-developed protoconus of the third upper premolar (3, 6). Other distinctive traits for the species are the pink face, shape of the penis, short tragus and short snout (6).

M. alcaethoe is regarded as a forest specialist, and is most often observed in moist and old

growth deciduous forest during summer (7, 8). Summer roost sites are generally situated in tree cavities, and are difficult to find (8). As do many other vespertilionid bat species, *M. alcaethoe* visits caves and similar underground sites in autumn to swarm, a behaviour linked with mating (9). Very little is known about the hibernation behaviour of the species. As species identification often requires handling – and thus disturbance – of the bat, species of the 'whiskered bat'-complex are most often not identified to the species level during winter surveys. However, *M. alcaethoe* has been recorded at caves during winter (e.g. 10, 11).

The species was originally described from specimens from Greece (3), but soon found to have a widespread – but patchy – distribution in a large part of Europe (7). In northwest Europe, *M. alcaethoe* has been recorded in Germany (7), France (7, 12), the UK (13) and the Grand Duchy of Luxembourg (14). There have also been observations close to the Belgian border in Luxembourg and France (departments Pas de Calais, Ardennes and Meuse), and its presence in Belgium could thus be expected (7). In this short note, we present the first records of *M. alcaethoe* in Belgium.

Table 1

Measurements, age (based on the ossification of epiphyseal joints) and sex of *Myotis alcaethoe* caught in Belgium. FA = forearm length. Individuals used for genetic analyses are indicated with ** (cyt b and ND1) and * (cyt b).

Capture date	Site	Age	Sex	Sexual status	Mass (g)	FA (mm)	
11/07/11	Bois de Saint-Rémy	Adult	F	Post lactating	5	32	
30/07/11	Bois de Saint-Rémy	Adult	M		5	31,9	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	4	33,5	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	5,5	32,6	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	5,5	33,1	
04/10/11	Grotte touristique de Rochefort	Adult	F	non lactating	6	32,4	
25/09/12	Grotte touristique de Rochefort	Juvenile	M		5	31,5	
12/10/12	Grotte touristique de Rochefort	Adult	M		4	31,6	
12/10/12	Grotte touristique de Rochefort	Adult	M		5	32,7	
15/08/14	Etang des Prés de Virelles	Adult	M		5	33,1	
02/09/14	Grotte touristique de Rochefort	Juvenile	M		4,5	32,8	**
02/09/14	Grotte touristique de Rochefort	Juvenile	M		4,6	32,5	*
02/09/14	Grotte touristique de Rochefort	Juvenile	F		4,3	32,4	*

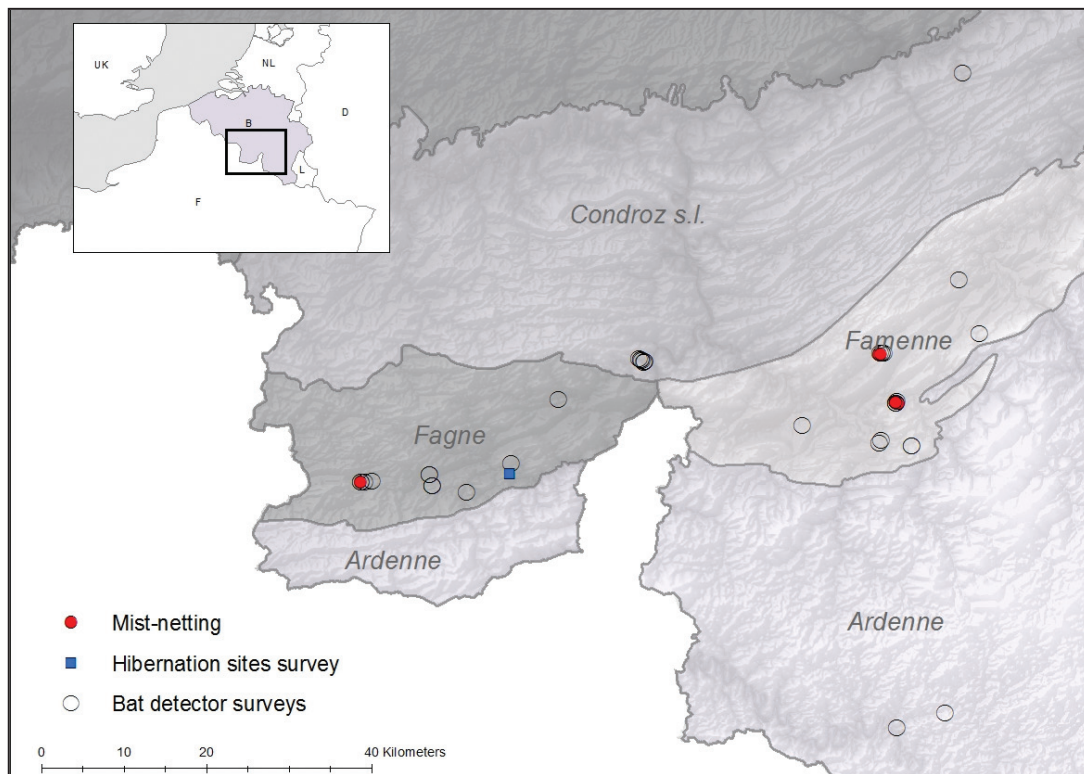


Fig. 1. – Map showing the locations of the Belgian records of *Myotis alcaethoe*. Red circles: mist-net captures; open circles: bat detector records; blue squares: hibernation records.

Between 2011 and 2014, 13 *M. alcaethoe* were captured during mist netting surveys in Wallonia (Table 1; Fig. 1). These individuals were all identified based on morphological characteristics (6). The capture of a post-lactating female and several juvenile bats (based on epiphyseal plates and secondary sexual characteristics) shows that a reproducing population is present in this region. During summer, the species has been captured in two old growth deciduous woodlands in Rochefort (forêt de Saint-Rémy; Province of Namur) (Fig. 2a) and Chimay (étang des prés de Virelles; Province of Hainaut). During the autumn swarming season the species has been captured at the entrance of a large natural cave in Rochefort from 2011 to 2014 (Grotte touristique de Rochefort; Province of Namur). This natural cave is a very important site for bats in this region, both for hibernation and for swarming. Eleven species have been captured there during the swarming season, including *M. mystacinus* and *M. brandtii*.

To confirm the morphological identification genetically, we collected faeces of three caught bats at this site in 2014 (Table 1). Bat faeces

can be used for non-invasive genotyping to identify species (e.g. 15, 16). Each dropping was individually placed in a tube with silica gel to absorb humidity and hence preserve DNA (17) or in pure ethanol. Droppings that were stored in pure ethanol were air dried first for half an hour on a tissue paper prior to DNA extraction. DNA was extracted using the QIAamp Fast DNA Stool Mini kit (Qiagen), following the manufacturer's protocol except for some steps that were modified as described below (17) (step numbers correspond to the QIAamp Fast DNA Stool Mini Handbook, pp14-16, ver. 03/2014). A single dropping was placed individually in a 2 ml microtube as Step 1. After addition of the InhibitEX Buffer the dropping was squashed using a disposable pestle (Eppendorf) during Step 2 until completely homogenized. Incubation at 70 °C in Step 7 lasted 15 minutes in a Thermomixer at 750 rpm (Eppendorf). For Steps 9 and 11 centrifugation was performed at 7200 rpm. Step 13 was omitted. DNA was finally eluted in 100 µl Buffer ATE during Step 14 and this step was repeated by pipetting the eluate back on the column membrane to increase DNA yield.

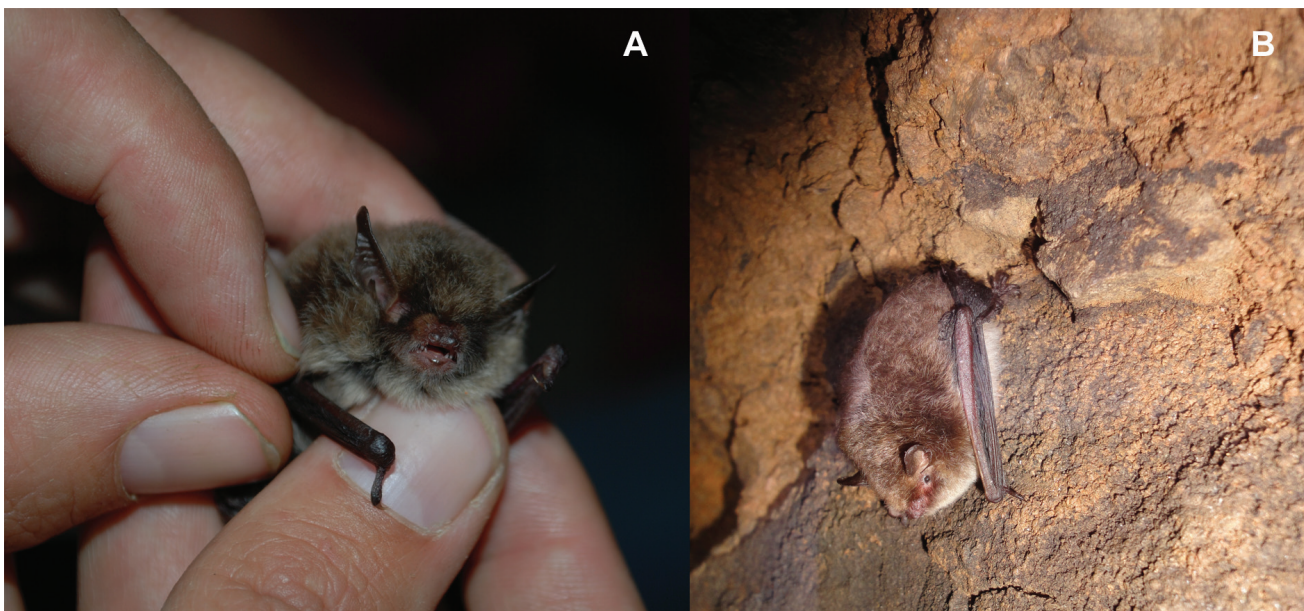


Fig. 2. – **A.** Mist-netted *M. alcaethoe* in Forêt de Saint-Rémy (photo Pierrette Nyssen) **B.** *M. alcaethoe* roosting in an underground tunnel (photo Bob Vandendriessche).

We amplified a 220 bp fragment containing 153 bp of the mitochondrial Cytochrome b gene using forward primer Bat_trnE31F (5'-TGACACGAAAAATCAYCGTTGT-3') and reverse primer Bat_cytb176R (5'-GTRTCTGATGTRTAGTGTATRGC-3'). PCRs were performed in 52 µL of reaction mixture containing 12 µL of extracted DNA, 0.4 µM of each primer, 1x Taq buffer with KCl, 2 mM MgCl₂, 200 µM of each dNTPs and 1.6 U Taq polymerase (Thermo Fisher Scientific). Each PCR was composed of an initial denaturation at 94 °C for 3 min; followed by 35 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), followed by a final elongation at 72 °C for 10 min. Amplified DNA was purified using the ExoSAP-IT method (Affymetrix Inc.). Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) in a 10 µL volume containing 4.4 ng of purified DNA, 0.4 µM of forward or reverse primer, 0.5x Ready Reaction mix and 0.5x Sequencing buffer. Sequencing of both strands was performed with a cycling profile of 35 cycles of 10 s denaturation at 96 °C, 5 s annealing at 50 °C and 4 min elongation at 60 °C. After purification with the BigDye XTerminator Purification kit (Life Technologies) products were analyzed on an ABI 3500 genetic analyzer (Life Technologies). Sequences for the three bats were identical (EMBL Accession No. LN864496) and an NCBI BLAST search showed that the 153 bp Cytb part was only identical to a set of *M. alcaethoe* haplotypes (EU541661, EU541662, EU541663; 7).

Secondly, we sequenced 1365 bp of a 1460 bp fragment containing the complete ND1 gene (3) for one of these individuals (table 1, EMBL Accession No. LN864497) following JAN et al. (2010) (13). Across the sequenced region, the haplotype was identical to the ND1 haplotype obtained from the Hungarian samples of *M. alcaethoe* (AY027835 and AY027836; 3). This haplotype has been recorded in the Iberian peninsula (18) and across Western and central Europe (7, 9, 12, 13, 19), while a number of

different closely related haplotypes have been observed in the Balkans and Asia minor (3, 20).

Up to now, only one roost site of *M. alcaethoe* has been recorded. At the beginning of April (8/4/2012) a torpid individual was observed in an old tunnel in Viroinval (Province of Namur; Fig. 2b). This site – situated in an old growth riparian woodland – is likely used as a hibernation site or a transit roost by the species. Annually, up to 10 bat species are counted here during hibernation, among them ca. 5-10 individuals of ‘whiskered bat’ (*M. mystacinus/brandtii/alcaethoe*). During a preliminary survey in the swarming season both *M. mystacinus* and *M. brandtii* were already captured at this tunnel (Dekeukeleire D, unpublished data).

Furthermore, several bat detector recordings in southern Belgium can be attributed to *M. alcaethoe*. The first observation was made on the 29th of May 2008, in the wooded river valley between Hermeton-sur-Meuse and Soulme (Province of Namur). Using a night vision camera (Night Mariner 150, ITT, New York, US), a small *Myotis* bat could be observed foraging a few meters above the river Hermeton under overhanging branches. Ultrasound recordings were made with a D1000x bat detector (Pettersson Elektronik AB, Uppsala, Sweden) and analysed in the BatSound Pro 3.3 software package (Pettersson Elektronik AB, Uppsala, Sweden). Signals (n: 20) had the following characteristics (mean ± SD): duration 2.87 ± 0.45 ms, pulse interval 65.85 ± 15.95 ms, start frequency 118.65 ± 2.89 kHz, end frequency 42.55 ± 2.80 kHz, peak frequency 59.70 ± 8.11 kHz, sigmoid (‘S’-) shape with upper and lower inflexion points at 59.70 ± 2.28 and 50.60 ± 2.24 kHz respectively (Fig. 3). These characteristics correspond well with the description by von HELVERSEN et al. (2001) (3) and BARATAUD (2012) (21). The only other European species using echolocation calls that consistently end above 40 kHz is the Geoffroy’s bat (*M. emarginatus*) (21, 22), but this species generally uses very high starting frequencies, up to 160 kHz (23). Moreover, *M. emarginatus* has linear-shaped echolocation

calls (duration < 3 ms) in confined spaces. The presence of sigmoid shapes and the absence of very high start frequencies, even though the bat flew within close proximity of the detector, points to *M. alcaethoe* (3, 21). Additional detector surveys in 2011 - 2013 confirmed flight activity of *M. alcaethoe* in the valley of Hermeton from April to October. Similar bat detector recordings were made in Bertrix (Province of Luxembourg), Villers-le-Temple (Province of Liège) and the wider surroundings of the capture sites in Rochefort (Province of Namur) and Virelles (Province of Hainaut) (Fig. 1).

These findings led us to re-identify all Belgian specimens of *M. mystacinus* (n: 124) and *M. brandtii* (n: 53) in the collection of the Museum of Natural Sciences (IRSNB) based on skull characteristics (3, 19, 24). However, no additional *M. alcaethoe* specimens could be discovered.

Our records indicate that *M. alcaethoe* is a resident species in the southern part of Belgium. Its presence has most likely gone undetected due to its similarity to other small *Myotis* species and its relatively recent description.

Recently, BOGDANOWICZ et al. (2012) (8) indicated possible high levels of hybridization between *M. mystacinus*, *M. brandtii* and *M. alcaethoe* at swarming sites in Poland. Nuclear microsatellite markers indicated that 6.5 to

30.4 % of the *M. alcaethoe* identified based on mtDNA were possible hybrids. Morphologically, the majority of these hybrids followed their mtDNA identification, although some showed intermediate phenotypes. As in other studies (e.g. 7, 18, 23) we have only used mitochondrial markers and morphological characteristics, and thus we cannot rule out the presence of hybrids. However, *M. alcaethoe* is widely distributed in Europe and occurs in neighboring regions (6), and moreover, our observations show reproduction in Belgium. The probability that *M. alcaethoe* does not occur in Belgium, and that our records only represent hybrids, thus seems very small.

The habitat where *M. alcaethoe* has been observed in Belgium – natural old growth deciduous forests and caves - is very similar to their habitat in other European regions (eg. 7, 8, 13). Up to now, most of the records are from the southern part of the Fagne-Famenne region in Wallonia (Fig. 1). This region – characterized by the presence of Devonian limestone – is a biodiversity hotspot in Belgium, and several plant and arthropod species from Mediterranean and continental biogeographic regions occur here (e.g. 25, 26). This occurrence pattern is quite similar to the distribution in Saxony-Anhalt (Germany), where the most northeastern German records of *M. alcaethoe* have been noted (7). However, there are also bat detector records in the Condroz and in a wooded river valley in

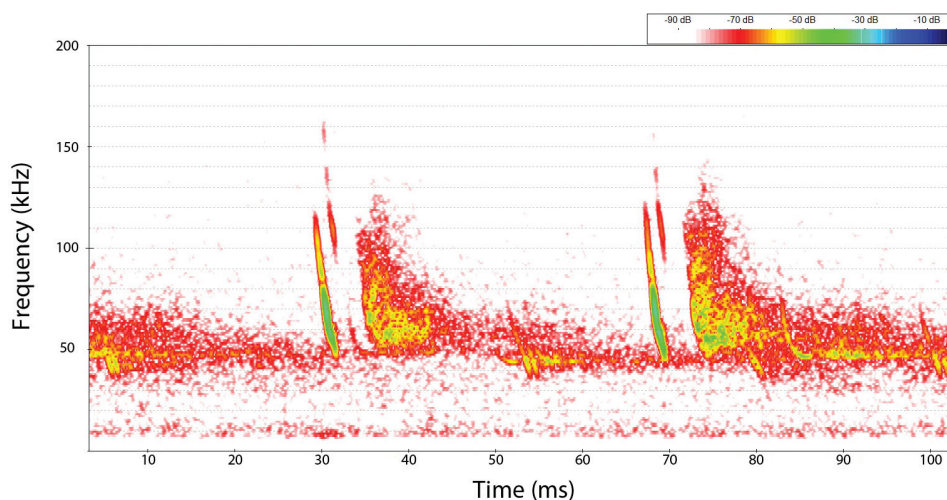


Fig. 3. – Echolocation signals of *M. alcaethoe* recorded in Hermeton-sur-Meuse on 29/05/2008 (recording nr M00037 Marc Van de Sijpe).

the Ardennes. Additional surveys in old growth forests and mist netting at swarming sites could reveal additional observations and clarify the range of this species in Belgium.

Bats are considered to be highly threatened due to habitat loss, pesticide use and anthropogenic disturbances (6). In southern Belgium, hibernation census data indicate a strong decrease in both species diversity and bat abundance at underground sites over the past 50 years (27). At this point, it is too early to determine the conservation status of *M. alcathoe* in Belgium, but it appears to be rare. As a forest specialist with a limited distribution, *M. alcathoe* could be regarded as a priority species for conservation plans.

ACKNOWLEDGEMENTS

All bats were captured and handled under license from the Belgian authorities (SPW-DNF-DGO3 permit réf déro 2013/RS/n°15 and déro 2014/RS/n°12). We would like to thank the many volunteers that helped with the fieldwork, especially those from Plecotus – Natagora, Jeunes & Nature and Vleermuizenwerkgroep – Natuurpunt. The coordination of bat field monitoring is funded by the public service of Wallonia (DGO3 - Contract “Convention pour l’aide au suivi des populations de chiroptères, la mise en place d’actions en faveur des chauves-souris et la sensibilisation et l’information du public”). Bat monitoring in Wallonia is conducted in close collaboration between Plecotus – Natagora and the public service of Wallonia.

We are very grateful to the Genetic Diversity lab technicians (INBO), Bart Hellemans and Filip Volckaert (KU Leuven) for assistance with the genetic analyses. We would also like to thank Christian Dietz for confirming the identification of the hibernating bat, and Alex Lefevre and Michel Barataud for their help with acoustic bat identification. Furthermore, we would like to thank Thierry Kervyn and Sébastien Puechmaille and an anonymous reviewer for useful comments on the first draft of the manuscript.

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Received: January 30th, 2015

Accepted: July 15th, 2015

Branch editor: Peter Galbusera