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SHORT COMMUNICATION

Molecular evidence of bird-eating behavior in *Nyctalus aviator*

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Abstract
Insectivorous bats consume a large variety of food items. Previous observations of feathers found in feces led to the hypothesis that the birdlike noctule (*Nyctalus aviator*, Vespertilionidae) could prey on birds. To test the hypothesis, we analyzed fecal samples from six species (*Barbastella pacifica*, *Murina hilgendorfi*, *Myotis frater*, *N. aviator*, *Plecotus sacrimontis*, and *Vespertilio sinensis*) collected from central Hokkaido, Japan, via DNA barcoding. We identified the presence of the Middendorff's grasshopper warbler (*Locustella ochotensis*) in the diet of a pregnant individual of *N. aviator*. All the other samples proved negative regarding bird prey DNA. This is the first time that the consumption of a bird by *N. aviator* is confirmed with molecular evidence. Our findings add invaluable insight into the diet of this bat and its potentially opportunistic foraging behavior.

Keywords *Nyctalus aviator* · *Locustella ochotensis* · Bird prey · DNA barcoding · Sanger sequencing

Introduction

There are about 1300 species of bats worldwide, of which the vast majority are insectivorous (Fenton and Simmons 2015; Vesterinen et al. 2013; Vesterinen et al. 2016; Vesterinen et al. 2018). Although invertebrate prey is usually widely available, many insectivorous bats are known to occasionally forage on other, rarer prey, including fish or birds (e.g., Aizpurua et al. 2013; Fukui et al. 2013; Ibáñez et al. 2016, Ma et al. 2003). While the frequency and significance of this behavior remain unstudied, it may serve as an important nutritional strategy during key life stages, such as reproduction (see Popa-Lisseanu et al. 2007).

In Japan, Fukui et al. (2013) reported the occasional consumption of birds by *N. aviator*, but the prey species were not identified. Here, we use molecular tools to detect the presence of a bird in the diet of *N. aviator* and to confirm the prey species.

Material and methods

Samples were obtained from 2015 to 2017 within the framework of a bigger study on the diet of Japanese bats (unpublished data). All the trapping and DNA extraction methods were carried out in the same way for all the samples as explained below.

Study area

The undulating landscape of the study region in central Hokkaido, Japan, is characterized by a mixture of arable land and urban areas in the valleys, while mixed forests cover the hills (Fig. 1). The study was conducted during the summer months (May to September) of 2015–2017. In the humid continental climate, average temperatures range from 9 to 17 °C in May, which is the coolest month of the study period, and from 19 to 26 °C in August, which is the warmest month of the

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year (Japan Meteorological Agency 2018). The mean precipitation from May to September ranges between 50 and 150 mm (Japan Meteorological Agency 2018).

Bat trapping and fecal sampling

Trapping sites were located in multiple locations in Hokkaido. Samples for *Barbastella pacifica* Kruskop, Kawai & Tiunov, 2019, *Murina hilgendorfi* Peters, 1880, *Myotis frater* G.M. Allen, 1923, *Plecotus sacrimontis* Allen, 1908, and *Vespertilio sinensis* Peters, 1880 were collected between 2015 and 2017 using harp traps or mist nets during nighttime. *Nyctalus aviator* Thomas, 1911, was captured from a bat box during daytime in 2017 (Fig. 1). Basic morphometric measurements were recorded for all bats. Bat feces were collected, either directly from the individual while handling, or from the cotton bags used to hold the bats while processing, resulting in one to ten fecal pellets per individual (Table 1). Fecal pellets were stored in 70% ethanol at − 20 °C until laboratory analysis.

Laboratory work and data analysis

DNA was extracted using QIAamp PowerFecal DNA Kit (Qiagen(/MoBio) cat. nr 12830-50, Qiagen, Hilden, Germany) following the manual (MoBio "Protocol: Detailed"; version 12192013) with following modifications (numbers refer to the step numbers in the manual). In step 1, we used whole fecal droppings as starting material (samples were dried briefly on clean paper). In step 6, we used a Tissue Lyser II (Cat No. 85300, Qiagen, Hilden, Germany) 2 × 1 min at full speed. In step 14, we transferred 700 µl of the supernatant into a clean collection tube and added 1125 µl of solution C4 in step 15. DNA was eluted into 100 µL of C6 buffer as recommended in the protocol and stored at − 20 °C until subsequent analysis.

We used a single bat-specific primer pair targeting the DNA barcode region of the mitochondrial cytochrome oxidase subunit I (COI; Walker et al. 2016) to confirm the presence of bird DNA in the bat feces. The PCR protocol followed Kaunisto et al. (2017), except that we used the MyTaq HS Red Mix (product nr BIO-25048, Bioline, UK). All the blank control reactions proved negative, that is, free of contamination. Successful PCR products were purified using A'SAP clean kit (product nr 80350, ArcticZymes, Trømssa, Norway) and sequenced by Sanger sequencing (5′ direction) at Macrogen Europe (Macrogen Inc., Seoul, South Korea). Sequences were trimmed for poor quality regions and primers were removed as described in Sorvari et al. (2012) using Geneious R6 (Kearse et al. 2012). Trimmed sequences were identified using BOLD systems (Ratnasingham and Hebert 2007) and blasted against the GenBank database (Altschul et al. 1990). As one of the sequences from *N. aviator* matched bird sequences in BOLD (*Locustella ochotensis*, 100% similarity), we re-analyzed the DNA from the fecal samples of the larger bats (forearm length over 40 mm; $n = 25$; Table 1) because we assumed that they are more likely to prey upon birds. For this re-analysis, we used three bird-specific primer pairs targeting mitochondrial *cytochrome oxidase subunit I* and *cytochrome b* genes (Pastor-Beviá et al. 2014; Online Resource 1: Table S1). Successful reactions were purified using A'SAP clean kit and sequenced by Sanger sequencing (5′ direction) at Macrogen Europe. Even when the amplification of short CytB products could not be confirmed in the gels, we purified the PCR products and sequenced them. The resulting sequences were trimmed and identified as described above. To further confirm the bird species, we downloaded trace files for the three closest matches for COI sequence from BOLD (species and ProcessID for each record: *Locustella ochotensis*, KFIP031-07; *L. pleskei*, KBBI031-07; and *L. certhiola*, KBPBR487-07) and four closest matches from GenBank for CytB-short

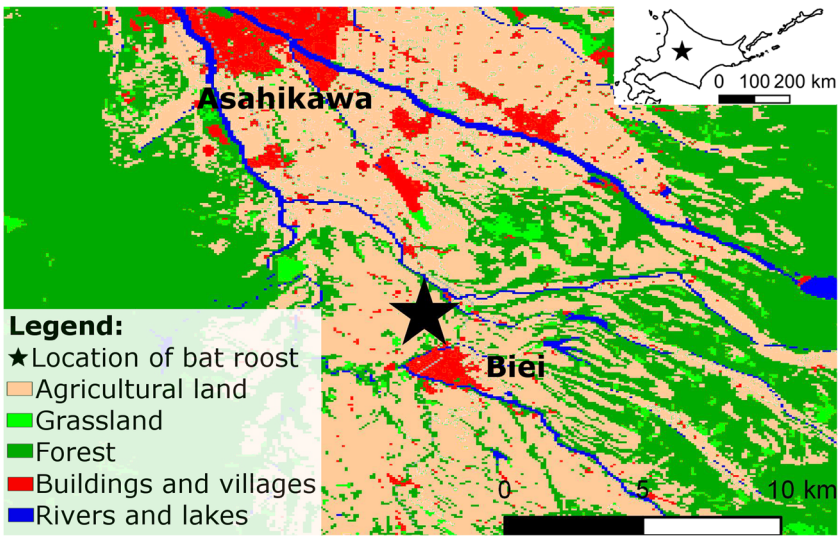

Fig. 1 The main map depicts the trapping site located north of Biei, while the inset map provides an overview of the site within Hokkaido.
Source: Main map: based on National Land numerical information: Land Use Fragmented Mesh Data "L03-b-14_6542", year 2014, provided by the Ministry of Land, Infrastructure, Transport and Tourism; Inset map: based on a map provided by the Geospatial Information Authority of Japan, http://www.gsi.go.jp/kankyochiri/gm_japan_e.html, accessed: 6th of September 2018

(accession numbers *L. ochotensis*, HQ706157; *L. pleskei*, AB261530; *L. certhiola*, HQ706154; and *Megalurus pryeri*, AB261528) and CytB-long (*L. ochotensis*, HQ706156; *L. pleskei*, AB261532; *L. certhiola*, HQ608848; and *M. pryeri*, AB261528). We then aligned our trimmed sequences with each corresponding reference set to see the actual nucleotide-level similarity. Detailed methods and alignments are available in Online Resource 1.

Results

The presence of bird DNA (Passeriformes, Sylvidae, *L. ochotensis*) in one sample of a female *N. aviator* was confirmed with the three primer pairs (COI, CytB-short, and CytB-long). The rest of the analyzed samples did not show evidence of bird DNA. We assigned the sequences from the one successful sample to a single bird species with 100% similarity. The sequences produced in this study are stored in GenBank with accession numbers MK900675 (COI), MK900676 (CytB-short), and MK900677 (CytB-long).

Discussion

Earlier evidence of predation of passerine birds by *N. aviator* was provided by Fukui et al. (2013), who sampled maternity colonies on Hokkaido and found bird feathers in fecal pellets from mid-May to June and September to late December. In this study, we found DNA of *L. ochotensis* in the feces of one pregnant *N. aviator*.

Table 1 Bat species, sampling year, sex, age, and number of samples (with one to ten fecal pellets per sample) analyzed in this study for each group

Genus	Species	Year	Sex	Age	*n*
Barbastella	*pacifica*	2015	M	ad	2
Murina	*hilgendorfi*	2015	M	ad	5
Plecotus	*sacrimontis*	2015	M	ad	3
Vespertilio	*sinensis*	2015	F	j	1
Murina	*hilgendorfi*	2016	F	ad	1
Murina	*hilgendorfi*	2016	M	NA	1
Murina	*hilgendorfi*	2017	M	ad	2
Myotis	*frater*	2017	M	ad	1
Nyctalus	*aviator*	2017	F	ad	7
Plecotus	*sacrimontis*	2017	F	ad	1
Vespertilio	*sinensis*	2017	F	j	1

Abbreviations: M = male, F = female, ad = adult, j = juvenile, NA = not available

With a size range of 13.5–14.5 cm and a weight range of 19–23 g, *L. ochotensis* (Brazil 2009) is of similar size, but about half the body weight of *N. aviator* (Ohdachi et al. 2015). Similarly, *N. lasiopterus* has been reported to feed on *Erithacus rubecula*, *Parus caeruleus* and other migratory passerines in Italy and Spain during autumn (Dondini and Vergari 2000; Ibáñez et al. 2001; Popa-Lisseanu et al. 2007). These birds are about half the size of *N. lasiopterus* and highlight the ability of these bats to capture prey that is half their weight while flying (Ibáñez et al. 2016).

Locustella ochotensis is a migratory passerine that passes through the Aomori prefecture south of Hokkaido between early-June and late-June and arrives a few days later at its breeding grounds in Hokkaido (Ishizawa 1960). Since the breeding period of these birds starts with the arrival of females in June (Nagata 1986) and the sampling of *N. aviator* took place in early June, *N. aviator* was feeding on *L. ochotensis* between the end of the bird's migration period and beginning of its breeding period. *Nyctalus lasiopterus* and *Ia io* have been reported to feed on passerine birds largely during the migratory periods, when bats are thought to capture their prey at high altitudes (Ibáñez et al. 2016). These bat species, as well as *N. aviator*, are adapted to foraging in open space, with long and narrow wings that allow for fast flight and echolocation calls at low frequencies (Fukui et al. 2004) to detect objects at a large distance (Norberg and Rayner 1987). As *L. ochotensis* may fly at high altitudes at night during migration (Ishizawa 1960), it is probable that *N. aviator* also catches these birds by aerial-hawking.

In light of previous reports about bird predation during periods of bird migration, it appears unlikely that *N. aviator* would catch these warblers during their breeding period, as the warblers exhibit nocturnal flight activity only during migration (Ishizawa 1960).

Although our finding confirms the hypothesis that *N. aviator* feeds on passerine birds, this study is based on a single positive sample, which highlights the need for further, more extensive, and specific diet studies.

As Japan lies within the East Asian flyway—a migratory route for many species of passerines (Brazil 2009)—we hypothesize that *N. aviator* forages on other passerine bird species in order to fulfill similar dietary requirements as *N. lasiopterus*. In contrast, *N. aviator* may opportunistically feed on passerine birds as they represent a rich food source that is available only during a short seasonal period. To test these hypotheses, more studies are necessary.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

Ethical approval The capture survey was conducted under the permission from the Ministry of Environment (Nos. 21-27-0077, 21-28-0087, and 21-29-0131) and Hokkaido Prefecture (Nos. 26-35 and 57), Japan. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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