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Concealed by darkness: interactions between predatory bats and nocturnally migrating songbirds illuminated by DNA sequencing

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Abstract

Recently, several species of aerial hawking bats have been found to prey on migrating songbirds, but details on this behavior and its relevance for bird migration are still unclear. We sequenced avian DNA in feather-containing scats of the bird-feeding bat *Nyctalus lasiopterus* from Spain collected during bird migration seasons. We found very high prey diversity, with 31 bird species from 8 families of Passeriformes, almost all of which were nocturnally flying sub-Saharan migrants. Moreover, species using tree hollows or nest boxes in the study area during migration periods were not present

in the bats' diet, indicating that birds are solely captured on the wing during nighttime passage. Additional to a generalist feeding strategy, we found that bats selected medium sized bird species, thereby assumingly optimizing their energetic cost-benefit balance and injury risk. Surprisingly, bats preyed upon birds half their own body mass. This shows that the 5% prey to predator body mass ratio traditionally assumed for aerial hunting bats does not apply to this hunting strategy or even underestimates these animals' behavioral and mechanical abilities. Considering the bats' generalist feeding strategy and their large prey size range, we suggest that nocturnal bat predation may have influenced the evolution of bird migration strategies and behavior.

Introduction

Predation has long captured the attention of ecologists for its power to affect prey demography and evolution (Anderson 1986; Zaretzky *et al.* 2011), influence ecosystem nutrient cycling (Schmitz 2010) and eventually lead to cascading changes in ecosystems (Ferretti *et al.* 2010). A recently discovered predator-prey interaction that has excited interest is predation by bats upon nocturnally migrating songbirds (Ibáñez *et al.* 2001, 2003; Bontadina & Arlettaz 2003; Popa-Lisseanu *et al.* 2007). Three bat species have so far been discovered to occupy this feeding niche in different parts of the world: *Nyctalus lasiopterus* in the Western Palearctic (Dondini & Vergari 2000; Ibáñez *et al.* 2001), *la io* in Southeastern Asia (Thabah *et al.* 2007), and *Nyctalus aviator* in Northeastern Asia (Fukui *et al.* 2013). The three species are among the largest representatives of the family Vespertilionidae, but belong to two not closely related genera (Thabah *et al.* 2007). This suggests that the novel feeding behavior evolved independently in insectivorous bats at least twice. Around a dozen other bat species feed on small terrestrial vertebrates – also complementing their diets with invertebrates to some extent – but they are all found in the tropics and typically show a gleaning hunting strategy (Norberg & Fenton 1988). *N. lasiopterus*, *N. aviator* and *la io*, by contrast, have a temperate – subtropical distribution

(Simmons 2005) and are aerial, open space hunters, as indicated by their wing morphology and echolocation (Ibáñez *et al.* 2001; Thabah *et al.* 2007; Estók & Siemers 2009; Fukui *et al.* 2013). Morphological analyses of droppings throughout the year of the two *Nyctalus* representatives show that bird predation is restricted to bird migration periods and that these bats are insectivorous the rest of the year (Ibáñez *et al.* 2001; Fukui *et al.* 2013). It has been suggested that bats capture birds in open flight (Ibáñez *et al.* 2001; Thabah *et al.* 2007; Fukui *et al.* 2013). This hypothesis indeed challenges previous knowledge on bat hunting dynamics. For example, according to comparative dietary studies on bats feeding in open space, bats capture airborne prey that do not exceed 5% of their own body mass (Fenton 1990). This is assumed to reflect mechanical limitations associated with size (Fenton 1990), although no experimental studies have addressed this issue. Paradoxically, even a 5 g songbird would account for as much as 10% of the bat's body mass (average weight is ca. 50 g for *N. lasiopterus*, (Ibáñez *et al.* 2001)). This disparity may explain why the discovery of this feeding strategy has been contested (Bontadina & Arlettaz 2003, Andreas 2010). Alternatively, it has been suggested that birds are captured while resting in tree hollows or nest boxes also used by bats (Dondini & Vergari 2000).

Optimal foraging theory predicts that predator decisions become more relevant as prey size increases relative to predator size (Charnov & Orians 1973). Indeed, the larger the typical prey consumed by a predator, the greater both the energy invested in the attack and the risk of injury. The comparatively large bird/bat body mass ratio would suggest an evolutionary advantage of selecting prey for bats hunting birds, at least at high encounter rates during the postnuptial songbird migration. At this time, bird densities peak, and ca. 50% of droppings of *N. lasiopterus* were found to contain only feathers (Ibáñez *et al.* 2001). For example, bats may select birds based on size, preferring those within a particular size range so as to optimize the balance between benefits (larger prey providing more energy per capture unit) and costs (larger prey may be more difficult to overpower and manipulate). Additionally, certain bird characteristics may be particularly conspicuous

to the bats (e.g. specific sensorial attributes) and be passively selected. Besides its intrinsic scientific interest, the dietary niche breadth of this feeding strategy may indeed affect migratory prey bird demographics.

The advent of molecular tools has allowed detailing diet composition to a species level and addressing new questions in trophic studies of bats (Clare *et al.* 2009; Razgour *et al.* 2011; Zeale *et al.* 2011; Alberdi *et al.* 2012). In this study, we have investigated the avian diet of the Western Palearctic bat *N. lasiopterus* by selectively sequencing avian DNA obtained from fecal remains collected along wide temporal and spatial windows. This has allowed us to 1) establish species composition and niche breadth of the bat's carnivorous diet, 2) settle the question whether bats hunt birds on the wing vs. capture them at the roosts, 3) discover a new prey size threshold for aerial-hawking bats, and 4) test whether bats select prey based on size. Based on our findings, we reflect on the impact of predation by bats on populations of migratory birds.

Materials and methods

Study sites and feces collection

We analyzed 226 feather-containing scats of *N. lasiopterus*. A first set of pellets (N = 154) was collected between 1998 and 2009 from flying bats netted over water courses, mainly from La Rioja, northern Spain (N=115) or as they returned to their tree roosts in a city-park in Seville in Andalusia, Southern Spain (N=39). All these bats were individually kept in cloth bags and thereafter sexed, weighed, measured, and released at the site of capture, and droppings were collected from the bag. Only one scat was selected per bat and night for analyses. A second set of pellets (N = 72) was collected daily under roosts between 2003 and 2010, either under palms (*Washingtonia filifera*) in a city-park in Seville (N = 32) or under bat-boxes in Doñana National Park (DNP) in Andalusia, Southern Spain (N = 40). For this set of pellets only one individual dropping per day and roost was analyzed, to

ensure it represented the diet of only one bat so as to render the two data sets comparable. Fecal pellets were kept either frozen at -20 °C or dried in paper bags at room temperature until processed.

DNA extraction, PCR amplification and sequencing, and prey quantification

A preliminary study showed a high proportion (>90 %) of bat pellets with a single bird prey (Pastor-Beviá *et al.* 2014) suggesting as the most appropriate lab strategy the combination for each dropping of single Sanger sequencing and cloning of a homogenized mix rather than a multiple sequencing of different bits, as has been done in other bat diet studies (Clare *et al.* 2009, 2011). Total DNA was extracted in a sterile DNA laboratory (LEM-EBD), starting from 0.01 – 0.05 g of fecal pellet unit, using a modified guanidinium thiocyanate (GuSCN) method by Rohland & Hofreiter (2007). Accordingly, each fecal pellet unit was pulverized using steel balls after a liquid nitrogen immersion and washed several times with ethanol, dried and resuspended with water (Pastor-Beviá *et al.* 2014).

Three mitochondrial fragments were amplified from avian DNA using different combinations of primer sets: two fragments (160 and 380 bp) of the Cytochrome b gene (Cytb) using the primer sets CytbSPrey (FW/RW) and CytbLPrey (FW/RW) respectively, and one fragment (380 bp) of the Cytochrome Oxidase gene subunit I (COI) using primer sets COIPrey (FW/RW) (Pastor-Beviá *et al.* 2014). Amplification products were sequenced in an ABI 3100 automated sequencer (PE Biosystems, Warrington, UK) following manufacturer's protocols. Obtained sequences were checked visually with SEQUENCHER v. 4.9 (Gene Codes Corp, MI, USA). Double peak patterns in the inspection of electropherograms suggested the co-amplification of different sequences in 25 amplification products. In 10 of them, the sequences could be ascribed unambiguously to two different bird species. The remaining 15 were cloned using pGEM-T Easy Vector System and high-efficiency competent cells (Promega, www.promega.com) after selecting 16 clones per plate and following Pastor-Beviá *et al.* (2014). The obtained sequences were compared to available sequences in the

GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank>) using the BLAST tool and species identification was based on a >98% of similitude threshold.

To quantify prey consumption we assumed a conservative approach in which the presence of DNA from one bird species in one dropping corresponds to only one individual prey. Since we never analyzed more than one fecal pellet of the same bat and day, the same individual bird could not appear in more than one pellet. We thus defined “prey items” for each bird species as the number of fecal pellets in which the species was identified. DNA from two different species in the same pellet would indicate the consumption of (at least) two individual birds.

Dietary niche breadth, prey characteristics and prey choice

Taxonomic arrangement, migratory behavior (by categories of long-distance migrant, partial migrant, and sedentary) and information regarding the use of hollows and nest boxes for each bird species identified were obtained from Cramp & Perrins (1977-1994) and Del Hoyo *et al.* (2004-2006), whereas information on migration patterns (primarily diurnal vs. nocturnal) was obtained from Newton (2008). Prey size was estimated from the average body mass of 20 individuals (10 males and 10 females) of each bird species captured during the postnuptial migration ringing campaigns in DNP (except for *Sylvia hortensis* with 11 individuals and *S. conspicillata* with 3).

To explore dietary niche breadth of *N. lasiopterus*, we considered bat scats as sampling units of the bird prey population, and estimated the total number of prey species consumed by calculating and extrapolating the sample-based rarefaction curve until reaching an asymptote (EstimateS, Colwell 2013).

To investigate prey size selection of *N. lasiopterus*, we compared body mass (bm) distribution of prey items consumed during postnuptial periods (n = 168) with bm distribution of all available prey. To estimate available prey, we used data of birds mist-netted in DNP during postnuptial ringing

campaigns from 2000 to 2010 that belonged to any of the species identified in feces of *N. lasiopterus*. We assumed that this set (N = 30,915) was representative of the distribution of total songbirds migrating at night across the Iberian Peninsula in the postnuptial period. Two species identified in the feces, *Calandrella brachydactyla* and *Carduelis cannabina* (both contributing with only 1 prey item) were not represented in the bird ringing data and therefore, since availability could not be estimated, were not used for the analysis. We also excluded *Cettia cetti*, a sedentary species found in only one scat, from the analysis. Since the species is resident in the area, it was overrepresented in the ringing data, biasing therefore estimates of availability.

We grouped available prey (expected) and actual prey (observed) data in three bm categories (<10 g, 10 – 15 g and >15 g). By bootstrapping (9999 draws) in R (R Development Core Team, 2015, version 3.2.3) the DNP postnuptial migrating bird capture dataset (N = 30,915), we obtained expected average frequencies, standard deviations (SD) and 99% confidence intervals (CI) for the three bm categories for a subset of 168 samples (Table 2). Additionally, we performed a Chi-square test of goodness of fit to compare bm values in the two datasets. We also compared observed vs. expected proportions within each bm category using the Z-test statistic derived from the Pearson Chi-Square statistic, calculated with IBM SPSS Statistics 21 (IBM Corp, Armonk, NY, USA).

Results

Avian DNA was amplified and sequenced from 215 out of 226 analyzed fecal samples (95% success). Only 15 scats had DNA from two bird species, and 1 scat had DNA from three species. Thus, a total of 232 prey items were identified unequivocally at species level based on a > 98% similarity with the DNA of a single species in the BLAST comparisons.

Identified prey belonged to 31 species of 8 families of Passeriformes (Alaudidae,

Hirundinidae, Motacillidae, Muscicapidae, Turdidae, Sylviidae, Regulidae and Fringilidae) (Table 1, Fig. 1). The sample-based rarefaction curve for the estimated prey species richness did not reach an asymptote within the number of samples analyzed, but only after extrapolating total sample number by a factor of five, estimating total richness at ca. 43 prey species, with 95% CI (25, 61) (Fig. 2).

The family Sylviidae was by far the most represented in the diet (16 species, and 75% of total prey items) with all 8 Iberian representatives of the genus *Sylvia* identified in feces. The family Muscicapidae accounted for as much as 13% of the total prey items despite being represented by only two species. By contrast, the family Turdidae accounted for only 6% of the prey items despite its higher diversity in the diet (6 species identified). The four remaining families contributed with 1-2 prey items each, summing up to only 2.2% of bird prey (Table 1, Fig. 1).

Species composition of the diet varied over the year (Table S1). The 5 species most preyed upon (*Sylvia cantillans*, *Phylloscopus trochilus*, *S. borin*, *S. communis* and *Ficedula hypoleuca*) represented almost 60% of total prey items while 10 species appeared only once. Most species (74.2%) and prey items (93.5%) were long-distance migrants (sub-Saharan migrants) that fly primarily by night (Table 1). Only two species considered exclusively sedentary (*Cettia cetti* and *Sylvia undata*) were found in the droppings, but they only contributed with 3 prey items, whereas three other species that show both sedentary and migrant populations were represented scarcely by 4 prey items (Table 1). Proportion of sub-Saharan vs. partial migrants vs. sedentary birds changed over the year (Fig. 3). The majority of species (87.1% of all species identified, and comprising 74.9% of prey items) found in the droppings do not use tree holes and/or nest-boxes to roost during the sampling period (Table 1).

The average bm of the 31 prey species consumed was 14.0 ± 4.9 g, ranging from 5 g for *Regulus ignicapilla* to 24.7 g for *Anthus trivialis*. The average bm for the 232 identified prey items was 12.6 ± 4.1 g (Table 1), with 82% weighing between 5 and 15 g, and none over 25g. 168 prey items

were identified in feces collected during postnuptial migration and were used for testing preferences in prey bm. Expected average frequencies, standard deviations (SD) and 99% confidence intervals (CI) for the three bird bm categories defined in Materials and methods are presented in Table 2. Our observed frequency for size class 1 was very near the average bootstrapped frequency, while it was outside the 99% confidence interval (CI) of the bootstrapped frequencies for size classes 2 and 3 (over, and below, respectively; Fig. 4). Bm values of observed prey items was significantly different from that of available prey as estimated from ringing data in DNP ($\chi^2 = 10.555$, $d.f. = 2$, $p = 0.005$). Our analyses indicate that birds with bm between 10 and 15 g were consumed in autumn more than expected ($Z = 2.67$, $p = 0.008$), while birds >15g were consumed less than expected ($Z = 2.98$, $p = 0.003$). Proportion of birds with bm < 10 g consumed was not significantly different from expected ($Z = 0.032$, $p = 0.972$) (Fig. 4).

Discussion

Prey identification by means of DNA sequencing

We used Sanger DNA sequencing to identify avian prey in fecal remains of *N. lasiopterus* collected over more than 10 years (1998-2010) at different locations in Spain. Although Sanger DNA sequencing in its application to dietary studies is increasingly being displaced by next generation sequencing (NGS) (e.g. bat diets: Razgour *et al.* 2011; Vesterinen *et al.* 2013; Clare *et al.* 2014), in combination with cloning it can still be recommended when targeted DNA has relatively low diversity, samples have heterogeneous origins, or have undergone different conservation procedures (Pastor-Beviá *et al.* 2014). In these cases, it produces a lower cost, comparably high rate of successful DNA amplification to NGS or even higher (95%) (e.g. see Razgour *et al.* 2011). Achieving an amplification success of 95%, we found that *N. lasiopterus* preys on an outstanding diversity of bird prey, at least 31 bird species belonging to 8 different families.

Bat hunting strategy: aerial predators of migratory birds vs. nest predators

Most of the species (74.2%) and individual prey (93.5%) as estimated conservatively (“prey items”, see Materials and methods) identified in feces are nocturnal migrants (Table 1). This indicates that bats capture birds engaged in nocturnal flights, mostly during migration. Bird families are traditionally categorized as either diurnally or nocturnally migratory. There are however frequent exceptions within families, with species that do not fit the general strategy, and even within species (Martin 2010). These inconsistencies may explain our infrequent findings of not typically nocturnally migrating species in the bat’s diet. For instance, *Anthus trivialis* and *A. pratensis* – representing 3.5% of prey items – are considered, as other Motacillidae, diurnal migrants, but are frequently captured at night during ringing campaigns in the Alps (Dorka 1966). Similarly, the anecdotal report in the bat’s diet of *Calandrella brachydactyla*, *Serinus serinus*, *Carduelis cannabina* and *Riparia riparia* (1.7% of prey items and each appearing only in one scat), belonging to families of mostly diurnal migrants (Families Alaudidae, Fringillidae, and Hirundinidae, respectively), may correspond to occasional night flights of these birds (Dorka 1966; Martin 2010; Watson *et al.* 2011). The presence of the mainly sedentary species *Cettia cetti* and *Sylvia undata* (belonging to the typically migratory family Sylviidae and making up the remaining 1.3% of prey items) may be attributed to dispersal flights known to be undertaken by these species (Cramp & Perrins 1977-1994).

If birds were captured in nest boxes or roosts (Dondini & Vergari 2000), birds that typically use boxes or tree hollows should be present in the diet. However, no members of the family Paridae or the genera *Sitta* and *Certhia* that exhibit this behavior (Cramp & Perrins 1977-1994) appear in any droppings. Furthermore, identified prey species that may use these roosts, such as *Ficedula hypoleuca* and *Erithacus rubecula*, do so only during the breeding season in late spring/early summer (Cramp & Perrins 1977-1994), when bats do not consume birds (Ibáñez *et al.* 2001). In agreement

with predictions based on the eco-morphological characteristics of *N. lasiopterus*, our results clearly support the hypothesis that birds are captured, and presumably consumed (Ibáñez et al. 2001, 2003), in the air during high altitude migratory or dispersal movements (>500m; Popa-Lisseanu & Ibáñez 2007), and not while resting (Ibáñez et al. 2001, 2003). No evidence was collected in favor of bats catching birds encountered at their roosts, but this cannot be entirely ruled out (Dondini & Vergari 2000; Smirnov & Vekhnik 2013). If so, however, these captures would be rare and unlikely to result from an active search, in contrast to avian nest predators such as woodpeckers (Nilsson 1984; Weidinger 2009).

Dietary niche breadth

The high diversity of species consumed suggests that *N. lasiopterus* behaves as a generalist predator adjusting consumption largely to availability. For example, four out of the five species most frequently preyed upon (*Phylloscopus trochilus*, *Sylvia borin*, *S. communis* and *Ficedula hypoleuca*) (Table 1) are included within the 6 most abundant long-distance night-migrants of European passerines (Hahn et al. 2009). Diet composition along the postnuptial period matches the general phenology of migration during the same time window, starting in August with exclusively trans-Saharan migrants and ending in November with a majority of partial migrants and/or sedentary species with nocturnal dispersal movements (Table S1, Fig. 3) (Cramp & Perrins 1977-1994). Finally, up to 7 different bird species were consumed in one night at the same spot. Furthermore, the number of species identified relative to the number of samples analyzed did not reach an asymptote, suggesting that real prey species richness is higher than reflected by our sampling. Extrapolating the curve to over 1000 samples, we estimated prey species richness to be about 43 (Fig. 2). Considering only birds under 25 g (estimated prey upper size limit, see next section), there are ca. 50-55 bird species that have nocturnal flying habits and migrate over Iberia (Martin 2000; BirdLife International

2016; DNP ringing data), a figure that falls within the 95% CI of our estimated total prey diversity. Some of these species are rare or even very rare in the region, which would explain why it takes so many samples for the rarefaction curve to reach an asymptote (Fig. 2). It follows that *N. lasiopterus* preys on basically every available bird species within its prey size threshold. This further supports that the bat is a generalist bird predator that captures birds as they fly across the region.

Prey size window and the 5% paradigm

N. lasiopterus consumed prey ranging ca. 5 – 25 g (Table 1), 25 g thus possibly representing the upper size limit of its prey. It is however worth noting that the three families of birds that mainly migrate at night (Muscicapidae, Sylviidae and Turdidae) and that make up the largest part of the diet show discontinuous body mass distributions, with very few species weighing between 25-35g. In fact, in Iberia only *Acrocephalus arundinaceus* within Sylviidae has an average body mass over 25g. Among Turdidae there is a big gap between the body mass of the large *Turdus* (ca. 50-100 g) and the much smaller *Oenanthe oenanthe* (ca. 22 g). The absence of species >25 g in the diet could thus reflect low availability rather than size limitations. Swifts, however, despite showing a constant night flight activity over the whole breeding period (Bruderer & Weitnauer 1972), were absent from the diet, which may suggest that prey over 30 g (bm of *Apus apus* and *A. pallidus* is > 30 g) is inaccessible to the bats, since neither flight altitude of swifts nor their speed (Henningsson et al. 2009) seem to be limiting factors against predation by bats.

With an estimate of 5 to 25 g as the prey body mass window of *N. lasiopterus*, the ratio bird prey to bat body mass, ranging 10 - 50% and averaging 25%, widely exceeded the 5% threshold in relation to the flight ability of bats hunting airborne prey (Fenton 1990). Most probably, the rule remains valid for bats hunting airborne insects, but not for these bats hunting birds at high altitude (Ibáñez et al. 2003). *N. lasiopterus* does not appear to have particular adaptations to bird hunting: it

is very similar in its morphology and ecology to the smaller species in the genus, and its particularly low call frequencies can be explained simply by allometric scaling (Estók & Siemers 2009). There is therefore no objection to applying a 25% prey size threshold to smaller but morphologically and ecologically similar bat species (e.g. those with a strong jaw, generalist diet, long echolocation detection, or able to fly high). A bat of 20 g may consequently be able to capture birds between 5 to 10 g if it happens to encounter them while foraging. This prediction suggests a reinterpretation of the discovery by Gloor, Stutz and Ziswiler (Gloor *et al.* 1989) of feather remains in droppings of *Nyctalus noctula*, very similar and closely related to *N. lasiopterus* but with a lower bm of ca. 28.5 g (Jones *et al.* 2009), in Switzerland (Bontadina & Arlettaz 2003). Keeping in mind how long carnivory in *N. lasiopterus* remained undetected, we venture that predation by bats upon nocturnally flying birds, even when rare and opportunistic, may be more widespread among aerial bats than presently acknowledged.

Prey choice

With a prey to predator body mass ratio as high as 50%, optimal foraging theory would predict prey selection to be sufficiently advantageous to have evolved in *N. lasiopterus*. It has been suggested that bats' rapid flight and the short prey detection range permitted by echolocation prevents them from actually making economical decisions when foraging (Barclay & Brigham 1994; but see Vesterinen *et al.* 2016). However, *N. lasiopterus*, with its high intensity, low frequency echolocation calls and its unique tendency to skip up to three calls (Holderied & von Helversen 2003) would be able to detect a flying passerine at a distance of up to 29 m using echolocation alone (Estók & Siemers 2009). Such large distances may well allow enough time for target discrimination and decision-making.

Our results support a positive size selection of *N. lasiopterus* during autumn (period of maximum bird availability) towards medium sized birds (10 to 15 g; Fig. 4), possibly those

representing the most convenient cost/benefit balance in terms of energy and risk avoidance. Large birds (>15 g) may have been selected against (Fig. 4) because of the major challenges and risks involved in their manipulation during flight. Although our results are consistent with *N. lasiopterus* actively choosing prey from those available as predicted, a non-random diet relative to availability could also result from sensory bias or from differential success rates. For example, given their larger size, birds 10-15 g could be easier to spot relative to birds <10 g and consequently receive more attacks. The negative selection of birds >15 g does not support, however, detection bias towards larger birds, but it could be a result of lower attack success rates for larger, stronger birds.

The generalistic feeding strategy of *N. lasiopterus* does not suggest particular sensorial characteristics of the prey (e.g. plumage coloration, calling during flight, specific flight behaviors, etc.) to be decisive cues when locating prey. However, a few species were consumed more than expected according to their abundance. *Sylvia cantillans* for example appeared in more droppings in certain months during postnuptial migration than *Phylloscopus trochilus* (Table S1), despite that their migrating populations in Europe are 30 times smaller (Hahn *et al.* 2009). High predation figures for particular species may also be related to the recent and increasingly found behavior in some nocturnal migratory songbirds of short nocturnal movements unrelated to migration (Mukhin *et al.* 2009).

Bat predation and bird migration

We show that *N. lasiopterus* is a generalist predator of migrating birds up to 25 g. The majority of songbirds in migration fall within this size category (e.g. 93% of all birds captured in nets in DNP during migration) and are thus potentially exposed to predation by bats. The new prey to bat size threshold further suggests that bat predation upon migrating birds may be more widespread among open space aerial bat hunters. When more precise data on bat population numbers and feeding

strategy become available, it may be possible to quantify bats' total predation on bird populations, and to compare it with that of diurnal specialists on migrating birds such as the Eleanora's falcon. Even local changes in predation risk have been shown to generate immediate responses in the behavior of migrating birds (Ydenberg *et al.* 2004; Jonker *et al.* 2010) and therefore, predation by bats may have been a contributing factor in shaping bird migration strategies and driving the evolution of nocturnal anti-predator behaviors.

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References

- Alberdi A, Garin I, Aizpurua O, Aihartza J (2012) The foraging ecology of the mountain long-eared bat *Plecotus macbullaris* revealed with DNA mini-barcodes. *PLoS ONE*, **7**, e35692.
- Anderson CM (1986) Predation and primate evolution. *Primates*, **27**, 15–39.
- Andreas M (2010) Study of diet in Middle East and African bats. In: *A Tribute to Bats* (eds Horacek I, Uhrin M), pp. 265–267. Lesnická práce s.r.o. Armonk, NY, Kostelec nad Černými lesy.
- Barclay RM, Brigham RM (1994) Constraints on optimal foraging: a field test of prey discrimination by echolocating insectivorous bats. *Animal Behaviour*, **48**, 1013–1021.
- BirdLife International (2016) Species search. Available at: <http://www.birdlife.org/datazone/species/search> [Accessed February 24, 2016].
- Bontadina F, Arlettaz R (2003) A heap of feathers does not make a bat's diet. *Functional Ecology*, **17**, 141–142.
- Bruderer B, Weitnauer E (1972) Radarbeobachtungen über Zug und Nachtflüge des Mauerseglers (*Apus apus*). *Revue Suisse de Zoologie*, **79**, 1190–1200.
- Charnov E, Orians GH (1973) *Optimal foraging: Some theoretical explorations*. University of Washington, Washington.
- Clare EL, Fraser EE, Braid HE, Fenton MB, Hebert PDN (2009) Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*): using a molecular approach to detect arthropod prey. *Molecular Ecology*, **18**, 2532–2542.
- Clare EL, Barber BR, Sweeney BW, Hebert PDN, Fenton MB (2011) Eating local: influences of habitat on the diet of little brown bats (*Myotis lucifugus*). *Molecular Ecology*, **20**, 1772–1780.
- Clare EL, Symondson WO, Fenton MB (2014) An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). *Molecular Ecology*, **23**, 3633–3647.
- Colwell RK (2013) EstimateS: Statistical estimation of species richness and shared species from samples (Version 9 and earlier)[Software]. Available from <http://viceroy.eeb.uconn.edu/estimates/>
- Cramp S, Perrins CM eds (1977–1994) *The Birds of the Western Palearctic, Vols. 1–9*. Oxford University Press, Oxford.
- Del Hoyo J, Elliot A, Christie DA eds (2004–2006) *Handbook of the birds of the world, Vols. 9-11*. Lynx Edicions, Barcelona.
- Dondini G, Vergari S (2000) Carnivory in the greater noctule bat (*Nyctalus lasiopterus*) in Italy. *Journal of Zoology*, **251**, 233–236.
- Dorka V (1966) Das jahres- und tageszeitliche Zugmuster von Kurz- und Langstreckenziehern nach Beobachtungen auf den Alpenpässen Cou/Bretolet (Wallis). *Ornithologische Beobachter*, **63**, 165–223.

- Estók P, Siemers BM (2009) Calls of a bird-eater: the echolocation behaviour of the enigmatic greater noctule, *Nyctalus lasiopterus*. *Acta Chiropterologica*, **11**, 405–414.
- Fenton MB (1990) The foraging behaviour and ecology of animal-eating bats. *Canadian Journal of Zoology*, **68**, 411–422.
- Ferretti F, Worm B, Britten GL, Heithaus MR, Lotze HK (2010) Patterns and ecosystem consequences of shark declines in the ocean. *Ecology Letters*, **13**, 1055–1071.
- Fukui D, Dewa H, Katsuta S, Sato A (2013) Bird predation by the birdlike noctule in Japan. *Journal of Mammalogy*, **94**, 657–661.
- Gloor S, Stutz HPB, Ziswiler V (1989) Nutritional habits of the noctule bat *Nyctalus noctula* (Schreber, 1774) in Switzerland. *Myotis*, **32–33**, 231–242.
- Hahn S, Bauer S, Liechti F (2009) The natural link between Europe and Africa – 2.1 billion birds on migration. *Oikos*, **118**, 624–626.
- Henningsson P, Karlsson H, Bäckman J, Alerstam T, & Hedenström A (2009) Flight speeds of swifts (*Apus apus*): seasonal differences smaller than expected. *Proceedings of the Royal Society of London B*, **276**, 2395–2401.
- Holderied MW, von Helversen O (2003) Echolocation range and wingbeat period match in aerial-hawking bats. *Proceedings of the Royal Society B*, **270**, 2293–2299.
- Ibáñez C, Juste J, García-Mudarra JL, Agirre-Mendi PT (2001) Bat predation on nocturnally migrating birds. *Proceedings of the National Academy of Science USA*, **98**, 9700–9702.
- Ibáñez C, Juste J, García-Mudarra JL, Agirre-Mendi PT (2003) Feathers as indicator of a bat's diet: a reply to Bontadina & Arlettaz. *Functional Ecology*, **17**, 143–145.
- Jones KE, Bielby J, Cardillo M, *et al.* (2009) PanTHERIA: a species-level database of life history, ecology, and geography of extant and recently extinct mammals: Ecological Archives E090-184. *Ecology*, **90**, 2648.
- Jonker RM, Eichhorn G, van Langevelde F, Bauer S (2010) Predation danger can explain changes in timing of migration: the case of the barnacle goose. *PLoS ONE*, **5**, e11369.
- Martin G (2010) *Birds by night*. Poyser, London.
- Mukhin A, Grinkevich V, Helm B (2009) Under cover of darkness: nocturnal life of diurnal birds. *Journal of Biological Rhythms*, **24**, 225–231.
- Newton I (2008) *The migration ecology of birds*. Academic Press, London.
- Nilsson SG (1984) The evolution of nest-site selection among hole-nesting birds: The importance of nest predation and competition. *Ornis Scandinavica*, **15**, 167–175.
- Norberg UM, Fenton MB (1988) Carnivorous bats? *Biological Journal of the Linnean Society*, **33**, 383–394.
- Pastor-Beviá D, Ibáñez C, García-Mudarra JL, Juste J (2014) A molecular approach to the study of avian DNA in bat feces. *Acta Chiropterologica*, **16**, 451–460.

- Popa-Lisseanu AG, Delgado-Huertas A, Forero MG *et al.* (2007) Bats' conquest of a formidable foraging niche: the myriads of nocturnally migrating songbirds. *PLoS ONE*, **2**, e205.
- Popa-Lisseanu AG, Ibáñez C. (2007) Ecología espacial y trófica del nóctulo gigante (*Nyctalus lasiopterus*) en el Parque Nacional de Doñana y su entorno. In: *Proyectos de investigación en parques nacionales: 2003-2006*, pp. 261-279. Organismo Autónomo de Parques Nacionales, Ministerio de Medio Ambiente, Madrid.
- Razgour O *et al.* (2011) High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. *Ecology and Evolution*, **1**, 556–570.
- Rohland N, Hofreiter M (2007) Comparison and optimization of ancient DNA extraction. *Biotechniques*, **42**, 343–352.
- Schmitz OJ, Hawlena D, Trussell GC (2010) Predator control of ecosystem nutrient dynamics. *Ecology Letters*, **13**, 1199–1209.
- Simmons NB (2005) Order Chiroptera. In: *Mammal species of the World: a taxonomic and geographic reference*, Third Edition, Volume 1 (eds Wilson DE, Reeder DM), pp. 312–529. Johns Hopkins University Press, Baltimore.
- Smirnov DG, Vekhnik VP (2013) Trophic ecology and predation of the greater noctule bat (*Nyctalus lasiopterus*) in Russia. *Biology Bulletin*, **40**, 206–212.
- Thabah A, Li G, Wang Y *et al.* (2007) Diet, echolocation calls, and phylogenetic affinities of the great evening bat (*Ia io*; Vespertilionidae): another carnivorous bat. *Journal of Mammalogy*, **88**, 728–735.
- Vesterinen EJ, Lilley T, Laine VN, Wahlberg N (2013) Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread insectivorous predator Daubenton's Bat (*Myotis daubentonii*) in Southwestern Finland. *PLoS ONE*, **8**, e82168.
- Vesterinen EJ, Ruokolainen L, Wahlberg N, *et al.* (2016) What you need is what you eat? Prey selection by the bat *Myotis daubentonii*. *Molecular Ecology*, **25**, 1581–1594.
- Watson ML, Wells JV, Bavis RW (2011) First detection of night flight calls by pine siskins. *Wilson Journal of Ornithology*, **123**, 161–164.
- Weidinger K (2009) Nest predators of woodland open-nesting songbirds in central Europe. *Ibis*, **151**, 352–360.
- Ydenberg, R C, Butler, RW, Lank, DB, Smith, BD, Ireland, J (2004) Western sandpipers have altered migration tactics as peregrine falcon populations have recovered. *Proceedings of the Royal Society B*, **271**, 1263-1269.
- Zanette LY, White AF, Allen MC, Clinchy M (2011) Perceived predation risk reduces the number of offspring songbirds produce per year. *Science*, **334**, 1398–1401.
- Zeale MRK, Butlin RK, Barker GLA, Lees DC, and Jones G (2011) Taxon-specific PCR for DNA barcoding arthropod prey in bat feces. *Molecular Ecology Resources*, **11**, 236–244.

Data Accessibility:

Sample information (date, location, conservation procedure, etc.) and *Cytb* and *COI* sequences are provided in Dryad doi: <http://dx.doi.org/10.5061/dryad.00vg4>

Supporting Information

Table S1. Monthly distribution of bird prey items, defined as the number of fecal pellets in which the species was identified (see Materials and Methods), identified in feces of *Nyctalus lasiopterus* and percentage along 13 years of study.

Author Contributions

CI, AGP-L and JJ designed the study and collected the data, JLG-M and DP-B produced sequence and blast analyses, DP-B and AGP-L analyzed data, AGP-L led the writing with significant contributions from CI and JJ.

For Review Only

Table 1. Characteristics of the bird species identified in feces of *Nyctalus lasiopterus*. N: number of prey items, defined as the number of fecal pellets in which the species was identified (see Materials and Methods). Migration category: 1 Long-distance migrant; 2 Partial migrant; 3 Largely sedentary (Cramp & Perrins 1977-1994; 29; 50). Migration time: 1 = primarily by day; 2 = primarily by night (51).

Bird families and species	N	Migration category	Migration time	Hollows use	Body mass (g)
Alaudidae					
<i>Calandrella brachydactyla</i>	1	1	1	no	21.2 ± 1.75
Fringillidae					
<i>Carduelis cannabina</i>	1	2 + 3	1	no	13.4 ± 0.55
<i>Serinus serinus</i>	1	2 + 3	1	no	10.9 ± 0.79
Hirundinidae					
<i>Riparia riparia</i>	1	1	1	no	10.7 ± 0.76
Motacillidae					
<i>Anthus pratensis</i>	2	2	1	no	18.0 ± 1.42
<i>Anthus trivialis</i>	6	1	1	no	24.7 ± 3.92
Muscicapidae					
<i>Ficedula hypoleuca</i>	28	1	2	yes	13.0 ± 1.92
<i>Muscicapa striata</i>	3	1	2	yes	15.8 ± 1.86
Regulidae					
<i>Regulus ignicapilla</i>	1	2	2	no	5.0 ± 0.23
Sylviidae					
<i>Acrocephalus schoenobaenus</i>	3	1	2	no	12.1 ± 2.29
<i>Acrocephalus scirpaceus</i>	5	1	2	no	11.8 ± 2.26
<i>Cettia cetti</i>	1	3	-	no	12.3 ± 1.75
<i>Hippolais polyglota</i>	12	1	2	no	11.0 ± 1.50
<i>Locustella naevia</i>	14	1	2	no	13.9 ± 2.47
<i>Phylloscopus bonelli</i>	10	1	2	no	7.9 ± 1.29
<i>Phylloscopus collybita</i>	7	1 + 2	2	no	6.8 ± 1.06
<i>Phylloscopus trochilus</i>	24	1	2	no	8.5 ± 1.08
<i>Sylvia atricapilla</i>	5	1 + 2	2	no	17.4 ± 2.02
<i>Sylvia borin</i>	16	1	2	no	18.5 ± 2.85
<i>Sylvia cantillans</i>	46	1	2	no	9.9 ± 1.31
<i>Sylvia communis</i>	23	1	2	no	14.4 ± 1.67
<i>Sylvia conspicillata</i>	4	2	2	no	9.6 ± 0.86
<i>Sylvia hortensis</i>	1	1	2	no	19.7 ± 1.57
<i>Sylvia melanocephala</i>	2	2	2	no	11.4 ± 0.50
<i>Sylvia undata</i>	2	3	-	no	8.3 ± 0.27
Turdidae					
<i>Erithacus rubecula</i>	3	2	2	yes	14.9 ± 0.89
<i>Luscinia megarhynchos</i>	5	1	2	no	22.2 ± 2.20
<i>Oenanthe oenanthe</i>	1	1	2	yes	22.1 ± 2.70
<i>Phoenicurus phoenicurus</i>	1	1	2	yes	14.3 ± 1.90
<i>Saxicola rubetra</i>	2	1	2	no	18.8 ± 1.85
<i>Saxicola torquata</i>	1	2 + 3	2	no	14.5 ± 1.12

Table 2. Estimated average standard deviations and confidence intervals of available bird prey, grouped by size. Values were obtained after randomly resampling – for a subset of 168 samples, same number as the observed prey items – 9999 distributions created by bootstrapping the original postnuptial migratory bird capture data from Doñana National Park (period 2000 – 2010). Observed frequencies also shown for comparison.

Bootstrapped frequencies				
	Size class 1	Size class 2	Size class 3	Sum
Mean	50.83	70.86	46.31	168
SD	5.927	6.352	5.834	18.112
99% CI	(36, 66)	(55, 87)	(32, 62)	
Observed frequencies				
	Size class 1	Size class 2	Size class 3	Sum
n	51	88	29	168

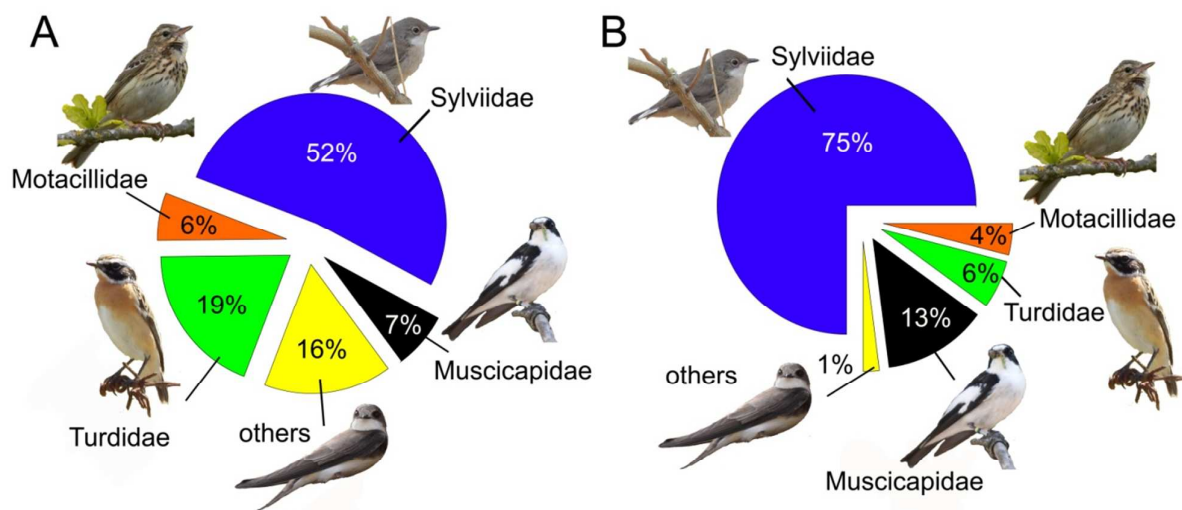


Fig. 1. Percentage grouped by family of bird species (A) and prey items (B) identified in feces of *Nyctalus lasiopterus*. Images by Lorenzo Pérez Rodríguez (*Ficedula hypoleuca*), Oscar Maganya (*Saxicola rubetra*), Carlos Ruiz (*Sylvia cantillans*), Chris Romeiks, Vogelartinfo (*Anthus trivialis*) (Wikimedia Commons GFDL 1.2, https://upload.wikimedia.org/wikipedia/commons/7/71/Anthus_trivialis_vogelartinfo_chris_romeiks_CHR4025.jpg), and Aiwok (*Riparia riparia*) (Wikimedia Commons, CC BY-SA 3.0, https://upload.wikimedia.org/wikipedia/commons/d/d4/Riparia_riparia_1.JPG).

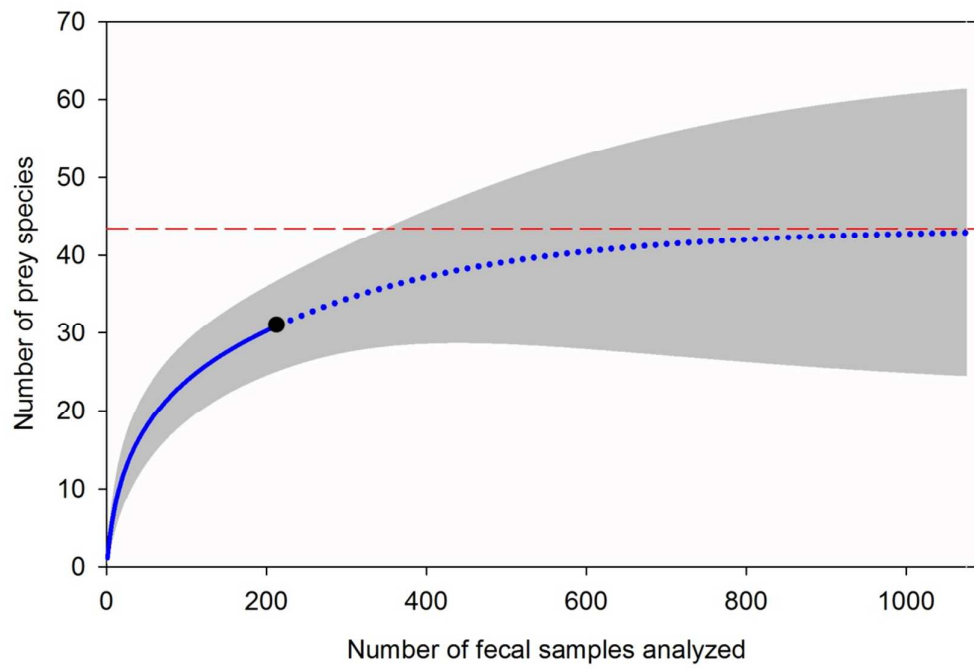


Fig. 2. Sampled-based rarefaction curve with 95% confidence intervals for the estimated species richness of bird prey of *Nyctalus lasiopterus*, calculated with EstimateS (52). Solid blue line: rarefaction curve; dotted line: extrapolation; solid black circle: reference samples; dashed red line: asymptote.

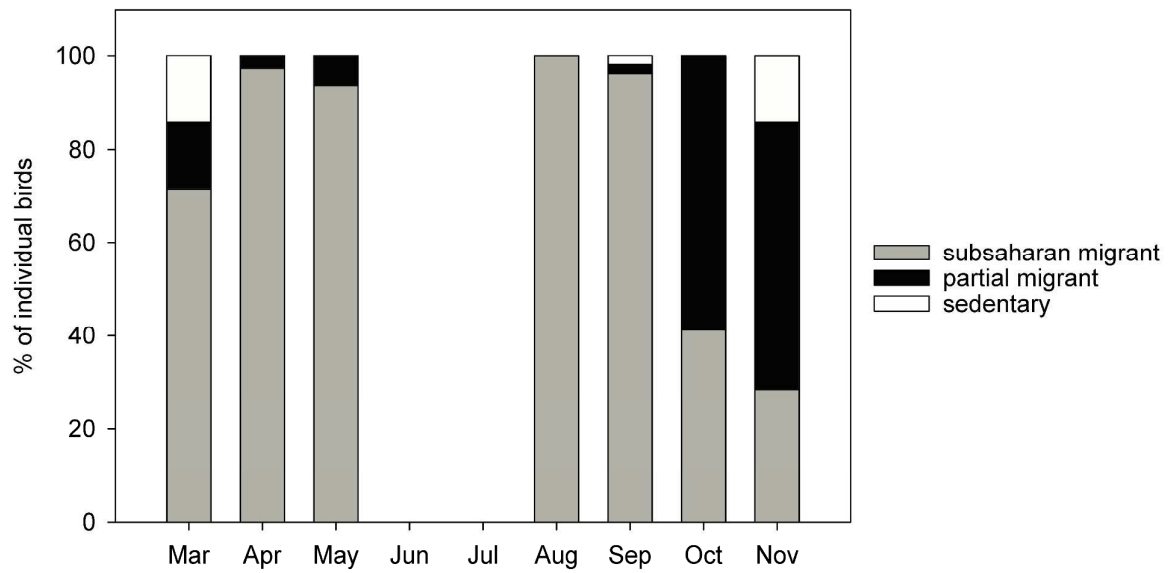


Fig. 3. Distribution of bird prey identified in feces of *N. lasiopterus* according to migration categories throughout the year.

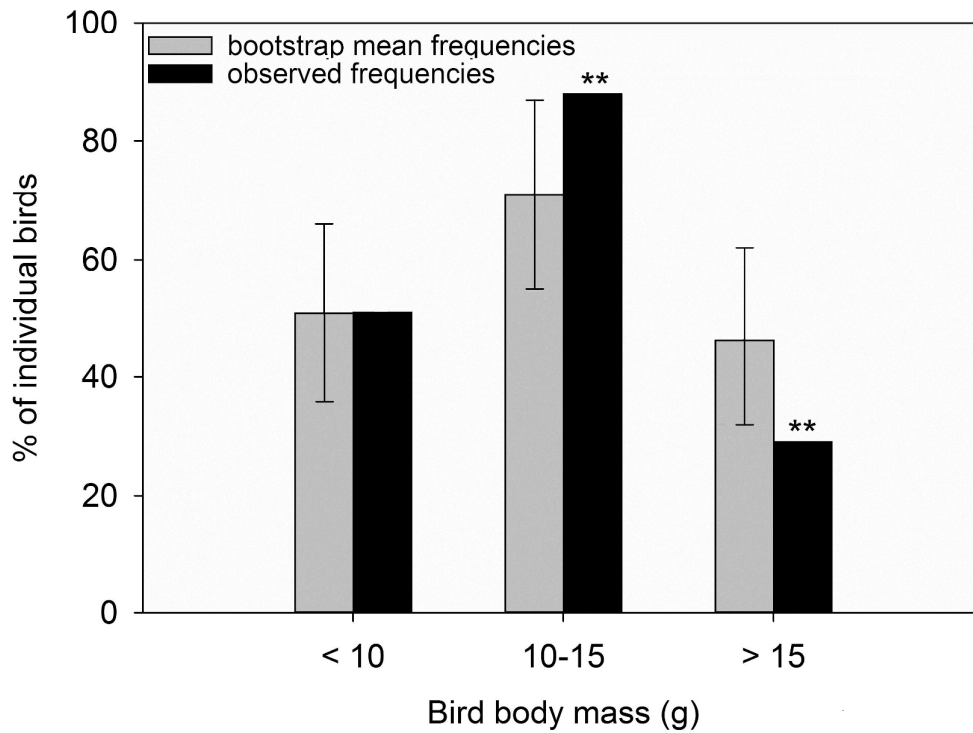


Fig. 4. Expected bird body mass distribution (black bars) during postnuptial migration obtained by bootstrapping a dataset of 30,915 birds ringed birds in Doñana National Park in the autumns of 2000-2010 and calculating frequencies for each category for repeated subsamples of $n=168$ (9999 draws) vs. observed frequencies in each category (total $n = 168$) for bird prey identified in feces of *N. lasiopterus* (grey bars). Vertical lines show the 99% CI for the expected frequencies. ** Difference is statistically significant at $p < 0.01$ (Z-test for proportions).

Supporting information

Table S1. Monthly distribution of bird prey items, defined as the number of fecal pellets in which the species was identified (see Materials and Methods), identified in feces of *Nyctalus lasiopterus* and percentage along 13 years of study.

	March		April		May		June	August		September		October		November		TOTAL
	n	%	n	%	n	%		n	%	n	%	n	%	n	%	
<i>Calandrella brachydactyla</i>										1	1.9					1
<i>Cettia cetti</i>										1	1.9					1
<i>Carduelis cannabina</i>												1	5.9			1
<i>Serinus serinus</i>												1	5.9			1
<i>Riparia riparia</i>										1	1.9					1
<i>Anthus pratensis</i>	1	14.3										1	5.9			2
<i>Anthus trivialis</i>			2	5.3	1	6.3				3	5.6					6
<i>Ficedula hypoleuca</i>					2	12.5		17	18.5	8	14.8	1	5.9			28
<i>Muscicapa striata</i>								1	1.1	2	3.7					3
<i>Phylloscopus bonelli</i>			2	5.3	3	18.8				4	7.4	1	5.9			10
<i>Phylloscopus collybita</i>												3	17.6	4	57.1	7
<i>Phylloscopus trochilus</i>	1	14.3	1	2.6				14	15.2	8	14.8					24
<i>Regulus ignicapilla</i>												1	5.9			1
<i>Acrocephalus schoenobaenus</i>								3	3.3							3
<i>Acrocephalus scirpaceus</i>								5	5.4							5

<i>Hippolais polyglota</i>			1	2.6	2	12.5		8	8.7			1	5.9			12
<i>Locustella naevia</i>								13	14.1			1	5.9			14
<i>Luscinia megarhynchos</i>					1	6.3		3	3.3	1	1.9					5
<i>Sylvia atricapilla</i>			1	2.6	1	6.3				1	1.9	2	11.7			5
<i>Sylvia borin</i>			2	5.3	1	6.3		12	13.0	1	1.9					16
<i>Sylvia cantillans</i>	3	42.8	24	63.2	3	18.8	1			14	25.9			1	14.3	46
<i>Sylvia communis</i>			1	2.6				16	17.4	5	9.3	1	5.9			23
<i>Sylvia conspicillata</i>	1	14.3	2	5.3	1	6.3										4
<i>Sylvia hortensis</i>										1	1.9					1
<i>Sylvia melanocephala</i>												1	5.9	1	14.3	2
<i>Sylvia undata</i>	1	14.3												1	14.3	2
<i>Erithacus rubecula</i>										2	3.7	1	5.9			3
<i>Oenanthe oenanthe</i>			1	2.6												1
<i>Phoenicurus phoenicurus</i>			1	2.6												1
<i>Saxicola rubetra</i>					1	6.3				1	1.9					2
<i>Saxicola torquata</i>												1	5.9			1
TOTAL (individuals)	7		38		16		1	92		54		17		7		232
TOTAL (species)	5		11		10		1	10		16		14		4		31