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Diet of the insectivorous bat *Pipistrellus nathusii* during autumn migration and summer residence

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Abstract

Migration is widespread among vertebrates, yet bat migration has received little attention and only in the recent decades has a better understanding of it been gained. Migration can cause significant changes in behaviour and physiology, due to increasing energy demands and aerodynamic constraints. Dietary shifts, for example, have been shown to occur in birds before onset of migration. For bats it is not known if a change in diet occurs during migration, although breeding season related dietary preference has been documented. It is known that a diet rich in fats and the accumulation of fat deposits do increase the flight range of migratory bats. Some bat species can be regarded as long-distance migrants, covering up to 2,000 km between summer and winter roosting areas. *Pipistrellus nathusii* (Vespertilionidae), a European long-distant migrant, travels each year along the Baltic Sea from north-eastern Europe to hibernate in central and southern Europe. This study presents

data on the dietary habits of migrating *Pipistrellus nathusii* compared with those during the breeding season. We analysed faecal samples from bats on fall migration caught at the Ornithological Field Station in Pape, Latvia and from samples collected in North-Latvian summer roosts. We applied both morphological identification and molecular methods, as morphological methods also recognize life stages of prey and can contribute frequency data. The diets of bats on migration and breeding bats were similar, with Diptera and Lepidoptera comprising the major prey categories. However, certain prey groups could be explained by the different hunting habitats exploited during migration vs. summer residence.

Introduction

Across the animal kingdom numerous species make annual movements of both short and long duration. In particular, bird migration has been intensively studied since the late 19th century. In the last decades these studies have been sophisticated both in methods and explanations and still are being developed further (Berthold 2001; Robinson *et al.* 2007; Wikelski *et al.*2007; Fiedler 2009). Many species of bats, the only volant mammals, are also known to migrate. Although the first interest in bat migration arose as early as the end of the 19th century (Merriam 1887), bat migration has been largely ignored until recently. Unlike birds, the elusive life strategies of bats, difficulties regarding visual observations, and low success in mark recapture programs, have made these species difficult to study. However, substantial recent advances have been made, which increase our understanding of orientation and physiology (Holland *et al.* 2006; Cryan & Brown 2007; McGuire & Guglielmo 2009; Voigt *et al.* 2010, 2012b). New techniques have contributed to our ability to track and infer actual range of movement, such as satellite tracking and stable isotope analysis (Cryan *et al.* 2004; Richter & Cumming 2008; Popa-Lisseanu & Voigt 2009; Voigt *et al.* 2012a; Tsoar *et al.* 2012)

Studies of bat migration can profit from previous work on bird migration (McGuire & Guglielmo 2009). Both birds and bats need to maintain a sufficient nutrient intake to meet the increased energy demand during migration over distances of sometimes several thousand kilometers between summer and winter habitats (Griffin 1970; Petersons 2004). As in birds, the scale of bat migration can vary considerably between short-distance, regional migrants (e.g., *Myotis daubentonii, M. lucifugus*) and long-distance migrants (e.g., *Pipistrellus nathusii, Lasioncyteris noctivagans*) (Fleming & Eby 2003; Dzal *et al.* 2009; Dzal *et al.* 2011). On their journeys birds and bats face similar tradeoffs between acquiring sufficient fat deposits (energy reserves) to fuel flight and maintaining body conditions (weight, size) optimal for flight with low energetic costs.

Birds are known to start to build up fat reserves before migratory flight and during stopovers at resting sites (e.g., Wadden Sea; McWilliams *et al.* 2004). Before the onset of migration birds show different adaptations to increase fat stores: they may become hyperphagous, their digestive and biosynthetic systems may alter, for example increase liver mass and liver activity (Egeler *et al.* 2000; Guglielmo & Williams 2003), and they may increase or reduce the size of their digestive systems (Piersma 1998; Piersma *et al.* 1999; McWilliams & Karasov 2004). Additionally birds are able to fly during the night and forage and refuel during the day.

Bats have to accomplish the dual task of both flying and refueling at night. Recent studies show that bats also become hyperphagous and increase their body fat and catabolic enzyme activity during pre-migration (Ewing *et al.* 1970; Bairlain 2001; McGuire *et al.* 2009, 2013a, b; Šuba *et al.* 2010). Furthermore, they are able to fuel their migration both directly from insects caught during flight and from stored fatty acid reserves to maintain both steady state and refill reserves (Voigt *et al.* 2010; Voigt *et al.* 2012b). The fly-and-forage hypothesis, which states that bats forage on the wing during migration, is supported by acoustical observations along migration routes (Ahlén *et al.* 2009; Valdez & Cryan 2009, Šuba *et al.* 2012). Yet, it is not clear to what extant bats are segregating foraging and migratory flight during these periods or whether they can truly hunt while migrating.

Another adaptation, the shift in diet towards different food items (e.g., from insects to fruit), helps some birds to gain sufficient energy during the pre-migration period (Bairlein 1990; Bairlein & Gwinner 1994; Bairlein 2001; McWilliams & Karasov 2005). It is not known if bats show similar behaviour. While most insectivorous bats use a generalist strategy, consuming prey in relation to their abundance (Anthony & Kunz 1977; Swift *et al.* 1985) within a given habitat (Clare *et al.* 2013a/ b in review, Special Issue), selective feeding and the ability to discriminate between food items have been demonstrated in some bat species (Von der Emde & Schnitzler 1990; Koselj *et al.* 2011). Dietary shifts over time have been

described in bats (Agosta 2002) and may be related to physiological state (pregnancy, lactation, preparation for hibernation) or changes in insect abundance (Clare *et al.* 2009, 2011, 2013a/b in press, Special Issue).

Here we tested the hypothesis that bat diet differs between summer roosting and fall migration. We used high throughput sequencing which yields detailed species-level data on prey in predator diets (Symondson 2002; King *et al.* 2008), and has been particularly successful in insectivores such as bats, (Razgour *et al.* 2011; Bohmann *et al.* 2011; Clare *et al.* 2013a/b in review) and shrews (Brown *et al*. in press, Special Issue). From species-level data (DNA sequences) we can draw conclusion on differences in prey items, apparent energy values or fat content and on putative foraging area differences between summer and migration habitats. We focused on a long-distance migrating bat, *Pipistrellus nathusii* (Keyserling & Blasius 1839), a generalist pipistrelle bat, which feeds to a large extent on insects connected to aquatic habitats, mainly on Diptera, particular Chironomidae (Beck 1994-1995; Vaughan 1997; Arnold *et al.* 2000; Flaquer *et al.* 2006). This species is known to travel up to 2000 km between the summer roosting grounds and hibernacula (Petersons 2004).

Methods

Study sites and sample collection

We collected samples for the purpose of dietary analysis at the Pape Ornithological Research Station at the southern Baltic coast of Latvia (56.165° N, 21.017° E) during the fall migration between 11 August and 12 September 2012 (Fig. 1). The station has been a central site for intensive research on bird and bat migration, particularly during the past twenty years (Petersons 2004). The surrounding areas are dominated by low sand dunes, partly covered by unmanaged pine woodlands (*Pinus sylvestris*) and grasslands. In the vicinity of the station is Pape Nature Park with Lake Pape, a 12km² shallow coastal lagoon lake, and a mosaic of marshland, reeds, meadows, forests and peat bogs. We caught bats from dusk until 01:00am using a Helgoland funnel trap following Petersons (2004). Bats were placed in a clean soft cotton bags and held for approximately 1h to collect faecal samples. Samples of *P. nathusii* faeces from summer colonies were collected from nursery colonies situated in buildings, at Vecpiebalga (57.058° N; 25.815° E) and artificial roosts with male groups at Garkalne (57.048° N; 24.382° E), Latvia during June 2013 (Fig. 1). Both sites are located in a mosaic landscape of forests, pasture and in proximity to large lakes.

Molecular diet analysis from faecal samples

For the molecular analysis we extracted DNA from faecal samples from individual migrating bats (n= 35 faecal samples) and from summer colony bulk samples, non-individually collected from under the roosting bats (n = 21 faecal samples) using the QIAamp DNA Stool Mini Kit (Qiagen, UK) following Zeale *et al.* (2011). To amplify the arthropod prey DNA we used modified primers based on the universal COI primer ZBJ-ArtF1c and ZBJ-ArtR2c. PCR (following the protocol of Zeale *et al.* 2011) produce a 157bp amplicon at the 5' end of the 658bp COI barcode region (Hebert *et al.* 2004). DNA was sequenced via a high throughput Ion Torrent sequencing platform (Life Technology) at the University of Bristol Genomics facility (School of Biological Sciences, Bristol, UK). For the adjustment,

trimming and organisation of sequences by MIDs after sequencing we used the Galaxy V platform (https://main.g2.bx.psu.edu/root; Giardine *et al.* 2005; Blankenberg *et al.*2005; Blankenberg *et al.*2010; Goecks *et al.*2010). To allow niche analysis procedure for all sequences, we clustered the sequences into molecular operational taxonomic units (MOTU) using the program jMOTU (Jones *et al.*2011). We tested grouping thresholds from 1-10bp and selected a 4bp threshold as the most appropriate for this data set (see Razgour *et al.* 2011). We extracted representative sequences for each MOTU and compared sequences against references within the Barcode of Life Data System (Ratnasingham & Hebert 2007; Clare *et al.* 2009). If sequences matched completely to a reference sequence without matching any other arthropod, we regarded the sequence as belonging to the same species. However, the short amplicon length also constrains some species assignments. We used a modified version of the criteria used by Razgour *et al.* (2011) as follows:

1a. True species match (>99 % similarity)

1b. Likely species match (>98% similarity)

2. Match (>98%) to more than one species, only one of which belongs to local assemblage

3. Match (>98%) to several species or genera – genus or family level assignment considered provisional.

Morphological identification from faecal samples

For morphological faecal analysis, we dried samples after DNA extraction (see Zeale *et al.* 2011) at room temperature and stored them at -20°C to avoid coprophagous insects. Before analysis, we soaked the pellets for 48 h in 70% ethanol and then dissected them under a binocular vision microscope (x 40 – 60). We separated characteristic fragments and mounted them in Euparal for further examination. We identification taxa to class, order, family, or genus level (where possible), by comparison of fragments with whole collected insects, arthropod identification keys from the literature (Medvedev 1989; Savage 1990; Shiel

et al. 1997; Osterbroek *et al.* 2005) and fragment photos from earlier studies (Krüger *et al.* 2012).

For every sample we calculated the frequency of each prey group relative to all samples, to estimate relative importance of prey groups (Shiel *et al.* 1997; Vaughaun 1997; Krüger *et al.* 2012).

Statistical analysis

As molecular and morphological analysis produce presence-absence data and frequency data, respectively, we analysed the two data sets in different ways.

We used the Hamming distance and Bray-Curtis index (Equation 1) for similarity to analyze the dietary differences between the migratory group and the summer group as measured by molecular data. Both indices use binomial data. The Hamming distance is related to the number of changes needed to adjust two strings of same length to each other (Hamming 1950). A smaller value for Hamming distances reflects high overlap in dietary choices. The Bray-Curtis index (Bray & Curtis 1957) measures the dissimilarity between the dietary data sets, where C_{ij} is the sum of the lesser value for only those items which both data sets have in common. S_i and S_j are the total number of items counted in both data sets. If the data sets are identical, then both predators feed on the same prey and the Bray–Curtis index is 0. If the two data sets do not share any prey items then the index is 1 (Bloom 1981).

$$BC_{ij} = \frac{2C_{ij}}{S_i + S_j} \qquad \text{(Equation 1)}$$

$$\mathrm{D} = 1 - \sum_{i=1}^{s} \frac{n_i\,(n_i - 1)}{n\,(n-1)} \qquad \text{(Equation 2)}$$

$$O_{jk} = \frac{\sum p_{ij} p_{ik}}{(\sum p_{ij}^2 \sum p_{ik}^2)^{1/2}} \qquad \text{(Equation 3)}$$

To assess dietary niche breadth based on the morphological diet data, we used the Simpson's index for diversity and heterogeneity (Equation 2), where n_i is the relative proportion of a prey item *i (*with *i* = 1…*n*) of a total of *n* prey items. Thus, D is 0, if all eaten prey belongs to one prey group. The higher the diversity, the closer D gets to 1.As an additional niche parameter we calculated Pianka`s index of niche overlap (Equation 3), where p_i is the frequency of occurrence of prey item i in the diets of species j and k (Pianka 1973):

We used a non-metric multi-dimensional scaling (NMDS) with Jaccard distance to visualize the degree of similarity or dissimilarity of the diet. The resulting two-dimensional ordination plot shows the samples sorted relative to their dissimilarity, with similar samples in close proximity and dissimilar samples further apart. We used a threshold (=stress value) of < 0.2 for ecological interpretation of the NMDS plot (Clark & Warwick 2001).

We conducted indices calculation, Adonis, and NMDS using the *vegan* R library (Oksanen *et al.* 2011). We applied generalized linear models (GLM) with a binomial distribution and a logit link function (Zuur *et al.* 2007) , to assess level of significance of differences between the two data sets regarding the presence or absence of prey groups, using *multcomp* R library (Hothorn *et al.* 2008).

Results

Molecular analysis

We found 220 MOTUs, of which 148 could be assigned to insect species (Table 1). For 72 MOTUs we found no matches in the BOLD System. We rejected 1 MOTU, because it contained only very short reads. Of the MOTUs 32% could be assigned to species level, 28% to genus level, 23% to family and 17% only to order level. We found 108 MOTUs in samples from migrating bats, whereas 58 MOTUs were assigned to samples from summer roosts. 19 MOTUs were found in both groups.

Hamming Distances between migratory bats and bats at summer colonies was 197. Additionally overall Bray-Curtis similarity between migratory bats and bats at summer colonies was 0.84. Both suggest low dietary similarity.

Morphological analysis

We found that the diet was significantly different between bats from summer roosts and on migration, indicated by the conducted permutational analysis of variance (ADONIS: $F = 4.371$, $df = 1$, $p<0.001$). Comparing diversity and species richness in the diet of *P. nathusii* between the two sites, we found no differences (Table 2). The trophic niche overlap, indicated by Pianka index, was relatively high (Table 2). The ordination plot (NMDS) shows samples spread out evenly along the two dimensions, overlapping to a great extent. The slight clustering along the first dimension has to be interpreted cautiously, as a stress value of 0.2 was reached (Fig. 2).

Based on GLMs we found significant difference between certain prey groups. *P. nathusii* from summer roosts appear to feed more often on Chironomidae than migrating *P. nathusii*. In contrast Tipulidae occurred more often in the diet of migrating bats (Table 3).

Discussion

The high values for Hamming distance and Bray-Curtis similarity we found for the molecular diet data indicate that diets of *P. nathusii* during migration and at summer colonies show low similarity. Despite this we found similar diversity indices for both groups, based on the morphological analysis, and a relatively high niche overlap. Yet, if compared to the niche overlap between different species foraging in similar habitats, for example *Myotis dasycneme* and *Myotis daubentonii*, the niche overlap between *P. nathusii* appears less strong (Krüger *et al.* 2012; Krüger *et al.* in press). Subtle but significant differences appear regarding Chironomidae and Tipulidae occurrence in the diet of migrating and summer bats, respectively. Chironomid species, especially in areas between latitude of 50° and 60°, can have two or more generations per year with diverging peaks from April to October. Several species (up to 15) can form groups which emerge in synchrony and cause an increase in potential prey biomass. As different groups follow different emergence patterns, alternating peaks of different Chironomid groups exist, replacing each other during the season and forming a more or less constant food resource (Oliver 1971, Pinder 1986, Berg and Hellenthal 1992, Tokeshi 1995, Benke 1998). The higher occurrence of Chironomidae in summer roosting bats might be explained by the reproductive state of bats and hence their needs for easily accessible prey, such as swarming Chironomidae. The higher occurrence of Tipulidae in migrating bats could be related to a peak in Tipulidae during that time at Pape, coinciding with migration paths. Diptera too are thought to migrate (Hogsette & Ruff 1985), and the tracks of migrating *P. nathusii* and tipulids may have coincided. Alternatively the bats may have been hunting more often in terrestrial habitats during this period.

A significant issue here is whether the difference is due to “migration” or “location”. Clare *et al.* (In press) demonstrated the degree of location, season and inter-annual variation in bat diet across landscapes. Since most bats forage among prey in their immediate vicinity it is not clear to what degree shifts in diet observed here are caused by changes in insect

253 phenology, bat physiological demands and habitat-insect relationships. We report here that
254 there is variation between summer colonies and migrating individuals, though the underlying
255 cause is very likely a mix of these competing factors. We suggest that the next logical step is
256 to expand this type of study to include multiple summer and migration sites so that
257 comparisons can be made across locations.

258 The higher resolution of molecular diet analyses of prey species, compared with
259 morphological analyses, provides valuable information on associations between prey, habitat
260 and predator (Clare *et al.* 2010; Razgour *et al.* 2011, Clare *et al.* 2013a in press; Krüger *et al.*
261 in press). While our morphological observations suggest that diet was strongly overlapping
262 between summer and migratory groups, we did observe a higher species richness in the
263 migratory diet based on the molecular data. There are two potential drivers of increased
264 diversity in the diet of migrating *P. nathusii*. First, migrating bats cover more space and more
265 potential habitat types. This may expose them to a higher diversity of potential prey as a
266 consequence. Second, insect diversity is general reduced later in the summer. At this point a
267 reduced availability of prey may force the *P. nathusii* to become more flexible in the prey
268 they consume (Clare *et al*. 2013a/b in press).

269 We also found that the diet of migrating *P. nathusii* contained higher occurrence of
270 insect species inhabiting aquatic habitats like the beetles *Cyphon phragmiteticola* and *Agonum*
271 *piceum*. This probably reflects the fact that *P. nathusii* forage in the adjacent bog and marsh
272 lands of Lake Pape. The moths *Epinotia immundana*, *Epinotia nisella* and *Phyllonorycter*
273 *apparella* are associated with riverine forests and trees in marshes, supporting the inference
274 that bats forage in the vicinity of aquatic habitats. Further indications for aquatic foraging
275 habitats are the occurrences of Trichoptera and Megaloptera. By contrast the moth
276 *Malacosoma castrensis* indicates foraging over dunes, as this is the major habitat of this moth.
277 The dunes at Pape spread out parallel to the coastline, and are also used by *P. nathusii* as a

major flight corridor during migration (Šuba *et al.*2012) suggesting prey habitat and predator habitat overlap at this point.

In the diet of *P. nathusii* from summer colonies we found prey species which are typically associated with forested areas, like *Bupalus pinaria*, a pine pest species, or *Promethes sulcator*, an ichneumon wasp. These species were not identified in samples from migrating *P. nathusii*. As the colony sites are also within a few kilometres of lakes, we also found prey species associated with aquatic habitats, like Chironomidae or Ephemeroptera. Overall we can observe how the foraging habitat of *P. nathusii* determines the diet and thus differences between migrating and summering bats might be explained. In birds it has been shown that during migration sedge warblers (*Acrocephalus schoenobaenus*) select stop-over sites with high abundance of aphids (Bibby & Green 1981). Insectivorous bats, like *P. nathusii*, are known to forage particularly in habitats with high insect abundance like riverine and semi-aquatic habitats.

Many insect species are also known to migrate (e.g. Hummingbird Hawk-moth *Macroglossum stellatarum*, Monarch butterfly *Danaus plexippus*). The beet army worm, *Spodoptera exigua*, originally distributed in the Americas, now occurring globally, is also a known long-distance migrant (Westbrook 2008). In Europe this species has been observed to travel long distances, from Russia over Fennoscandia towards Denmark and the British Isles (Mikkola 1970). The occurrence of a migrating insect in the diets of migrating bats may be a coincidental overlap of migration routes and the opportunistic foraging behaviour of pipistrelles, which has been also observed in other species such as *Tadarida brasiliensis* which feeds opportunistically on migrating moths (Lee & McCracken 2005). Hoary bats are believed to time migration with the mass emergence of moths, its major prey (Valdez & Cryan 2009). In Europe the noctula bat *Nyctalus lasiopterus* has been found to exploit migrating songbirds during spring and autumn migration (Ibáñez *et al.* 2001; Popa-Lisseanu

303 *et al.* 2007). Similar behavior has been also reported for the birdlike noctula, *Nyctalus aviator*,
304 in Japan (Fukui *et al.* 2013).

305 Energetic values of insect prey might influence foraging behaviour and diet preference
306 in migrating bats. Due to the high costs of migration flights, bats might prefer prey with high
307 fat content and high nutritional value, to maintain energy flow and fat deposits. The variation
308 in insect nutritional values is high, with large moths or beetles showing relatively higher fat
309 content than many other groups (Verkerk *et al.* 2007). In addition, some migrating insect also
310 accumulate fat to survive long dispersal flights, for example noctuid moths (Beall 1948;
311 Angelo & Slansky 1984; Kevan & Kendall 1997). The occurrence of *Spodoptera exigua*
312 (Noctuidae), and other Lepidoptera and Coleoptera in the diet of migrating *P. nathusii*,
313 suggest that these bats feed on prey with high fat content. Voigt *et al.* (2010, 2012b) showed
314 how *P. nathusii* and also other bat species fuel their flight during migration with endogenous
315 fatty acids from adipocytes in combination with proteins and carbohydrates directly
316 metabolised from exogenous sources, such as insects. This process is determined by a limited
317 capacity for energy storage and primarily saves energy during the costly process of converting
318 macronutrients to lipids for storage. Nevertheless, it can be regarded as a beneficial digestive
319 adaptation to flying and migration. Hence, the selective exploitation of prey with high fat
320 content would enable *P. nathusii* to balance its fat reserves despite their high energy demands
321 from long-distance and foraging flights. Birds are known to alter their dietary preference and
322 select different food sources shortly before or during migration. Geese have been shown to
323 select for certain plants species during migration (Gwinner 1990). Insectivorous migratory
324 birds like garden warbler (*Sylvia borin*) switch from arthropod based diet to fruit based diets
325 (Bairlein 1990) and furthermore select for fruit with certain fatty acid compositions prior to
326 migration (McWilliams *et al*. 2004). Other birds like willow warblers switch to insect prey
327 high in sugars, like aphids (Berthold 2001). Adaptive alteration of diet selectivity during
328 migration seems to be a valuable trait in migrants. .

This kind of selectivity would require the bats to discriminate between prey of different energy values. In bats selective behaviour and prey discrimination based on size has previously been demonstrated only for horseshoe bats (Koselj *et al.* 2011). Whether the same ability exists for other bats, like *Pipistrellus*, which, in contrast to horseshoe bats, use short frequency-modulated (FM) calls and mainly feed on Diptera on the wing, is not clear. Also like for frugivory in insectivorous birds during migration, the differences in diet between summer and migratory *P. nathusii* may result from the seasonal changes in availability of certain food items, insects and fruit, respectively.

The fat stores of migrating bats have higher proportions of polyunsaturated fatty acids (PUFAs) (McGuire *et al.* 2013b). Thus PUFAs may be an important resource during pre-migration and migration itself. Naturally, the diet of *Pipistrellus nathusii* is often dominated by Diptera, particularly Chironomidae, which are rich in highly unsaturated fatty acids (Thompson 1982; Hanson *et al.* 1985). Thus increased lipid biosynthesis capacity and additional intake of bigger, or fatter prey, may not be required during migration (McGuire & Guglielmo 2009).

In general, our results demonstrate differences in the diet of *P. nathusii* in summer roosts and on migration. *P. nathusii* is a generalist predator and feeds on prey groups thought to be rich in important fatty acids (e.g. Chironomidae) thus the need to select for prey with particularly high fat content during migration might be low. Yet, there is no evidence that endogenous triggered selectivity can be observed in insectivorous bats as is the case with some insectivorous and grazing birds.

Additionally, the ability of pipistrelle bats to discriminate between prey of differing energetic values might be poor and hamper shifts in prey selection. Diet of migrating bats like *P. nathusii* might rather depend on the availability of prey at the respective stop-over site and the differences between migrating and summering individuals found in prey groups are likely to be related to habitat differences along migratory routes and in the summering grounds. For

the future it would beneficial to find and add more migratory stop-over sites, where species can be studied.

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Data Accessibility

Molecular (sequences) and morphological (binary presence-absence) dietary data will be provided on a DRYAD account (doi: 10.5061/dryad.2d38f).

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Table 1 Taxa which were assigned to MOTU utilising the BOLD search system (V.3). The confidence levels signify 1a) perfect match to one genus or species (>99%), 1b) match to one genus or species (>98%), 2) match to more than one species, of which only one was a local species, 3) match >98% to several species of different genera or to reference sequences only identified to family level. Presence and absence of prey items in the diet of bat groups is indicated by 1 and 0, respectivley.

Order	Family	Species	Conf	Migration	Summer
Diptera	Asilidae	unknown	3	1	0
	Chaoboridae	unknown	3	1	0
	Chironomidae	unknown	3	1	1
		Glyptotendipes sp.	3	0	1
		Microtendipes sp.	3	1	0
		Microtendipes brevitarsis	1b	1	0
		Parachironomus tenuicaudatu	1b	1	0
		Paracladopelma winnelli	1b	1	1
		Procladius sp.	1b	1	1
		Synenotendipes impar	1b	0	1
		Tanytarsus mendax	1a	1	0
		Xenochironomus xenolabis	1a	1	0
		unknown	2	1	0
	Culicidae	*Aedes sp.*	2	1	0
		Anopheles sp.	3	0	1
		Culiseta sp.	2	1	0
		Ochlerotatus annulipes	1a	1	0
	Dolichopodidae	unknown	3	1	0
	Empididae	unknown	3	1	0
	Limoniidae	*Dicranomyia frontalis*	1a	1	1
		Dicranomyia sp.	1b	1	1
		Erioptera sp.	2	1	0
		Helius flavus	1a	1	0
		Limonia nubeculosa	1b	1	0
		Metalimnobia sp.	1a	1	1
		Molophilus sp.	1a	0	0
		Phylidorea ferruginea	3	1	0
		Phylidorea fulvonervosus	1b	0	1
		Rhipidia maculata	1a	1	0
		Helina impunctata	1a	0	1
	Muscidae	unknown	3	0	1
	Mycetophilidae	*Mycetophila luctuosa*	1a	0	1

		unknown	3	1	0
	Pedicidae	*Psychodae phalaenoides*	1a	0	1
	Psychodidae	unknown	3	1	0
	Sciaridae	*Anticheta sp.*	1b	0	1
	Sciomyzidae	*Haematopota pluvialis*	1a	0	1
	Tabanidae	*Hybomitra lurida*	1a	1	0
		Nephrotoma sp.	1b	1	0
	Tachinidae	unknown	3	1	0
	Tipulidae	*Tipula sp.*	1a	1	1
Lepidoptera	Amphisbatidae	*Pseudatemelia josephinae*	1a	0	1
	Argyresthiidae	*Argyresthiago edartella*	1a	0	1
	Blastobasidae	unknown	3	1	0
	Coleophoridae	*Coleophora glitzella*	1a	0	1
		Coleophora limosipennella	1a	1	0
	Elachistidae	*Agonopterix sp.*	1b	1	0
		Semioscopis sp.	1a	1	0
	Gelechiidae	*Exoteleia dodocella*	1a	1	1
		Coleotechnite spiceaella	1b	1	0
	Geometridae	*Hydriomena sp.*	1a	1	0
		Bupalus pinaria	1a	0	1
	Gracillariidae	*Phyllonorycter apparella*	1a	1	0
	Lasiocampidae	*Malacosoma castrensis*	1b	1	0
		Dendrolimus pini	1a	0	1
	Noctuidae	*Spodoptera exigua*	1b	1	0
	Tortricidae	*Acleris emargana*	1a	1	0
		Adoxo phyesorana	1a	0	1
		Cnephasia sp.	1a	1	1
		Epinotia immunda	1a	1	0
		Epinotia nisella	1a	1	0
		Eudemis porphyrana	1a	0	1
		Lozotaenia forsterana	1a	0	1
		Rhopobota naevana	1a	1	0
		Sparganothis sp.	1b	1	0
Ephemeroptera	Baetidae	*Cloeon dipterum*	1a	1	0
	Caenidae	*Caenis sp.*	1a	1	1
	Ephemerellidae	*Eurylophella temporalis.*	1b	1	1
	Ephemeridae	unknown	3	1	0

	Heptageniidae	unknown	1a	1	0
	Isonychiidae	*Heptagenia sp.*	1a	1	1
		Isonychia sp.	1b	1	0
Trichoptera	Leptoceridae	unknown	3	1	0
	Glossosomatidae	*Glossosoma intermedium*	1a	1	0
Neuroptera	Chrysopidae	*Chrysoperla sp.*	1a	1	0
	Hemerobiidae	*Hemerobius sp.*	1a	1	1
Hemiptera	Notonectidae	*Notonecta sp.*	1b	1	0
Coleoptera	Carabidae	*Agonom sp.*	1b	1	0
		Agonom piceum	1a	1	0
		Dromius sp.	1b	1	0
	Scirtidae	*Cyphon sp.*	1b	1	0
		Cyphon phragmiteticola	1a	1	0
Megaloptera	Sialidae	*Sialis sp.*	1b	1	0
Hymenoptera	Ichneumonidae	*Promethes sulcator*	1a	0	1

Table 2 Indices for richness, diversity and niche overlap for diet of bats from summer colonies and migration, derived from morphological analysis of faecal samples.

	Summer	Migration
Simpson's index	0.88	0.87
Species richness	14	13
Pianka's index	0.76	

Table 3 The frequency of prey groups in the diet of *P. nathusii* from summer colonies and migration based on morphological presence / absence data. We tested data with generalized liner model (GLM) and Tukey post-hoc test. Significant differences are indicated in bold.

	Migration	Summer	z	p<
Nematocera	73.3%	47.8%	-1.531	0.126 -
Anisopodidae	0.0%	21.7%	0.007	0.995
Chironomidae	**40.0%**	**91.3%**	**3.034**	**0.00241**
Culicidae	0.0%	17.4%	0.006	0.995
Tipulidae	**86.7%**	**30.4%**	**-3.051**	**0.00228**
Brachycera	40.0%	52.2%	0.732	0.464
Hemiptera	6.7%	0.0%	-0.003	0.998
Corixidae	0.0%	8.7%	0.004	0.997
Cicada	0.0%	4.3%	0.004	0.997
Aphidoidea	13.3%	26.1%	0.927	0.354
Trichoptera	26.7%	17.4%	-0.681	0.496
Lepidoptera	53.3%	30.4%	-1.396	0.163-
Ephemeroptera	26.7%	0.0%	-0.005	0.996
Neuroptera	46.7%	34.8%	-0.73	0.465
Coleoptera	13.3%	0.0%	-0.005	0.996
Hymenoptera	6.7%	4.3%	-0.311	0.756
Araneae	13.3%	4.3%	-0.957	0.338
Simuliidae	0.0%	4.3%	0.004	0.997
Formicidae	26.7%	0.0%	-0.005	0.996

Figure 1 Overview of the sampling locations for faecal samples of *Pipistrellus nathusii* (Picture) in Latvia. Summer colonies were sampled in Garkalne and Vecpiebalga. Migrating bats were caught and sampled in Pape, Ornithological Station, situated within the migration route of *P. nathusii* (Species photo by Viesturs Vintulis).

Figure 2 Plot of a non-metric two-dimensional ordination scale (NMDS) based on the presence-absence prey data derived from the morphological diet analysis on migrating *P. nathusii* (circle) and *P. nathusii* from summer colonies (cross) (n= 50, stress = 0.20).

Diet of the insectivorous bat *Pipistrellus nathusii* during autumn migration and summer residence

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Running title: Diet of the migrating bat Pipistrellus nathusii

Abstract

Migration is widespread among vertebrates~~-~~, y~~Y~~et bat migration has received little attention and only in the recent decades ~~knowledge~~ has a better understanding of it ~~has~~ been gained. Migration can cause significant changes in behaviour and physiology, due to increasing energy demands and aerodynamic constraints. Dietary shifts, for example~~s~~, have been shown to occur in birds before onset of migration. For bats it is not known if a change in diet occurs during migration, although ~~especially~~ breeding season related dietary preference has been documented. It is known that a diet rich in fats and the ~~fat-rich diets, and subsequent~~ accumulation of ~~high~~ fat deposits~~,~~ do increase the flight range of migratory bats. Some bat species can be regarded as long-distance migrants, covering up to 2,000 km ~~on their way~~ between summer and winter roosting areas. *Pipistrellus nathusii* (Vespertilionidae), a European long-distant migrant, travels each year along the Baltic Sea from north-eastern

Europe to hibernate in central and southern Europe. This study presents data on the dietary habits of migrating *Pipistrellus nathusii* compared with ~~dietary habits~~ those during the breeding season. We analysed faecal samples from bats on fall migration caught at the Ornithological Field Station in Pape, Latvia and from samples collected in North-Latvian summer roosts. We applied both morphological identification and molecular methods, as morphological methods also recognize life stages of prey and can contribute frequency data. The diets of bats on migration and breeding bats were similar, with Diptera and Lepidoptera comprising the major prey categories. However, certain prey groups could be explained by the different hunting habitats ~~used~~ exploited during migration vs. summer residence.

Introduction

Across the animal kingdom numerous species ~~Thousands of insects, fish, birds and mammals~~ make annual movements of both short and long duration. In particular, bird migration ~~between hemispheres~~ has been ~~observed~~ intensively ~~and~~ studied since the late 19th century. In the last decades these studies have been sophisticated both in methods and explanations and still are being developed further (Berthold 2001; Robinson *et al.* 2007; ~~Cryan *et al.*2004;~~ Wikelski *et al.*2007; Fiedler 2009). Many species of bats, the only volant mammals, are also known to migrate. Although the first interest~~s~~ in bat migration arose as early as the end of the 19th century ~~by~~ (Merriam ~~(~~1887), bat migration has been largely ignored until recently. Unlike birds, the elusive life strategies of bats, difficulties regarding visual observations, and low success in mark recapture programs, have made these species difficult to study. However, substantial recent advances have been made, which increase our understanding of orientation and physiology (Holland *et al.* 2006; Cryan & Brown 2007; ~~Richter & Cumming 2008;~~ McGuire & Guglielmo 2009; Voigt *et al.* 2010, 2012b). New techniques have contributed to our ability to track and infer actual range of movement, such as satellite tracking and stable isotope analysis (Cryan *et al.* 2004; Richter & Cumming 2008; Popa-Lisseanu & Voigt 2009; Voigt *et al.* 2012a; Tsoar *et al.* 2012)

Studies of bat migration can profit from previous work on bird migration (McGuire & Guglielmo 2009). Both birds and bats need to maintain a ~~steady~~ sufficient nutrient intake to meet the increased energy demand during migration ~~state (energy in - energy out)~~ over distances of sometimes several thousand kilometers between summer and winter habitats (Griffin 1970; Petersons 2004). As in birds, the scale of bat migration can vary considerably between short-distance, regional migrants (e.g., *Myotis daubentonii, M. lucifugus*) and long-distance migrants (e.g., *Pipistrellus nathusii, Lasioncyteris noctivagans*) (Fleming & Eby 2003; Dzal *et al.* 2009; Dzal *et al.* 2011). On their journeys birds and bats face similar tradeoffs between acquiring sufficient fat deposits (energy reserves) to fuel flight and

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maintaining ~~optimal~~ body conditions (weight, size) optimal for ~~aerodynamic constitution~~ flight with low energetic costs.

Birds are known to start to build up fat reserves before migratory flight and during stopovers at resting sites (e.g., Wadden Sea; McWilliams *et al.* 2004). Before the onset of migration birds show different adaptations to increase fat ~~storages~~ stores~~and decrease mass~~: they may become hyperphagous, their digestive and biosynthetic systems may alter, ~~e.g.,~~for example increase ~~in~~ liver mass and liver activity (Egeler *et al.* 2000; Guglielmo & Williams 2003), and they may increase or reduce the size of their digestive systems (Piersma 1998; Piersma *et al.* 1999; McWilliams & Karasov 2004). Additionally birds are able to fly during the night and forage and refuel during the day.

Bats have to accomplish the dual task of both flying and refueling at night. Recent studies show that bats also become hyperphagous and increase their body fat and catabolic enzyme activity during pre-migration (Ewing *et al.* 1970; Bairlain 2001; McGuire *et al.* 2009, 2013a, b; Šuba *et al.* 2010). Furthermore, they are able to fuel their migration both directly from insects caught during flight and from stored fatty acid reserves to maintain both steady state and refill reserves (Voigt *et al.* 2010; ~~Suarez & Welch 2011;~~ Voigt *et al.* 2012b). The fly-and-forage ~~strategy~~ hypothesis, which states that bats forage on the wing during migration, is ~~also~~ supported by acoustical observations along migration routes (Ahlén *et al.* 2009; Valdez & Cryan 2009, Šuba *et al.* 2012). Yet, it is not ~~for certainclaer~~clear to ~~which~~what extant ~~or if at all~~ bats are segregating~~e~~ foraging and migratory flight during these periods or whether they can truly hunt while ~~commuting~~migrating. ~~-~~

Another adaptation, the shift in diet towards different food items (e.g., from insects to fruit), helps some birds to gain sufficient energy during the pre-migration period (Bairlein 1990; Bairlein & Gwinner 1994; Bairlein 2001; McWilliams & Karasov 2005). It is not known if bats show similar behaviour. While most insectivorous bats use a generalist strategy, consuming prey in relation to their abundance (Anthony & Kunz 1977; Swift *et al.* 1985)

within a given habitat (Clare *et al.* 2013a/ b in review, Special Issue), selective feeding and the ability to discriminate between food items have been demonstrated in some bat species (Von der Emde & Schnitzler 1990; Koselj *et al.* 2011). Dietary shifts over time have been described in bats (Agosta 2002) and may be related to physiological state (pregnancy, lactation, preparation for hibernation) or changes in insect abundance (Clare *et al.* 2009, 2011, 2013a/b in ~~review~~press, Special Issue).

Here we tested the hypothesis that bat diet differs between summer roosting and fall migration. We used high throughput sequencing which yields detailed species-level data on prey in predator diets (Symondson 2002; King *et al.* 2008), and has been particularly successful in insectivores such as bats, (Razgour *et al.* 2011; Bohmann *et al.* 2011; Clare *et al.* 2013a/b in review) and shrews (Brown *et al*. in press, Special Issue). From species-level data (DNA sequences) we can draw conclusion on differences in prey items, apparent energy values or fat content and on putative foraging area differences between summer and migration habitats. We focused on a long-distance migrating bat, *Pipistrellus nathusii* (Keyserling & Blasius 1839), a generalist pipistrelle bat, which feeds to a large extent on insects connected to aquatic habitats, mainly on Diptera, particular Chironomidae (Beck 1994-1995; Vaughan 1997; Arnold *et al.* 2000; Flaquer *et al.* 2006). This species is known to travel up to 2000 km between the summer roosting grounds and hibernacula (Petersons 2004).

Methods

Study sites and sample collection

We collected samples for the purpose of dietary analysis at the Pape Ornithological Research Station at the southern Baltic coast of Latvia (56.165° N, 21.017° E) during the fall migration between 11 August and 12 September 2012 (Fig. 1). The station has been a central site for intensive research on bird and bat migration, particularly during the past twenty years (Petersons 2004). The surrounding areas are dominated by low sand dunes, partly covered by unmanaged pine woodlands (*Pinus sylvestris*) and grasslands. In the vicinity of the station is Pape Nature Park with Lake Pape, a 12km² shallow coastal lagoon lake, and a mosaic of marshland, reeds, meadows, forests and peat bogs. We caught bats from dusk until 01:00am using a Helgoland funnel trap following Petersons (2004). Bats were placed in a clean soft cotton bags and held for approximately 1h to collect faecal samples. Samples of *P. nathusii* faeces from summer colonies were collected from nursery colonies situated in buildings, at Vecpiebalga (57.058° N; 25.815° E) and artificial roosts with male groups at Garkalne (57.048° N; 24.382° E), Latvia during June 2013 (Fig. 1). Both sites are located in a mosaic landscape of forests, pasture and in proximity to large lakes.

Molecular diet analysis from faecal samples

For the molecular analysis we extracted DNA from faecal samples from individual migrating bats (n= 35 faecal samples) and from summer colony bulk samples, non-individually collected from under the roosting bats (n = 21 faecal samples) using the QIAamp DNA Stool Mini Kit (Qiagen, UK) following Zeale *et al.* (2011). To amplify the arthropod prey DNA we used modified primers based on the universal COI primer ZBJ-ArtF1c and ZBJ-ArtR2c. PCR (following the protocol of Zeale *et al.* 2011) produce a 157bp amplicon at the 5' end of the 658bp COI barcode region (Hebert *et al.* 2004). DNA was sequenced via a high throughput Ion Torrent sequencing platform (Life Technology) at the University of Bristol Genomics facility (School of Biological Sciences, Bristol, UK). For the adjustment,

trimming and organisation of sequences by MIDs after sequencing we used the Galaxy V platform (https://main.g2.bx.psu.edu/root; Giardine *et al.* 2005; Blankenberg *et al.*2005; Blankenberg *et al.*2010; Goecks *et al.*2010). To allow niche analysis procedure for all sequences, we clustered the sequences into molecular operational taxonomic units (MOTU) using the program jMOTU (Jones *et al.*2011). We tested grouping thresholds from 1-10bp and selected a 4bp threshold as the most appropriate for this data set (see Razgour *et al.* 2011). We extracted representative sequences for each MOTU and compared sequences against references within the Barcode of Life Data System (Ratnasingham & Hebert 2007; Clare *et al.* 2009). If sequences matched completely to a reference sequence without matching any other arthropod, we regarded the sequence as belonging to the same species. However, the short amplicon length also constrains some species assignments. We used a modified version of the criteria used by Razgour *et al.* (2011) as follows:

1a. True species match (>99 % similarity)

1b. Likely species match (>98% similarity)

2. Match (>98%) to more than one species, only one of which belongs to local assemblage

3. Match (>98%) to several species or genera – genus or family level assignment ~~made by~~ considered provisional.

Morphological identification from faecal samples

For morphological faecal analysis, we dried samples after DNA extraction (see Zeale *et al.* 2011 ~~notes~~) at room temperature and stored them at -20°C to avoid coprophagous insects. Before analysis, we soaked the pellets for 48 h in 70% ~~Ethanol~~ ethanol and then dissected them under a binocular vision microscope (x 40 – 60). We separated characteristic fragments and mounted them in Euparal for further examination. We identification taxa to class, order, family, or genus level (where possible), by comparison of fragments with whole collected insects, arthropod identification keys from the literature (Medvedev 1989; Savage

1990; Shiel *et al.* 1997; Osterbroek *et al.* 2005) and fragment photos from earlier studies (~~McAney *et al.* 1991;~~ Krüger *et al.* ~~2012~~2012).

For every sample we calculated the frequency of each prey group relative to all samples, to estimate relative importance of prey groups (~~McAney~~ Shiel *et al.* ~~1991~~1997; Vaughaun 1997; Krüger *et al.* 2012).

Statistical analysis

As molecular and morphological analysis produce presence-absence data and frequency data, respectively, we analysed the two data sets in different ways.

We used the Hamming distance and Bray-Curtis index (Equation 1) for similarity to analyze the dietary differences between the migratory group and the summer group as measured by molecular data. Both indices use binomial data. The Hamming distance is related to the number of changes needed to adjust two strings of same length to each other (Hamming 1950). A smaller value for Hamming distances reflects high overlap in dietary choices. The Bray-Curtis index (Bray & Curtis 1957) measures the dissimilarity between the dietary data sets, where C_{ij} is the sum of the lesser value for only those items which both data sets have in common. S_i and S_j are the total number of items counted in both data sets. If the data sets are identical, then both predators feed on the same prey and the Bray–Curtis index is 0. If the two data sets do not share any prey items then the index is 1 (Bloom 1981).

$$BC_{ij} = \frac{2C_{ij}}{S_i + S_j} \qquad \text{(Equation 1)}$$

$$D = 1 - \sum_{i=1}^{S} \frac{n_i\,(n_i - 1)}{n\,(n-1)} \qquad \text{(Equation 2)}$$

$$O_{jk} = \frac{\sum p_{ij} p_{ik}}{(\sum p_{ij}^2 \sum p_{ik}^2)^{1/2}} \qquad \text{(Equation 3)}$$

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To assess dietary niche breadth based on the morphological diet data, we used the Simpson's index for diversity and heterogeneity (Equation 2), where n_i is the relative proportion of a prey item *i* (with $i = 1 \ldots n$) of a total of *n* prey items. Thus, D is 0, if all eaten prey belongs to one prey group. The higher the diversity, the closer D gets to 1.As an additional niche parameter we calculated Pianka`s index of niche overlap (Equation 3), where p_i is the frequency of occurrence of prey item i in the diets of species j and k (Pianka 1973):

We used a non-metric multi-dimensional scaling (NMDS) with Jaccard distance to visualize the degree of similarity or dissimilarity of the diet. The resulting two-dimensional ordination plot shows the samples sorted relative to their dissimilarity, with similar samples in close proximity and dissimilar samples further apart. We used a threshold (=stress value) of < 0.2 for ecological interpretation of the NMDS plot (Clark & Warwick 2001).

We conducted indices calculation, Adonis, and NMDS using the *vegan* R library (Oksanen *et al.* 2011). We applied generalized linear models (GLM) with a binomial distribution and a ~~and a~~ logit link function (Zuur *et al.*, 2007) ~~general linear hypotheses (glht) with Tukey's post-hoc test~~, to assess level of significance of differences between the two data sets regarding the presence or absence of prey groups, using *multcomp* R library (Hothorn *et al.* 2008).

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Results

Molecular analysis

We found 220 MOTUs, of which 148 could be assigned to insect species (Table 1). For 72 MOTUs we found no matches in the BOLD System. We rejected 1 MOTU, because it contained only very short reads. Of the MOTUs 32% could be assigned to species level, 28% to genus level, 23% to family and 17% only to order level. We found 108 MOTUs in samples from migrating bats, whereas 58 MOTUs were assigned to samples from summer roosts. 19 MOTUs were found in both groups.

Hamming Distances between migratory bats and bats at summer colonies was 197. Additionally overall Bray-Curtis similarity between migratory bats and bats at summer colonies was 0.84. Both suggest low dietary similarity.

Morphological analysis

We found that the diet was significantly different between bats from summer roosts and on migration, indicated by the conducted permutational analysis of variance (ADONIS: F = 4.371, df = 1, p<0.001). Comparing diversity and species richness in the diet of *P. nathusii* between the two sites, we found no differences (Table 2). The trophic niche overlap, indicated by Pianka's index, was relatively high (Table 2).The ordination plot (NMDS) shows samples spread out evenly along the two dimensions, overlapping to a great extent. The slight clustering along the first dimension has to be interpreted cautiously, as a stress value of 0.2 was reached (Fig.~~1~~ 2).

Based on GLMs we found significant difference between certain prey groups. *P. nathusii* from summer roosts appear to feed more often on Chironomidae than migrating *P. nathusii*. In contrast Tipulidae occurred more often in the diet of migrating bats (Table 3).

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Discussion

The high values for Hamming distance and Bray-Curtis similarity we found for the molecular diet data indicate that diets of ~~migrating~~ *P. nathusii* ~~bats~~ during migration and ~~*P. nathusii* bats~~ at summer colonies show ~~high~~ low similarity. ~~We~~ Despite this, we~~also~~ found ~~very~~ similar diversity indices for both groups, based on the morphological analysis and a relatively high niche overlap. Yet, if compared to the niche overlap between different species foraging in similar habitats, for example *Myotis dasycneme* and *Myotis daubentonii*, the niche overlap between *P. nathusii* appears less strong (Krüger *et al.* 2012; Krüger *et al.* in press).~~In comparison to other data we found higher niche overlap between different species foraging in similar habitats, e.g. *Myotis dasycneme* and *Myotis daubentonii* (Krüger *et al.* 2012; Krüger *et al.* 2013 in reviewpress). However, s~~Subtle but significant differences appear regarding Chironomidae and Tipulidae occurrence in the diet of migrating and summer bats, respectively. Chironomid species, especially in areas between latitude of 50° and 60°, can have two or more generations per year with diverging peaks from April to October. Several species (up to 15) can form groups which emerge in synchrony and cause an increase in potential prey biomass. As different groups follow different emergence patterns, alternating peaks of different Chironomid groups exist, replacing each other during the season and forming a more or less constant food resource (Oliver 1971~~,~~; Pinder 1986~~,~~; Berg and Hellenthal 1992~~,~~; Tokeshi 1995~~,~~; Benke 1998). The higher occurrence of Chironomidae in summer roosting bats might be explained by the reproductive state of bats and hence their needs for easily accessible prey, ~~like~~ such as swarming Chironomidae. The higher occurrence of Tipulidae in migrating bats could be ~~either~~ related to a peak in Tipulidae during that time at Pape, coinciding with migration paths. Diptera too are thought to migrate (Hogsette & Ruff 1985), and the tracks of migrating *P. nathusii* ~~bats~~ and Diptera like tipulids may have coincided. Alternatively the bats may have been hunting more often in terrestrial habitats during this period. ~~(Hogsette & Ruff 1985).~~

A significant issue here is whether the difference is due to "migration" or "location". Clare *et al.* (In press) demonstrated the degree of location, season and inter-annual variation in ba~~d~~t diet across landscapes. Since most bats forage among prey in their immediate vicinity it is not clear to what degree shifts in diet observed here are caused by changes in insect phenology, bat physiological demands and habitat-insect relationships. We report here that there is variation between summer colonies and migrating individuals, though the underlying cause is very likely a mix of these competing ~~influences~~factors. We suggest that the next logical step is to expand this type of study to include multiple summer and migration sites so that comparisons can be made across location.

The higher resolution of molecular diet analyses ~~on~~of prey species, compared ~~contrasting~~ with morphological ~~analysis~~analyses, provides valuable information on associations between prey, habitat and predator (Clare *et al.* 2010; Razgour *et al.* 2011, Clare *et al.* 2013a in press; Krüger *et al.* in press). ~~White~~ While our morphological observations suggest that diet was strongly overlapping between summer and migratory groups, we did observe a higher species richness in the migratory diet based on the molecular data. There are two potential drivers of increased diversity in the diet of migrating *P. nathusii*~~bats~~. First, migrating ~~individuals~~ bats ~~migrating are covering~~ cover more space and more potential habitat types. This may expose them to a higher diversity of potential prey as a consequence. Second, insect diversity ~~, in general~~is general reduced later~~,~~ ~~falls later~~ in the summer. At this point a reduced availability of prey may force the *P. nathusii* ~~bats~~ to become more flexible in the prey they consume (Clare *et al.* 2013a/b in ~~review~~press).

We also found that the diet of migrating ~~pipistrelles~~ *P. nathusii* contained higher occurrence of insect species inhabiting aquatic habitats like the beetles *Cyphon phragmiteticola* and *Agonum piceum*. This probably reflects the fact that *P. nathusii* ~~bats~~ forage in the adjacent bog and marsh lands of Lake Pape. The moths *Epinotia immundana*, *Epinotia nisella* and *Phyllonorycter apparella* are associated with riverine forests and trees in

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marshes, supporting the inference that bats forage in the vicinity of aquatic habitats. Further indications for aquatic foraging habitats are the occurrences of Trichoptera and Megaloptera. By contrast the moth *Malacosoma castrensis* indicates foraging over dunes, as this is the major habitat of this moth. The dunes at Pape spread out parallel to the coastline, and are also used by *P. nathusii* ~~bats~~ as a major flight corridor during migration (Šuba~~r~~ *et al.*2012) suggesting prey habitat and predator habitat overlap at this point.

In the diet of *P. nathusii* ~~bats~~ from summer colonies we found prey species which are typically associated with forested areas, like *Bupalus pinaria*, a pine pest species, or *Promethes sulcator*, an ichneumon wasp. These species were not identified in samples from migrating *P. nathusii*~~bats~~. As the colony sites are also within a few kilometres of lakes, we also found prey species associated with aquatic habitats, like Chironomidae or Ephemeroptera. Overall we can observe how the foraging habitat of *P. nathusii* determines the diet and thus differences between migrating and summering bats might be ~~triggered~~explained. In birds it has been shown that during migration sedge warblers (*Acrocephalus schoenobaenus*) select for stop-over sites with high abundance of aphids (Bibby & Green 1981). Insectivorous bats, like *P. nathusii*, ~~-~~are known to forage particularly in habitats with high insect abundance like riverine and semi-aquatic habitats.

Many insect species are also known to migrate (e.g. ~~-~~Hummingbird Hawk-moth *Macroglossum stellatarum*, ~~-~~ Monarch ~~butterflies~~ butterfly *Danaus plexippus*). The beet army worm, *Spodoptera exigua*, originally distributed in the Americas, now occurring globally, is also a known long-distance migrant (Westbrook 2008). In Europe this species has been observed to travel long distances, from Russia over Fennoscandia towards Denmark and the British Isles (Mikkola 1970). The occurrence of a migrating insect in the diets of migrating bats may be a coincidental overlap of migration routes and the opportunistic foraging behaviour of pipistrelles, which has been also observed in other species such as *Tadarida brasiliensis* which feeds opportunistically on migrating moths (Lee & McCracken 2005). ~~Also~~

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~~for~~ Hoary bats ~~it is~~are believed ~~that it times its~~to time migration with the mass emergence of moths, its major prey (Valdez & Cryan 2009). In Europe the noctula bat~~,~~ *Nyctalus lasiopterus* ~~,~~ has been found to ~~a bat that predates bird, has adapted to~~ exploit ~~the occasional food source of~~ migrating songbirds during spring and autumn migration (Ibáñez *et al.* 2001; Popa-Lisseanu *et al.* 2007). Similar behavior has been also reported for ~~the case for~~ the birdlike noctula, *Nyctalus aviator*, in Japan (Fukui *et al.* 2013).

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Energetic values of insect prey might influence foraging behaviour and diet preference in migrating bats. Due to the high costs of migration flights, bats might prefer prey with high fat content and high nutritional value, to maintain energy flow and fat deposits. The variation in insect nutritional values is high, with large moths or beetles showing relatively higher fat content than many other groups (Verkerk *et al.* 2007). In addition, some migrating insect also accumulate fat to survive long dispersal flights, e.g. moths of the Noctuidae (Beall 1948; Angelo & Slansky 1984; Kevan & Kendall 1997). The occurrence of *Spodoptera exigua* (Noctuidae), and other Lepidoptera and Coleoptera in the diet of migrating *P. nathusii* suggest that these bats feed on prey with high fat content. Voigt *et al.* (2010, 2012b) ~~propose~~ showed how ~~that~~ *P. nathusii* and also other bat species ~~fuels its~~fuel their ~~migration~~ flight during migration with endogenous fatty acids from adipocytes in combination with proteins and carbohydrates ~~from~~ directly metabolised from exogenous sources, such as insects. This process is determined by a limited capacity for energy storage and primarily saves energy during the costly process of converting macronutrients to lipids for storage. Nevertheless, it can be regarded as beneficial digestive adaptation to flying and ~~hence~~ migration. ~~Similarly other bats are able to fuel flight with energy oxidized from both insect prey and fat deposits and are able to directly reload fat deposits with fatty acids from insect prey (Voigt *et al.* 2010)~~. Hence, the selective exploitation of prey with high fat content would enable *P. nathusii* to balance its fat ~~and energy~~ reserves despite their high energy demands from long-distance and foraging flights. Birds are known to alter their dietary preference and select different food

sources shortly before or during migration. Geese ~~are known~~have been shown to select for certain plants species during migration (Bairlein 1990). Insectivorous migratory birds like garden warbler (*Sylvia borin*) switch from arthropod based diet to fruit based diets (Bairlein 1990) and furthermore ~~-~~select for fruit with certain fatty acid compositions prior to migration (McWilliams *et al.* 2004). Other birds like willow warblers switch to insect prey high in sugars, like aphids (Berthold 2001). Adaptive alteration of diet selectivity during migration seems to be a valuable trait in migrants. ~~The latter is comparable to possible shifts in diets of migratory bats~~.

This kind of selectivity would require the bats to discriminate between prey of different energy ~~/ fat~~ values. In bats selective behaviour and prey discrimination based on size has previously been demonstrated only for horseshoe bats ~~selective behaviour and prey discrimination based on size has been demonstrated previously~~ (Koselj *et al.* 2011). Whether the same ability exists for other bats, like *Pipistrellus*, which, in contrast to horseshoe bats, use short frequency-modulated (FM) calls and mainly feed on Diptera on the wing, ~~-~~is not clear. Also like for frugivory in insectivorous birds during migration, the differences in diet between summer and migratory *P. nathusii* may result from the seasonal changes in availability of certain food items, insects and fruit, respectively.

The fat stores of migrating bats have higher proportions of polyunsaturated fatty acids (PUFAs) (McGuire *et al.* 2013b). Thus PUFAs ~~are seem to~~may be an important resource during pre-migration and migration itself. Naturally, the diet of *Pipistrellus nathusii* is often dominated by Diptera, particularly Chironomidae, which are rich in highly unsaturated fatty acids (Thompson 1982; Hanson *et al.* 1985). ~~Overall aquatic insects have higher PUFA content than do terrestrial insects, though this varies depending on life stage (Hanson *et al* 1985).The high occurrence of Chironomidae in the summer diet of *P. nathusii* demonstrates that bats already have good supply of fat resources, needed for building up reserves. Migrating bats still feed to large extent on Nematocera, often associated with high PUFA~~

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~~content.~~ Thus increased lipid biosynthesis capacity and additional intake of bigger, or fatter prey, may not be required during migration (McGuire & Guglielmo 2009).

In general, our results demonstrate differences in the diet of *P. nathusii* from summer roosts and *P. nathusii* on migration. *P. nathusii* is a generalist predator and feeds on prey groups thought to be rich in important fatty acids (e.g. Chironomidae) thus the need to select for prey with particularly high fat content during migration might be low. Yet, there is no evidence that endogenous triggered selectivity can be observed in insectivorous bats like it is the case for some insectivorous and grazing birds.

Additionally, ~~their~~ the ability of pipistrelle bats to discriminate between prey of differing energetic values might be poor and hamper shifts in prey selection. Diet of migrating bats like *P. nathusii* might rather depend on the availability of prey at the respective stop-over site and ~~T~~the differences between migrating and summering individuals found in prey groups ~~can~~ are likely to be related to habitat differences along migratory routes and in the summering grounds. For the future it would beneficial to find and add more migratory stop-over sites, where species can be studied.

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Data Accessibility

Molecular (sequences) and morphological (binary presence-absence) dietary data will be provided on a DRYAD account (doi: 10.5061/dryad.2d38f).

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Table 1 Taxa which were assigned to MOTU utilising the BOLD search system (V.3). The confidence levels signify 1a) perfect match to one genus or species (>99%), 1b) match to one genus or species (>98%), 2) match to more than one species, of which only one was a local species, 3) match >98% to several species of different genera or to reference sequences only identified to family level. Presence and absence of prey items in the diet of bat groups is indicated by 1 and 0, respectivley.

Order	Family	Species	Conf	Migration	Summer
Diptera	Asilidae	unknown	3	1	0
	Chaoboridae	unknown	3	1	0
	Chironomidae	unknown	3	1	1
		Glyptotendipes sp.	3	0	1
		Microtendipes sp.	3	1	0
		Microtendipes brevitarsis	1b	1	0
		Parachironomus tenuicaudatu	1b	1	0
		Paracladopelma winnelli	1b	1	1
		Procladius sp.	1b	1	1
		Synenotendipes impar	1b	0	1
		Tanytarsus mendax	1a	1	0
		Xenochironomus xenolabis	1a	1	0
		unknown	2	1	0
	Culicidae	*Aedes sp.*	2	1	0
		Anopheles sp.	3	0	1
		Culiseta sp.	2	1	0
		Ochlerotatus annulipes	1a	1	0
	Dolichopodidae	unknown	3	1	0
	Empididae	unknown	3	1	0
	Limoniidae	*Dicranomyia frontalis*	1a	1	1
		Dicranomyia sp.	1b	1	1
		Erioptera sp.	2	1	0
		Helius flavus	1a	1	0
		Limonia nubeculosa	1b	1	0
		Metalimnobia sp.	1a	1	1
		Molophilus sp.	1a	0	0
		Phylidorea ferruginea	3	1	0
		Phylidorea fulvonervosus	1b	0	1
		Rhipidia maculata	1a	1	0
		Helina impunctata	1a	0	1
	Muscidae	unknown	3	0	1
	Mycetophilidae	*Mycetophila luctuosa*	1a	0	1

		unknown	3	1	0
	Pedicidae	*Psychodae phalaenoides*	1a	0	1
	Psychodidae	unknown	3	1	0
	Sciaridae	*Anticheta sp.*	1b	0	1
	Sciomyzidae	*Haematopota pluvialis*	1a	0	1
	Tabanidae	*Hybomitra lurida*	1a	1	0
		Nephrotoma sp.	1b	1	0
	Tachinidae	unknown	3	1	0
	Tipulidae	*Tipula sp.*	1a	1	1
Lepidoptera	Amphisbatidae	*Pseudatemelia josephinae*	1a	0	1
	Argyresthiidae	*Argyresthiago edartella*	1a	0	1
	Blastobasidae	unknown	3	1	0
	Coleophoridae	*Coleophora glitzella*	1a	0	1
		Coleophora limosipennella	1a	1	0
	Elachistidae	*Agonopterix sp.*	1b	1	0
		Semioscopis sp.	1a	1	0
	Gelechiidae	*Exoteleia dodocella*	1a	1	1
		Coleotechnite spiceaella	1b	1	0
	Geometridae	*Hydriomena sp.*	1a	1	0
		Bupalus pinaria	1a	0	1
	Gracillariidae	*Phyllonorycter apparella*	1a	1	0
	Lasiocampidae	*Malacosoma castrensis*	1b	1	0
		Dendrolimus pini	1a	0	1
	Noctuidae	*Spodoptera exigua*	1b	1	0
	Tortricidae	*Acleris emargana*	1a	1	0
		Adoxo phyesorana	1a	0	1
		Cnephasia sp.	1a	1	1
		Epinotia immunda	1a	1	0
		Epinotia nisella	1a	1	0
		Eudemis porphyrana	1a	0	1
		Lozotaenia forsterana	1a	0	1
		Rhopobota naevana	1a	1	0
		Sparganothis sp.	1b	1	0
Ephemeroptera	Baetidae	*Cloeon dipterum*	1a	1	0
	Caenidae	*Caenis sp.*	1a	1	1
	Ephemerellidae	*Eurylophella temporalis.*	1b	1	1
	Ephemeridae	unknown	3	1	0

	Heptageniidae	unknown	1a	1	0
	Isonychiidae	*Heptagenia sp.*	1a	1	1
		Isonychia sp.	1b	1	0
Trichoptera	Leptoceridae	unknown	3	1	0
	Glossosomatidae	*Glossosoma intermedium*	1a	1	0
Neuroptera	Chrysopidae	*Chrysoperla sp.*	1a	1	0
	Hemerobiidae	*Hemerobius sp.*	1a	1	1
Hemiptera	Notonectidae	*Notonecta sp.*	1b	1	0
Coleoptera	Carabidae	*Agonom sp.*	1b	1	0
		Agonom piceum	1a	1	0
		Dromius sp.	1b	1	0
	Scirtidae	*Cyphon sp.*	1b	1	0
		Cyphon phragmiteticola	1a	1	0
Megaloptera	Sialidae	*Sialis sp.*	1b	1	0
Hymenoptera	Ichneumonidae	*Promethes sulcator*	1a	0	1

Table 2 Indices for richness, diversity and niche overlap for diet of bats from summer colonies and migration, derived from morphological analysis of faecal samples..

	Summer	Migration
Simpson's index	0.88	0.87
Species richness	14	13
Pianka's index	0.76	

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Table 3 The frequency of prey groups in the diet of *P. nathusii* from summer colonies and migration based on morphological presence / absence data. We tested data with generalized liner model (GLM) ~~and Tukey post-hoc test~~. Significant differences are~~The significance of differences between prey groups, estimated with GLM, is~~ indicated ~~with p values (~~in bold. ~~),~~

	Migration	Summer	z	p<
Nematocera	73.3%	47.8%	-1.531	-0.126
Anisopodidae	0.0%	21.7%	-0.007	0.995
Chironomidae	**40.0%**	**91.3%**	**3.034**	**0.00241**
Culicidae	0.0%	17.4%	0.006	0.995
Tipulidae	**86.7%**	**30.4%**	**-3.051**	**0.00228**
Brachycera	40.0%	52.2%	0.732	0.464
Hemiptera	6.7%	0.0%	-0.003	-0.998
Corixidae	0.0%	8.7%	0.004	0.997
Cicada	0.0%	4.3%	0.004	0.997
Aphidoidea	13.3%	26.1%	0.927	-0.354
Trichoptera	26.7%	17.4%	-0.681	0.496
Lepidoptera	53.3%	30.4%	-1.396	0.163
Ephemeroptera	26.7%	0.0%	-0.005	0.996
Neuroptera	46.7%	34.8%	-0.73	0.465
Coleoptera	13.3%	0.0%	-0.005	0.996
Hymenoptera	6.7%	4.3%	-0.311	-0.756
Araneae	13.3%	4.3%	-0.957	-0.338
Simuliidae	0.0%	4.3%	0.004	-0.997
Formicidae	26.7%	0.0%	-0.005	0.996

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Figure 1 Overview of the sampling locations for faecal samples of *Pipistrellus nathusii* (Picture) in Latvia. Summer colonies were sampled in Garkalne and Vecpiebalga. Migrating bats were caught and sampled in Pape, Ornithological Station, situated within the migration route of *P. nathusii* (Species photo by Viesturs Vintulis).

~~Figure 1 Overview of the sampling locations for faecal samples of *Pipistrellus nathusii* (Picture) in Latvia. Summer colonies were sampled in Garkalne and Vecpiebalga. Migrating bats were caught and sampled in Pape, Ornithological Station, situated within the migration route of *P. nathusii* (Species photo by Viesturs Vintulis).~~

Figure 2 Plot of a non-metric two-dimensional ordination scale (NMDS) based on the presence-absence prey data derived from the morphological diet analysis on migrating *P. nathusii* (circle) and *P. nathusii* from summer colonies (corss) ~~Non-metric two-dimensional ordination scale (NMDS) of morphological prey data~~ (n= 50, stress = 0.20).

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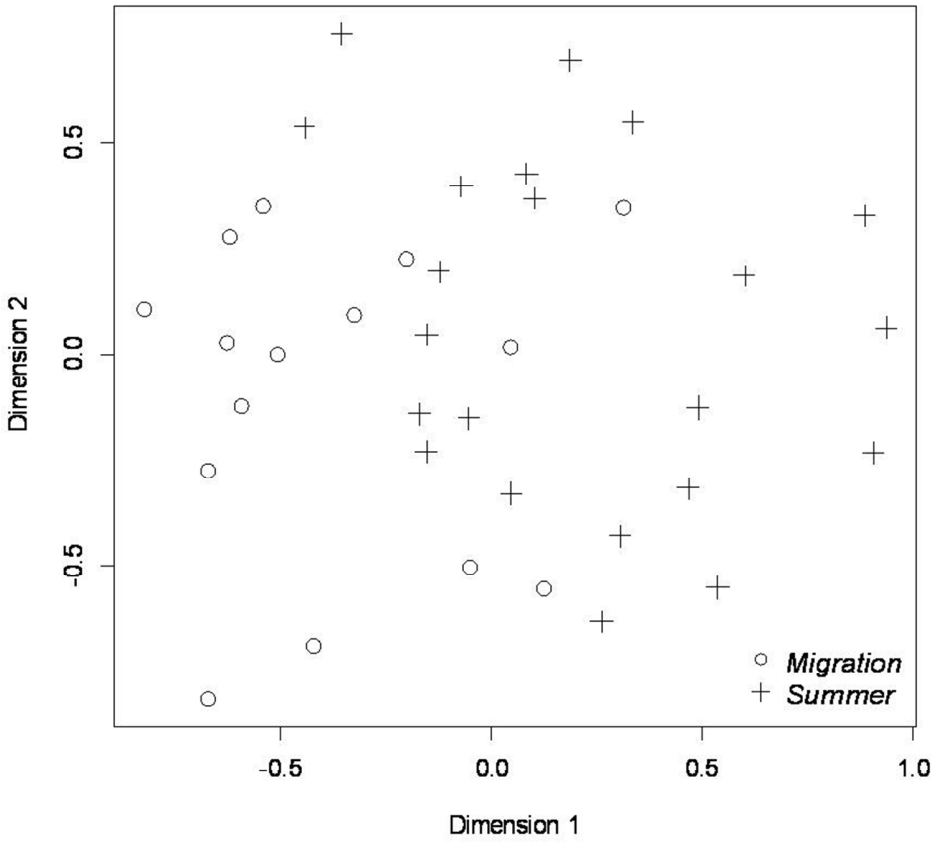

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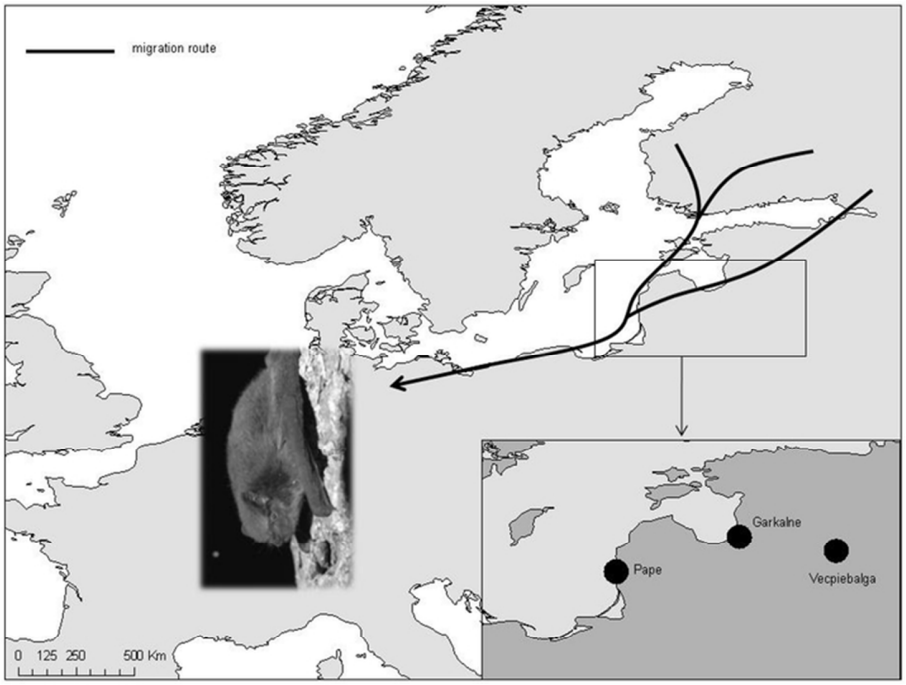

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