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

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Molecular Ecology

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

2	residence
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16	
17	Abstract
18	Migration is widespread among vertebrates, yet bat migration has received little
19	attention and only in the recent decades has a better understanding of it been gained.
20	Migration can cause significant changes in behaviour and physiology, due to increasing
21	energy demands and aerodynamic constraints. Dietary shifts, for example, have been shown
22	to occur in birds before onset of migration. For bats it is not known if a change in diet occurs
23	during migration, although breeding season related dietary preference has been documented.
24	It is known that a diet rich in fats and the accumulation of fat deposits do increase the flight
25	range of migratory bats. Some bat species can be regarded as long-distance migrants, covering
26	up to 2,000 km between summer and winter roosting areas. Pipistrellus nathusii
27	(Vespertilionidae), a European long-distant migrant, travels each year along the Baltic Sea
28	from north-eastern Europe to hibernate in central and southern Europe. This study presents

data on the dietary habits of migrating <i>Pipistrellus nathusii</i> 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.

Molecular Ecology

Introduction

40	Across the animal kingdom numerous species make annual movements of both short
41	and long duration. In particular, bird migration has been intensively studied since the late 19 th
42	century. In the last decades these studies have been sophisticated both in methods and
43	explanations and still are being developed further (Berthold 2001; Robinson et al. 2007;
44	Wikelski et al.2007; Fiedler 2009). Many species of bats, the only volant mammals, are also
45	known to migrate. Although the first interest in bat migration arose as early as the end of the
46	19 th century (Merriam 1887), bat migration has been largely ignored until recently. Unlike
47	birds, the elusive life strategies of bats, difficulties regarding visual observations, and low
48	success in mark recapture programs, have made these species difficult to study. However,
49	substantial recent advances have been made, which increase our understanding of orientation
50	and physiology (Holland et al. 2006; Cryan & Brown 2007; McGuire & Guglielmo 2009;
51	Voigt et al. 2010, 2012b). New techniques have contributed to our ability to track and infer
52	actual range of movement, such as satellite tracking and stable isotope analysis (Cryan et al.
53	2004; Richter & Cumming 2008; Popa-Lisseanu & Voigt 2009; Voigt et al. 2012a; Tsoar et
54	al. 2012)
55	Studies of bat migration can profit from previous work on bird migration (McGuire &
56	Guglielmo 2009). Both birds and bats need to maintain a sufficient nutrient intake to meet the
57	increased energy demand during migration over distances of sometimes several thousand
58	kilometers between summer and winter habitats (Griffin 1970; Petersons 2004). As in birds,
59	the scale of bat migration can vary considerably between short-distance, regional migrants
60	(e.g., Myotis daubentonii, M. lucifugus) and long-distance migrants (e.g., Pipistrellus nathusii,
61	Lasioncyteris noctivagans) (Fleming & Eby 2003; Dzal et al. 2009; Dzal et al. 2011). On
62	their journeys birds and bats face similar tradeoffs between acquiring sufficient fat deposits
63	(energy reserves) to fuel flight and maintaining body conditions (weight, size) optimal for
64	flight with low energetic costs.

65	Birds are known to start to build up fat reserves before migratory flight and during
66	stopovers at resting sites (e.g., Wadden Sea; McWilliams et al. 2004). Before the onset of
67	migration birds show different adaptations to increase fat stores: they may become
68	hyperphagous, their digestive and biosynthetic systems may alter, for example increase liver
69	mass and liver activity (Egeler et al. 2000; Guglielmo & Williams 2003), and they may
70	increase or reduce the size of their digestive systems (Piersma 1998; Piersma et al. 1999;
71	McWilliams & Karasov 2004). Additionally birds are able to fly during the night and forage
72	and refuel during the day.
73	Bats have to accomplish the dual task of both flying and refueling at night. Recent
74	studies show that bats also become hyperphagous and increase their body fat and catabolic
75	enzyme activity during pre-migration (Ewing et al. 1970; Bairlain 2001; McGuire et al. 2009,
76	2013a, b; Šuba et al. 2010). Furthermore, they are able to fuel their migration both directly
77	from insects caught during flight and from stored fatty acid reserves to maintain both steady
78	state and refill reserves (Voigt et al. 2010; Voigt et al. 2012b). The fly-and-forage hypothesis,
79	which states that bats forage on the wing during migration, is supported by acoustical
80	observations along migration routes (Ahlén et al. 2009; Valdez & Cryan 2009, Šuba et al.
81	2012). Yet, it is not clear to what extant bats are segregating foraging and migratory flight
82	during these periods or whether they can truly hunt while migrating.
83	Another adaptation, the shift in diet towards different food items (e.g., from insects to
84	fruit), helps some birds to gain sufficient energy during the pre-migration period (Bairlein
85	1990; Bairlein & Gwinner 1994; Bairlein 2001; McWilliams & Karasov 2005). It is not
86	known if bats show similar behaviour. While most insectivorous bats use a generalist strategy,
87	consuming prey in relation to their abundance (Anthony & Kunz 1977; Swift et al. 1985)
88	within a given habitat (Clare et al. 2013a/b in review, Special Issue), selective feeding and
89	the ability to discriminate between food items have been demonstrated in some bat species
90	(Von der Emde & Schnitzler 1990; Koselj et al. 2011). Dietary shifts over time have been

91	described in bats (Agosta 2002) and may be related to physiological state (pregnancy,
92	lactation, preparation for hibernation) or changes in insect abundance (Clare et al. 2009, 2011,
93	2013a/b in press, Special Issue).
94	Here we tested the hypothesis that bat diet differs between summer roosting and fall
95	migration. We used high throughput sequencing which yields detailed species-level data on
96	prey in predator diets (Symondson 2002; King et al. 2008), and has been particularly
97	successful in insectivores such as bats, (Razgour et al. 2011; Bohmann et al. 2011; Clare et al.
98	2013a/b in review) and shrews (Brown et al. in press, Special Issue). From species-level data
99	(DNA sequences) we can draw conclusion on differences in prey items, apparent energy
100	values or fat content and on putative foraging area differences between summer and migration
101	habitats. We focused on a long-distance migrating bat, Pipistrellus nathusii (Keyserling &
102	Blasius 1839), a generalist pipistrelle bat, which feeds to a large extent on insects connected
103	to aquatic habitats, mainly on Diptera, particular Chironomidae (Beck 1994-1995; Vaughan
104	1997; Arnold et al. 2000; Flaquer et al. 2006). This species is known to travel up to 2000 km
105	between the summer roosting grounds and hibernacula (Petersons 2004).
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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
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160 et al. 1997; Osterbroek et al. 2005) and fragment photos from earlier studies (Krüger et al.

161 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;
- 164 Krüger *et al.* 2012).
- 165 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

172 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_{ii} is the sum of the lesser value for only those items which both data sets have in

common. S_i and S_i 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).

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

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

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$$O_{jk} = \frac{\sum p_{ij} p_{ik}}{\left(\sum p_{ij}^2 \sum p_{ik}^2\right)^{1/2}}$$
 (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 $i = 1n$) 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).

201	Results
202	Molecular analysis
203	We found 220 MOTUs, of which 148 could be assigned to insect species (Table 1).
204	For 72 MOTUs we found no matches in the BOLD System. We rejected 1 MOTU, because it
205	contained only very short reads. Of the MOTUs 32% could be assigned to species level, 28%
206	to genus level, 23% to family and 17% only to order level. We found 108 MOTUs in samples
207	from migrating bats, whereas 58 MOTUs were assigned to samples from summer roosts. 19
208	MOTUs were found in both groups.
209	Hamming Distances between migratory bats and bats at summer colonies was 197.
210	Additionally overall Bray-Curtis similarity between migratory bats and bats at summer
211	colonies was 0.84. Both suggest low dietary similarity.
212	Morphological analysis
213	We found that the diet was significantly different between bats from summer roosts
214	and on migration, indicated by the conducted permutational analysis of variance (ADONIS: F
215	= 4.371, df = 1, p<0.001). Comparing diversity and species richness in the diet of P . nathusii
216	between the two sites, we found no differences (Table 2). The trophic niche overlap, indicated
217	by Pianka index, was relatively high (Table 2). The ordination plot (NMDS) shows samples
218	spread out evenly along the two dimensions, overlapping to a great extent. The slight
219	clustering along the first dimension has to be interpreted cautiously, as a stress value of 0.2
220	was reached (Fig. 2).
221	Based on GLMs we found significant difference between certain prey groups. P.
222	nathusii from summer roosts appear to feed more often on Chironomidae than migrating P.
223	nathusii. In contrast Tipulidae occurred more often in the diet of migrating bats (Table 3).
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Discu	ission

The high values for Hamming distance and Bray-Curtis similarity we found for the
molecular diet data indicate that diets of <i>P. nathusii</i> 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 <i>P. nathusii</i> 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 <i>P. nathusii</i> . 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 <i>P. nathusii</i> as a

278	major flight corridor during migration (Šuba et al.2012) suggesting prey habitat and predator
279	habitat overlap at this point.
280	In the diet of P. nathusii from summer colonies we found prey species which are
281	typically associated with forested areas, like Bupalus pinaria, a pine pest species, or
282	Promethes sulcator, an ichneumon wasp. These species were not identified in samples from
283	migrating <i>P. nathusii</i> . As the colony sites are also within a few kilometres of lakes, we also
284	found prey species associated with aquatic habitats, like Chironomidae or Ephemeroptera.
285	Overall we can observe how the foraging habitat of <i>P. nathusii</i> determines the diet and thus
286	differences between migrating and summering bats might be explained. In birds it has been
287	shown that during migration sedge warblers (Acrocephalus schoenobaenus) select stop-over
288	sites with high abundance of aphids (Bibby & Green 1981). Insectivorous bats, like P.
289	nathusii, are known to forage particularly in habitats with high insect abundance like riverine
290	and semi-aquatic habitats.
291	Many insect species are also known to migrate (e.g. Hummingbird Hawk-moth
292	Macroglossum stellatarum, Monarch butterfly Danaus plexippus). The beet army worm,
293	Spodoptera exigua, originally distributed in the Americas, now occurring globally, is also a
294	known long-distance migrant (Westbrook 2008). In Europe this species has been observed to
295	travel long distances, from Russia over Fennoscandia towards Denmark and the British Isles
296	(Mikkola 1970). The occurrence of a migrating insect in the diets of migrating bats may be a
297	coincidental overlap of migration routes and the opportunistic foraging behaviour of
298	pipistrelles, which has been also observed in other species such as Tadarida brasiliensis
299	which feeds opportunistically on migrating moths (Lee & McCracken 2005). Hoary bats are
300	believed to time migration with the mass emergence of moths, its major prey (Valdez &
301	Cryan 2009). In Europe the noctula bat Nyctalus lasiopterus has been found to exploit
302	migrating songbirds during spring and autumn migration (Ibáñez et al. 2001; Popa-Lisseanu

et al. 2007). Similar behavior has been also reported 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, for example noctuid moths (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) showed how P. nathusii and also other bat species fuel their flight during migration with endogenous fatty acids from adipocytes in combination with proteins and carbohydrates 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 a beneficial digestive adaptation to flying and migration. Hence, the selective exploitation of prey with high fat content would enable P. nathusii to balance its fat 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 have been shown to select for certain plants species during migration (Gwinner 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. .

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 <i>P. nathusii</i> 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 <i>Pipistrellus nathusii</i> 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 <i>P. nathusii</i> 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
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356 can be studied.

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363	Data Accessibility
364	Molecular (sequences) and morphological (binary presence-absence) dietary data will
365	be provided on a DRYAD account (doi: 10.5061/dryad.2d38f).
366	
367	
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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.

6	1	6
6	1	7

618		Migration	Summer	z	p<
619	Nematocera	73.3%	47.8%	-1.531	0.126 -
620	Anisopodidae	0.0%	21.7%	0.007	0.995
621	Chironomidae	40.0%	91.3%	3.034	0.00241
622	Culicidae	0.0%	17.4%	0.006	0.995
	Tipulidae	86.7%	30.4%	-3.051	0.00228
623	Brachycera	40.0%	52.2%	0.732	0.464
624	Hemiptera	6.7%	0.0%	-0.003	0.998
625	Corixidae	0.0%	8.7%	0.004	0.997
626	Cicada	0.0%	4.3%	0.004	0.997
627	Aphidoidea	13.3%	26.1%	0.927	0.354
628	Trichoptera	26.7%	17.4%	-0.681	0.496
629	Lepidoptera	53.3%	30.4%	-1.396	0.163-
630	Ephemeroptera	26.7%	0.0%	-0.005	0.996
631	Neuroptera	46.7%	34.8%	-0.73	0.465
	Coleoptera	13.3%	0.0%	-0.005	0.996
632	Hymenoptera	6.7%	4.3%	-0.311	0.756
633	Araneae	13.3%	4.3%	-0.957	0.338
634	Simuliidae	0.0%	4.3%	0.004	0.997
635	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 presenceabsence 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).



1	Diet of the insectivorous bat <i>Pipistrellus nathusii</i> during autumn migration and summer	
2	residence	
3	FraukeKrüger* ¹ , Elizabeth L. Clare², William O.C. Symondson³, Oskars Keišs⁴, Gunārs Pētersons⁵	
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12		
13	Key words: Chiroptera, Diet Analysis, Migration, Pipistrellus nathusii Migration, Chiroptera,	
14	Pipistrellus nathusii, Diet Analysis	
15	Running title: Diet of the migrating bat Pipistrellus nathusii	
16		
17	Abstract	
18	Migration is widespread among vertebrates-, vYet bat migration has received little	
19	attention and only in the recent decades knowledge has a better understanding of it has been	
20	gained. Migration can cause significant changes in behaviour and physiology, due to	
21	increasing energy demands and aerodynamic constraints. Dietary shifts, for examples, have	
22	been shown to occur in birds before onset of migration. For bats it is not known if a change in	
23	diet occurs during migration, although especially-breeding season related dietary preference	
24	has been documented. It is known that <u>a diet rich in fats and the fat rich diets</u> , and subsequent	
25	accumulation of high fat deposits, do increase the flight range of migratory bats. Some bat	
26	species can be regarded as long-distance migrants, covering up to 2,000 km on their way	
27	between summer and winter roosting areas. Pipistrellus nathusii (Vespertilionidae), a	
28	European long-distant migrant, travels each year along the Baltic Sea from north-eastern	

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

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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 interests 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 longdistance 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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67 flight with low energetic costs. 68 Birds are known to start to build up fat reserves before migratory flight and during 69 stopovers at resting sites (e.g., Wadden Sea; McWilliams et al. 2004). Before the onset of 70 migration birds show different adaptations to increase fat storages stores and decrease mass: 71 they may become hyperphagous, their digestive and biosynthetic systems may alter, e.g., for 72 example increase in-liver mass and liver activity (Egeler et al. 2000; Guglielmo & Williams 73 2003), and they may increase or reduce the size of their digestive systems (Piersma 1998; 74 Piersma et al. 1999; McWilliams & Karasov 2004). Additionally birds are able to fly during 75 the night and forage and refuel during the day. 76 Bats have to accomplish the dual task of both flying and refueling at night. Recent 77 studies show that bats also become hyperphagous and increase their body fat and catabolic 78 enzyme activity during pre-migration (Ewing et al. 1970; Bairlain 2001; McGuire et al. 2009, 79 2013a, b; Šuba et al. 2010). Furthermore, they are able to fuel their migration both directly 80 from insects caught during flight and from stored fatty acid reserves to maintain both steady 81 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, 82 83 is also supported by acoustical observations along migration routes (Ahlén et al. 2009; Valdez 84 & Cryan 2009, Suba et al. 2012). Yet, it is not for certainelaer clear to which what extant or if

maintaining optimal-body conditions (weight, size) optimal for aerodynamic constitution

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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)

at all-bats are segregatinge foraging and migratory flight during these periods or whether they

can truly hunt while commuting migrating. -

92	within a given habitat (Clare et al. 2013a/b in review, Special Issue), selective feeding and
93	the ability to discriminate between food items have been demonstrated in some bat species
94	(Von der Emde & Schnitzler 1990; Koselj et al. 2011). Dietary shifts over time have been
95	described in bats (Agosta 2002) and may be related to physiological state (pregnancy,
96	lactation, preparation for hibernation) or changes in insect abundance (Clare et al. 2009, 2011
97	2013a/b in reviewpress, Special Issue).
98	Here we tested the hypothesis that bat diet differs between summer roosting and fall
99	migration. We used high throughput sequencing which yields detailed species-level data on
100	prey in predator diets (Symondson 2002; King et al. 2008), and has been particularly
101	successful in insectivores such as bats, (Razgour et al. 2011; Bohmann et al. 2011; Clare et al.
102	2013a/b in review) and shrews (Brown et al. in press, Special Issue). From species-level data
103	(DNA sequences) we can draw conclusion on differences in prey items, apparent energy
104	values or fat content and on putative foraging area differences between summer and migration
105	habitats. We focused on a long-distance migrating bat, <i>Pipistrellus nathusii</i> (Keyserling &
106	Blasius 1839), a generalist pipistrelle bat, which feeds to a large extent on insects connected
107	to aquatic habitats, mainly on Diptera, particular Chironomidae (Beck 1994-1995; Vaughan
108	1997; Arnold et al. 2000; Flaquer et al. 2006). This species is known to travel up to 2000 km
109	between the summer roosting grounds and hibernacula (Petersons 2004).
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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.

128 Molecular diet analysis from faecal samples

For the molecular analysis we extracted DNA from faecal samples from individual migrating bats (n= 35 <u>faecal</u> samples) and from summer colony bulk samples, non-individually collected from under the roosting bats (n = 21 <u>faecal</u> 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,

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	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
l	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_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
l	collected insects, arthropod identification keys from the literature (Medvedev 1989; Savage
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trimming and organisation of sequences by MIDs after sequencing we used the Galaxy V

164 1990; Shiel et al. 1997; Osterbroek et al. 2005) and fragment photos from earlier studies 165

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(McAney et al. 1991; Krüger et al. 20122012).

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

169 Statistical analysis

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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 Cii is the sum of the lesser value for only those items which both data sets have in common. S_i and S_i 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).

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

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

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

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	To assess dietary niche breadth based on the morphological diet data, we used the
Ī	Simpson's index for diversity and heterogeneity (Equation 2), where n_i is the relative
l	proportion of a prey item i (with $i = 1n$) 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
ļ	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 hoe test, to assess level of significance of differences between the two data
ļ	sets regarding the presence or absence of prey groups, using <i>multcomp</i> R library (Hothorn <i>et</i>
	al. 2008).

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206	Results
207	Molecular analysis
208	We found 220 MOTUs, of which 148 could be assigned to insect species (Table 1).
209	For 72 MOTUs we found no matches in the BOLD System. We rejected 1 MOTU, because it
210	contained only very short reads. Of the MOTUs 32% could be assigned to species level, 28%
211	to genus level, 23% to family and 17% only to order level. We found 108 MOTUs in samples
212	from migrating bats, whereas 58 MOTUs were assigned to samples from summer roosts. 19
213	MOTUs were found in both groups.
214	Hamming Distances between migratory bats and bats at summer colonies was 197.
215	Additionally overall <u>Bray-Curtis</u> similarity between migratory bats and bats at summer
216	colonies was 0.84. Both suggest low dietary similarity.
217	Morphological analysis
218	We found that the diet was significantly different between bats from summer roosts
219	and on migration, indicated by the conducted permutational analysis of variance (ADONIS: F
220	= 4.371, df = 1, p<0.001). Comparing diversity and species richness in the diet of <i>P. nathusii</i>
221	between the two sites, we found no differences (Table 2). The trophic niche overlap, indicated
222	by Pianka's index, was relatively high (Table 2). The ordination plot (NMDS) shows samples
223	spread out evenly along the two dimensions, overlapping to a great extent. The slight
224	clustering along the first dimension has to be interpreted cautiously, as a stress value of 0.2
225	was reached (Fig. <u>4</u> 2).
226	Based on GLMs we found significant difference between certain prey groups. P.
227	nathusii from summer roosts appear to feed more often on Chironomidae than migrating P.
228	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, sSubtle 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).

258	A significant issue here is whether the difference is due to "migration" or "location".	
259	Clare et al. (In press) demonstrated the degree of location, season and inter-annual variation	Formatted: Font: Italic
260	in badt diet across landscapes. Since most bats forage among prey in their immediate vicinity	
261	it is not clear to what degree shifts in diet observed here are caused by changes in insect	
262	phenology, bat physiological demands and habitat-insect relationships. We report here that	
263	there is variation between summer colonies and migrating individuals, though the underlying	
264	cause is very likely a mix of these competing influences factors. We suggest that the next	
265	logical step is to expand this type of study to include multiple summer and migration sites so	
266	that comparisons can be made across location.	
267	The higher resolution of molecular diet analyses enof prey species, compared	
268	eontrasting with morphological analysisanalyses, provides valuable information on	
269	associations between prey, habitat and predator (Clare et al. 2010; Razgour et al. 2011, Clare	
270	et al. 2013a in press; Krüger et al. in press). White While our morphological observations	Formatted: Font: Italic
271	suggest that diet was strongly overlapping between summer and migratory groups, we did	
272	observe a higher species richness in the migratory diet based on the molecular data. There are	
273	two potential drivers of increased diversity in the diet of migrating <u>P. nathusii</u> bats. First,	
274	migrating individuals bats migrating are covering cover more space and more potential habitat	
275	types. This may expose them to a higher diversity of potential prey as a consequence. Second,	
276	insect diversity, in general is general reduced later, falls later in the summer. At this point a	
277	reduced availability of prey may force the <u>P. nathusii</u> bats-to become more flexible in the prey	
278	they consume (Clare <i>et al.</i> 2013a/b in reviewpress).	Formatted: Font: Italic
279	We also found that the diet of migrating pipistrelles P. nathusii contained higher	Formatted: English (U.S.) Formatted: Font: Italic
280	occurrence of insect species inhabiting aquatic habitats like the beetles Cyphon	
281	phragmiteticola and Agonum piceum. This probably reflects the fact that P. nathusii bats	
282	forage in the adjacent bog and marsh lands of Lake Pape. The moths Epinotia immundana,	
283	Epinotia nisella and Phyllonorycter apparella are associated with riverine forests and trees in 12	

284 marshes, supporting the inference that bats forage in the vicinity of aquatic habitats. Further 285 indications for aquatic foraging habitats are the occurrences of Trichoptera and Megaloptera. 286 By contrast the moth *Malacosoma castrensis* indicates foraging over dunes, as this is the 287 major habitat of this moth. The dunes at Pape spread out parallel to the coastline, and are also 288 used by *P. nathusii* bats as a major flight corridor during migration (Šubar et al.2012) 289 suggesting prey habitat and predator habitat overlap at this point. 290 In the diet of *P. nathusii* bats-from summer colonies we found prey species which are 291 typically associated with forested areas, like *Bupalus pinaria*, a pine pest species, or 292 Promethes sulcator, an ichneumon wasp. These species were not identified in samples from 293 migrating *P. nathusii* bats. As the colony sites are also within a few kilometres of lakes, we 294 also found prey species associated with aquatic habitats, like Chironomidae or 295 Ephemeroptera. Overall we can observe how the foraging habitat of *P. nathusii* determines 296 the diet and thus differences between migrating and summering bats might be 297 triggered explained. In birds it has been shown that during migration sedge warblers 298 (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 299 300 in habitats with high insect abundance like riverine and semi-aquatic habitats. 301 Many insect species are also known to migrate (e.g. Hummingbird Hawk-moth 302 Macroglossum stellatarum, Monarch butterflies butterfly Danaus plexippus). The beet army 303 worm, Spodoptera exigua, originally distributed in the Americas, now occurring globally, is 304 also a known long-distance migrant (Westbrook 2008). In Europe this species has been 305 observed to travel long distances, from Russia over Fennoscandia towards Denmark and the 306 British Isles (Mikkola 1970). The occurrence of a migrating insect in the diets of migrating 307 bats may be a coincidental overlap of migration routes and the opportunistic foraging 308 behaviour of pipistrelles, which has been also observed in other species such as Tadarida 309 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

336 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 337 garden warbler (Sylvia borin) switch from arthropod based diet to fruit based diets (Bairlein 338 339 1990) and furthermore -select for fruit with certain fatty acid compositions prior to migration 340 (McWilliams et al. 2004). Other birds like willow warblers switch to insect prey high in Formatted: Font: Italic 341 sugars, like aphids (Berthold 2001). Adaptive alteration of diet selectivity during migration 342 seems to be a valuable trait in migrants. The latter is comparable to possible shifts in diets of migratory bats. 343 344 This kind of selectivity would require the bats to discriminate between prey of 345 different energy / fat values. In bats selective behaviour and prey discrimination based on size 346 has previously been demonstrated only for horseshoe bats selective behaviour and prey 347 discrimination based on size has been demonstrated previously (Koselj et al. 2011). Whether 348 the same ability exists for other bats, like *Pipistrellus*, which, in contrast to horseshoe bats, 349 use short frequency-modulated (FM) calls and mainly feed on Diptera on the wing, is not 350 clear. Also like for frugivory in insectivorous birds during migration, the differences in diet 351 between summer and migratory P. nathusii may result from the seasonal changes in Formatted: Font: Italic 352 availability of certain food items, insects and fruit, respectively. Formatted: English (U.S.) 353 The fat stores of migrating bats have higher proportions of polyunsaturated fatty acids 354 (PUFAs) (McGuire et al. 2013b). Thus PUFAs are seem tomay be an important resource 355 during pre-migration and migration itself. Naturally, the diet of *Pipistrellus nathusii* is often 356 dominated by Diptera, particularly Chironomidae, which are rich in highly unsaturated fatty 357 acids (Thompson 1982; Hanson et al. 1985). Overall aquatic insects have higher PUFA 358 content than do terrestrial insects, though this varies depending on life stage (Hanson et al 359 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. 360 361 Migrating bats still feed to large extent on Nematocera, often associated with high PUFA

where species can be studied.

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eontent. 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 ocosts 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 Tthe differences between migrating and summering individuals found in prey groups ean-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.

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382	Data Accessibility
383	Molecular (sequences) and morphological (binary presence-absence) dietary data will
384	be provided on a DRYAD account (doi: 10.5061/dryad.2d38f).
385	
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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, respectively.

Order	Family	Species	Conf	Migration	Summer
Diptera	Asilidae	unknown	3	1	0
	Chaoboridae	unknown	3	11	0
	Chironomidae	unknown	3	1	1/
		Glyptotendipes sp.	3	0	1
		Microtendipes sp.	3	1	0
		Microtendipes brevitarsis	1b_	1	0
		Parachironomus tenuicaudatu	_1b_	11	0
		Paracladopelma winnelli	1b_	1	1
		Procladius sp.	<u>1b</u> _	11	1
		Synenotendipes impar	1b_	0	1/
		Tanytarsus mendax	1a_	11	0
		Xenochironomus xenolabis	<u>la</u> _	1	0
		unknown	2	11	0
	Culicidae	Aedes sp.	2	1	0
		Anopheles sp.	3	0	1
		Culiseta sp.	2	11	0
		Ochlerotatus annulipes	la_	<u> </u>	0
	Dolichopodidae	unknown	3	1	0
	Empididae	unknown	3	1	0
	Limoniidae	Dicranomyia frontalis	<u>la_</u> _	11	1
		Dicranomyia sp.	1b	1	1
		Erioptera sp.	2_	1	0
		Helius flavus	_ <u>la</u> _	1	0
		Limonia nubeculosa	<u>1b</u>	1	0
		Metalimnobia sp.	<u>la</u> _	1	1
		Molophilus sp.	<u>1a</u> _	0	0
		Phylidorea ferruginea.	3	1	0
		Phylidorea fulvonervosus	<u>1b</u>	0	1
		Rhipidia maculata	<u>la</u> _	1	0
		Helina impunctata	<u>la</u> _	0	1
	Muscidae	unknown	3	0	1
	Mycetophilidae	Mycetophila luctuosa	<u>l a</u> _	0	1

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Ephemeroptera	Baetidae Caenidae	Cloeon dipterum Caenis sp.	1a 1a	1	0
		Sparganothis sp.	1b	1	0
		Rhopobota naevana	<u>1 a</u>	1	0
		Lozotaenia forsterana	la	0	1_
		Eudemis porphyrana	la	0	1_
		Epinotia nisella	1a	1	0
		Epinotia immunda	la	1	0
		Cnephasia sp.	la	1	1
		Adoxo phyesorana	la	0	1
	Tortricidae	Acleris emargana	1a	1	_0_
	Noctuidae	Spodoptera exigua	1b	1	0_
		Dendrolimus pini	la	0	1
	Lasiocampidae	Malacosoma castrensis-	1b	11	0
	Gracillariidae	Phyllonorycter apparella	la	1	0
		Bupalus pinaria	1a	0	1
	Geometridae	Hydriomena sp.	la_	11	0
		Coleotechnite spiceaella	1b	11	0
	Gelechiidae	Exoteleia dodocella	la	11	1
		Semioscopis sp.	la_	1	0
	Elachistidae	Agonopterix sp.	1b	11	0
		Coleophora limosipennella	la	11	0
	Coleophoridae	Coleophora glitzella	1a	0	1
	Blastobasidae	unknown	3	1	0
	Argyresthiidae	Argyresthiago edartella	1a	0	1
_epidoptera	Amphisbatidae	Pseudatemelia josephinae	la	0	1
	Tipulidae	Tipula sp.	1a	1	1
	Tachinidae	unknown	3	1	0
		Nephrotoma sp.	1b	1	0
	Tabanidae	Hybomitra lurida	1a	1	0
	Sciomyzidae	Haematopota pluvialis	1a	0	1
	Sciaridae	Anticheta sp.	1b	0	1_
	Psychodidae	unknown	33	11	0
	Pedicidae	Psychodae phalaenoides	3 1a	0	1

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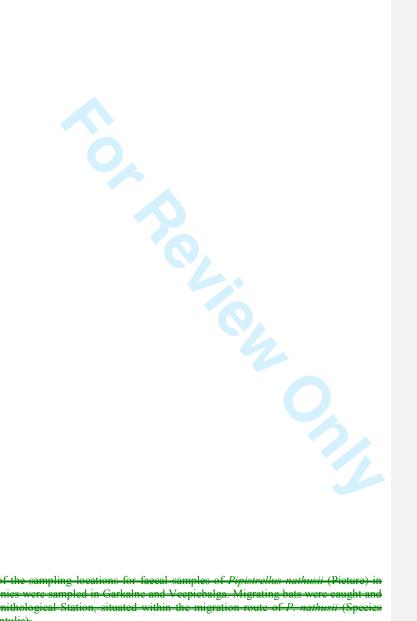
Table 3 The frequency of prey groups in the diet of *P. nathusii* from summer colonies and migration based on morphological presence / absence data. We tested data with generalized liner model (GLM) and Tukey post-hoc test. Significant differences are The significance of differences between prey groups,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

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646		Migration	Summer	z	p<
647	Nematocera	73.3 <u>%</u>	47.8 <u>%</u>	<u>-1.531</u> -	- <u>0.126 -</u>
648	Anisopodidae	0.0 <u>%</u>	21.7 <u>%</u>	- <u>0.007</u>	<u>0.995</u> -
649	Chironomidae	40.0 <u>%</u>	91.3 <u>%</u>	3.034	0.00241
650	Culicidae	0.0 <u>%</u>	17.4 <u>%</u>	<u>0.006</u> –	<u>0.995</u> -
	Tipulidae	86.7 <u>%</u>	30.4 <u>%</u>	-3.051	0.00228
651	Brachycera	40.0 <u>%</u>	52.2 <u>%</u>	0.732 -	0.464-
652	Hemiptera	6.7 <u>%</u>	0.0 <u>%</u>	- <u>0.003</u>	- <u>0.998</u>
653	Corixidae	0.0%	8.7 <u>%</u>	<u>0.004</u> -	<u>0.997</u> -
654	Cicada	0.0%	4.3 <u>%</u>	<u>0.004</u> -	<u>0.997</u> -
655	Aphidoidea	13.3 <u>%</u>	26.1 <mark>%</mark>	<u>0.927</u> -	<u>-0.354</u>
656	Trichoptera	26.7 <u>%</u>	17.4 <u>%</u>	<u>-0.681</u> -	0.496
657	Lepidoptera	53.3 <u>%</u>	30.4 <u>%</u>	<u>-1.396</u> -	<u>0.163</u> -
658	Ephemeroptera	26.7 <u>%</u>	0.0%	<u>-0.005</u> -	<u>0.996</u> -
	Neuroptera	46.7 <u>%</u>	34.8 <u>%</u>	- <u>0.73</u>	<u>0.465</u> -
659	Coleoptera	13.3 <u>%</u>	0.0 <u>%</u>	- <u>0.005</u>	<u>0.996</u> -
660	Hymenoptera	6.7 <u>%</u>	4.3 <mark>%</mark>	- <u>0.311</u>	- <u>0.756</u>
661	Araneae	13.3 <u>%</u>	4.3%	- <u>0.957</u>	<u>-0.338</u>
662	Simuliidae	0.0 <u>%</u>	4.3%	<u>0.004</u> -	<u>-0.997</u>
663	Formicidae	26.7 <u>%</u>	0.0%	- <u>0.005</u>	<u>0.996</u> -

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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).



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Latvia. Summer colonies were sampled in Garkalne and Veepiebalga. Migrating bats were caught and sampled in Pape, Ornithological Station, situated within the migration route of P. nathusii (Species photo by Viesturs Vintulis).

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Figure 2 Plot of a non-metric two-dimensional ordination scale (NMDS) based on the presenceabsence 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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Molecular Ecology

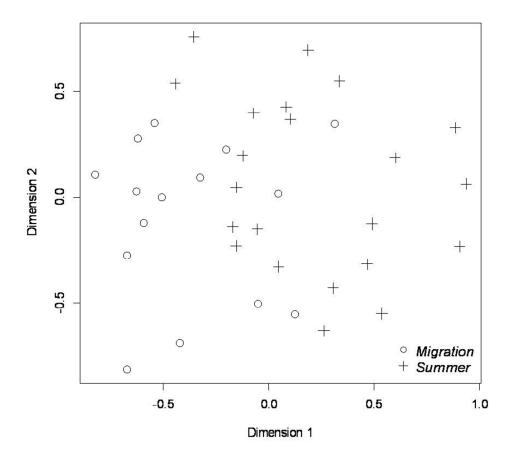
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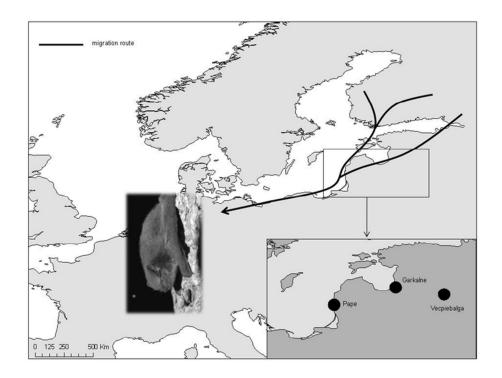
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