Stable isotope ratios of hydrogen separate mammals of aquatic and terrestrial food webs

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Summary

1. In food web studies, hydrogen stable isotope ratios (δ^2H) are increasingly used as endogenous markers to quantify the relative importance of allochthonous input of organic material into aquatic ecosystems. Yet, it is unclear if differences in δ^2H values between aquatic and terrestrial food webs translate into corresponding differences of δ^2H values of vertebrate consumers.

2. Using a triple-isotope approach, we asked if fur keratin of 12 sympatric, non-migratory bat species differ in stable isotope ratios (δ^2H_K, $\delta^{13}C_K$, $\delta^{15}N_K$) according to the ecosystem (aquatic or terrestrial) in which they predominantly forage. We conducted a study with two captive species beforehand that suggested species-specific differences in trophic discrimination for C and N, but not for H.

3. In wild bats, stable isotope ratios of two *Myotis* species that trawl insects in aquatic habitats differed from those of four congeneric species that glean terrestrial insects by about 40‰ for H and by about 5‰ for N, indicating that both δ^2H_K and $\delta^{15}N_K$ values are useful for identifying membership of aquatic and terrestrial food webs. Further, we assessed the relative association of aerial-hawking bat species to the terrestrial food web using trawling and gleaning bats as representatives of consumers in aquatic and terrestrial ecosystems, respectively. We found that the relative association of bats to terrestrial food webs varied largely among species, ranging from about 90% in *Rhinolophus hipposideros* to 43% in *Eptesicus serotinus*. Isotopic niche dimensions, as estimated by standardized ellipse areas using δ^2H_K and $\delta^{15}N_K$ values, varied largely among the 12 study species, with largest overlaps of isotopic niches among members of the aerial-hawking bat ensemble.

4. We conclude that δ^2H_K and $\delta^{15}N_K$ values are suitable parameters for evaluating the relative membership of mammals to aquatic and terrestrial food webs and also to evaluate isotopic niche dimensions and thus niche packing of species within consumer ensembles.

Key-words: aquatic ecosystems, bats, connectivity, migration, terrestrial ecosystems, trophic level

Introduction

Over past decades, stable hydrogen isotope ratios of metabolically inert tissues such as feather and fur keratin (δ^2H_K) have been used to infer the connectivity of breeding and wintering habitats of migratory birds (Hobson & Wassenaar 1997; Hobson 1999; Rubenstein *et al.* 2002; Hobson *et al.* 2004) and bats (Cryan *et al.* 2004; Popa-Lisseanu *et al.* 2012; Voigt *et al.* 2012, 2014; Cryan, Stricker & Wunder 2014; Lehnert *et al.* 2014). The approach of geolocating migratory animals using values of δ^2H_K is based on the assumption that δ^2H_K values largely depend on the stable isotope ratios of local surface or precipitation water during the time of moult (Hobson 1999; Wassenaar & Hobson 2003; Bowen, Wassenaar & Hobson 2005). Accordingly, δ^2H_K values should correlate positively with δ^2H values of environmental water. This relationship has been confirmed for various geographical areas and various taxa (birds: Hobson & Wassenaar 1997; Hobson 1999; Wolf, Bowen & Martinez del Rio 2011; bats: Cryan *et al.* 2004; Voigt *et al.* 2012, 2014; humans: O'Brien & Wooller 2007; Bowen *et al.* 2009). However, this correlation deviates sometimes from a 1 : 1 relationship (e.g. birds: Hobson 2008; bats: Ossa *et al.* 2012). Also, sympatric non-migratory species sometimes show significant differences in species-specific mean δ^2H_K values, indicating that δ^2H_K values may depend on microhabitat use or dietary specialization (Fraser *et al.* 2011; Voigt, Schneeberger & Luckner 2013). For example, Voigt, Schneeberger & Luckner (2013) found that δ^2H_K values of syntopic, non-migratory bats may vary over a range of about 70‰. Also, fur keratin of fruit-eating and insect-feeding bats may differ by about 44‰ (Voigt, Schneeberger & Luckner 2013), suggesting that δ^2H_K values largely depend on the specific feeding habits of consumers and presumably on isotopic differences in potential food or water sources. Based on these previous findings, we asked if δ^2H values of consumer keratin differ between members of aquatic and terrestrial food webs.

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Doucett *et al.* (2009) were among the first to observe that δ^2H values of organic matter from terrestrial habitats contrast strongly with that of aquatic food webs. They showed for stream ecosystems that δ^2H values of autochthonous organic matter were lower than allochthonous organic material from terrestrial food webs by about 150‰. Differences in δ^2H values between aquatic and terrestrial plant matter most likely originated from varying levels of kinetic fractionation of stable isotopes during evaporation (Smith & Ziegler 1990). Doucett *et al.* (2009) also observed that freshwater benthic invertebrates were enriched in ^2H by about 50‰ in relation to the autochthonous material of algae, suggesting a partial contribution of allochthonous material to the diet of benthic invertebrates. While variation of δ^2H values in plant and invertebrate tissue has already been used in studies of freshwater biology (Soto, Garcia & Catalan 2013; Yang *et al.* 2014), variation in δ^2H of consumer tissues has not yet been used in food web studies of terrestrial vertebrates.

We assumed that terrestrial mammals exhibit δ^2H_K values according to their relative association with aquatic or terrestrial food webs and that trophic discrimination is negligible for stable hydrogen isotopes (Hobson 2008). Accordingly, we inferred that it is possible to quantify the relative association of vertebrates to aquatic or terrestrial food webs according to their δ^2H_K values. The use of hydrogen for the study of food webs might be helpful because it might improve our understanding of ensembles, particularly in species-rich taxa and areas. For example, previous studies highlighted that more than 100 bat species may coexist in an Ecuadorian lowland rain forest (Rex *et al.* 2008) and that nitrogen isotopes of bats correlate with their trophic positions (Rex *et al.* 2010) and stable carbon isotopes on their spatial use (Rex *et al.* 2011). Yet, considering the relatively large overlap in stable isotope ratios across species, stable isotopes of only two elements may be insufficient in unravelling the details of niche packing within such species-rich ensembles. Here, we tested if δ^2H_K values may present a helpful additional marker for community studies by enabling researchers to assess the relative association of consumers to aquatic or terrestrial food webs. We consider bats as ideal candidates for studying mechanisms underlying species coexistence because they form diverse ensembles, because their feeding habits have been described by independent measures, such as wing morphology and echolocation call design (Arita & Fenton 1997), and because previous isotopic studies have already provided a good understanding of isotopic niche dimensions in this taxon (e.g. Siemers *et al.* 2011; Voigt, Schneeberger & Luckner 2013). In the remainder of the text, we use the term 'feeding ensemble' to describe a group of bats with similar ecological function, namely gleaning bats picking prey insects from the ground or leaves, aerial-hawking bats hunting prey insects in the open space and trawling bats skimming the water surface in search for prey insects.

To elucidate possible trophic discriminations of ^2H, ^{15}N and ^{13}C in relation to the corresponding light isotopes between fur keratin and diet, we first conducted a study with two captive bat species, *Myotis myotis* and *Rhinolophus ferrumequinum*. We then investigated if δ^2H_K values differ between non-migratory, wild bats foraging exclusively in terrestrial and those foraging exclusively in aquatic food webs, namely between species gleaning terrestrial insects from solid surfaces, such as ground and leaves, and those trawling insects from water surfaces. For this, we analysed δ^2H_K in fur samples of six insectivorous *Myotis* species collected shortly after their respective reproductive period in Bulgaria. By focusing on species of the same genus, we ensured phylogenetic independence (Price 1997). Four out of the six species, namely *Myotis bechsteinii, Myotis emarginatus, Myotis oxygnathus* and *M. myotis*, hunt insects in terrestrial ecosystems such as forests, agricultural fields and meadows (Dietz, Nill & von Helversen 2009). The other two species, namely *Myotis capaccinii* and *Myotis daubentonii*, hunt almost exclusively aquatic insects (Vaughan 1997; Dietz, Nill & von Helversen 2009; Krüger *et al.* 2014). We hypothesized that δ^2H_K values would permit differentiation of bats feeding on insects of aquatic and terrestrial ecosystems, because (i) organic matter of aquatic and terrestrial primary producers differs in δ^2H values (Doucett *et al.* 2009; Soto, Garcia & Catalan 2013; Yang *et al.* 2014) and (ii) because these differences should translate into corresponding tissue δ^2H_K values of aquatic and terrestrial insects (Schimmelmann & DeNiro 1986). Accordingly, we predicted that δ^2H_K values of bats hunting on terrestrial insects should exhibit higher δ^2H_K values than bats hunting on adult insects with aquatic larval stages. Lastly, we used the isotopic compositions of gleaning and trawling bats as reference points of terrestrial and aquatic food web consumers, respectively, to estimate the relative association of six aerial-hawking bat species to terrestrial food webs, assuming that the isotopic data of gleaning and trawling bats are typical for mammals feeding mostly on food items from terrestrial and aquatic food webs, respectively. By quantifying the relative isotopic niche overlap between species dyads, we aimed to elucidate if bat species forage in the same food web and thus may compete for similar resources.

Materials and methods

FEEDING EXPERIMENT

Before studying stable isotope ratios of fur keratin from free-ranging bats, we conducted a validation study in captive bats to estimate if stable isotope ratios differ between fur keratin, and the diet of bats. We used 11 *M. myotis* (10 males and one female) and four male *R. ferrumequinum* that were maintained in captivity for more than 7 years at the animal facilities at the Max Planck Institute for Ornithology in Seewiesen, Germany (permit 311.5-5682.1/1-2014-023). Animals were kept in separate rooms, but fed the same diet of mealworms with *ad libitum* access to tap water. In the summer of 2014, we collected hair samples from the dorsal fur of each bat. Further, we collected two samples of tap water in air-tight plastic containers (1 week apart) and three mealworms from the food dishes of bats. We shipped all samples to the stable isotope laboratory of the Leibniz Institute for Zoo and Wildlife Research (IZW).

Details of the stable isotope analyses are described in the Supporting information. Stable isotope ratios of non-exchangeable hydrogen in fur keratin (δ^2H_K) are given as δ-values, which express the parts per mille (‰) deviation of the sample isotope ratio from the international stan-

dard (V-SMOW for H, air nitrogen for N, and V-PDB for C). Tap water samples were analysed using a cavity ring-down spectrometer (Isotopic H_2O analyzer L1102-i; Picarro Ltd., Santa Clara, CA, USA) as described in Supporting information. Analytical precision based on the repeated analyses of international water standards was always better than 0·9‰ (1 SD).

The mealworms were euthanized in a freezer and stored separately in small, air-tight plastic vials. We then performed a sagittal slit into the abdomen, transferred the carcasses separately into glass tubes and evacuated air from these tubes. Closed tubes were then put on a warming plate for distillation. Water samples were analysed as described above.

To assess if stable isotope ratios differ between *M. myotis* and *R. ferrumequinum*, we calculated a Mann–Whitney *U*-test for each element separately with GRAPHPAD (Instat 3.0, La Jolla, CA, USA), assuming a level of significance of 0·05. We calculated mean values and standard deviation if not stated otherwise. To test if δ^2H_K values of bats differed from δ^2H of water samples, we used one random sample as reference and tested if bat δ^2H_K values deviated from this value using a Wilcoxon signed rank-sum test.

FIELD WORK

We captured bats of 12 sympatric, non-migratory species in Bulgaria (Ruse district, latitude 43.611537, longitude: 25.982123) under the licence of the Bulgarian authorities [Bulgarian Ministry of Environment and Water and Regional Inspectorate (RIOSV) Ruse, permits # 57/18.04.2006, 100/04.07.2007, 193/01.04.2009, 205/29.05.2009 and 465/29.06.2012]. More details regarding our field work and sample collection are provided in the Supporting information.

STABLE ISOTOPE AND DATA ANALYSES OF SAMPLES FROM FIELD WORK

Stable isotope ratios were analysed as described above. To test for differences among categories (species, ensemble), we used a two-way nested ANalysis Of SIMilarity (ANOSIM; PRIMER 6; Version 6-1-15; Primer-E Ltd.) where species as a factor is nested in the factor feeding ensemble. We then used a SIMPER analysis to quantify the percentage of isotopic dissimilarity between feeding ensembles and to depict the isotopes most relevant in explaining the dissimilarity between dyads (see Supporting information). All parameters are presented as means ± 1 standard deviation.

ISOTOPIC MIXING MODELS AND ISOTOPIC NICHES

We assessed the relative association of the six aerial-hawking bats to the terrestrial food web by applying a Bayesian isotope mixing model from the package SIAR version 4.1.3 Parnell & Jackson (2011) of the free statistical software R (R Core Team 2010). Further, we calculated and plotted isotopic ellipses for $\delta^{15}N_K$ and δ^2H_K values using the SIBER routine (Jackson *et al.* 2011) as part of the SIAR and CAR package in R (version 3.0.1; R Development Core Team 2013). Further details are provided in the Supporting information.

Results

FEEDING EXPERIMENT

We measured the isotopic composition of fur keratin in 11 *M. myotis* and four *R. ferrumequinum* after they shared the

same mineral-enriched mealworm diet over at least 7 years. We found no significant difference in δ^2H_K values between these species (Fig. 1a; Mann–Whitney test; $U' = 33$, $P = 0·177$), but differences in both $\delta^{13}C_K$ (Fig. 1b; $U' = 5$, $P = 0·026$) and $\delta^{15}N_K$ values (Fig. 1c; $U' = 0$, $P = 0·002$). In *M. myotis*, δ^2H_K, $\delta^{13}C_K$ and $\delta^{15}N_K$ values averaged $-68·8 \pm 5·2‰$, $-22·2 \pm 0·5‰$ and $9·3 \pm 0·2‰$, respectively, and in *R. ferrumequinum* $-58·0 \pm 15·8‰$, $-21·6 \pm 0·2‰$ and $8·8 \pm 0·2‰$, respectively. Significant isotopic differences between the two species averaged 0·6‰ for stable carbon and 0·5‰ for nitrogen isotopes.

δ^2H values of *ad libitum* offered tap water averaged $-98·4 \pm 0·3‰$ and $-99·9 \pm 0·8‰$, respectively (Fig. 1a). Since δ^2H_K values of the two species did not differ significantly, we pooled all bat data. The average δ^2H_K values of captive bats equalled $-66·0 \pm 9·9‰$, which was 32·4 and 33·9‰, respectively, higher than the δ^2H values of the tap water samples (one-sample *t*-test, $t_{14} = 12·7$, $P < 0·001$, $t_{14} = 13·3$, $P < 0·001$, respectively). Repeated measurements
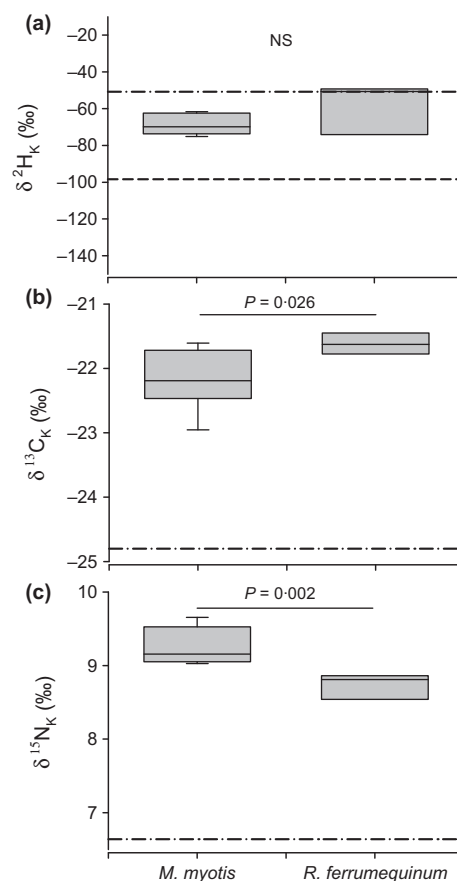

Fig. 1. Differences in stable isotope ratios in fur keratin for δ^2H_K (a), $\delta^{13}C_K$ (b) and $\delta^{15}N_K$ (c) values of captive *Myotis myotis* ($n = 11$) and *Rhinolophus ferrumequinum* ($n = 4$). Box plots depict the 25 and 75 percentiles, and whiskers, the standard deviation. Solid lines inside the boxes indicate the median and solid lines between boxes significant differences; ns, not significant. Dashed-dotted lines indicate the corresponding reference value for mealworm body water (a) and mealworm organic tissue (b, c). In graph (a), the dashed line indicates average δ^2H of a tap water sample.

of δ^2H values in water extracted from each of three meal-worms via vacuum distillation averaged $-49.7 \pm 0.3‰$, $-50.8 \pm 0.8‰$ and $-69.9 \pm 0.6‰$ (Fig. 1b). According to a one-sample *t*-test, δ^2H_K values of bats were significantly lower by 15‰ than that of the median δ^2H value of meal-worm body water ($t_{14} = 6.0$, $P < 0.001$).

STABLE ISOTOPE RATIOS OF FREE-RANGING BATS

In total, we captured 68 trawling (63 *M. capaccinii* and 5 *M. daubentonii*), 244 gleaning (10 *M. bechsteinii*, 12 *M. emarginatus*, 51 *M. oxygnathus* and 171 *M. myotis*) and 444 aerial-hawking bats (10 *Eptesicus serotinus*, 136 *Miniopterus schreibersii*, 113 *Rhinolophus euryale*, 37 *R. ferrumequinum*, 25 *Rhinolophus hipposideros* and 123 *Rhinolophus mehelyi*) (Table S1, Supporting information). We found differences in stable isotope ratios among species across all feeding ensembles (ANOSIM: global $r = 0.297$, $P < 0.001$) and also among feeding ensembles (global $r = 0.723$, $P < 0.001$). Isotopic differences were significant between gleaning and aerial-hawking species ($r = 0.58$, $P = 0.006$) and aerial-hawking and trawling bat species ($r = 0.909$,

$P < 0.022$). Isotopic differences tended to differ between gleaning and trawling bat species ($r = 1.0$, $P < 0.067$). According to a SIMPER analysis, stable isotope ratios of gleaning and aerial-hawking bats differed by 5.1%, which was foremost explained by variation in δ^2H_K (64.6% of dissimilarity explained by H) and $\delta^{15}N_K$ (26.2%). Aerial-hawking and trawling bats differed in isotopic composition by 5.9% with δ^2H_K explaining 62.4% and $\delta^{15}N_K$ 26.7% of the dissimilarity. Lastly, stable isotope ratios of gleaning and trawling bats differed by 9.7%, which was mostly attributable to variation in $\delta^{15}H_K$ (66.2%) followed by variation in $\delta^{15}N_K$ (27.4%). Since $\delta^{13}C_K$ values were not relevant in explaining variation among species, we focused on δ^2H_K and $\delta^{15}N_K$ in the remainder of the results section. δ^2H_K averaged $-105.9 \pm 13.8‰$ for trawling, $-65.8 \pm 9.0‰$ for gleaning and $-82.5 \pm 10.1‰$ for aerial-hawking insectivorous bats (Fig. 2). $\delta^{15}N$ averaged $12.5 \pm 1.9‰$ for trawling, $7.0 \pm 1.4‰$ for gleaning bats and $9.1 \pm 1.3‰$ for aerial-hawking insectivorous bats (Fig. 2). Trawling bats exhibited lower δ^2H_K values but higher $\delta^{15}N$ values than gleaning and aerial-hawking bats (Table S1). Pairwise differences ranged between 37‰ and 53‰ for δ^2H_K and 2.1 and 8.0 for $\delta^{15}N$ (Fig. 2).

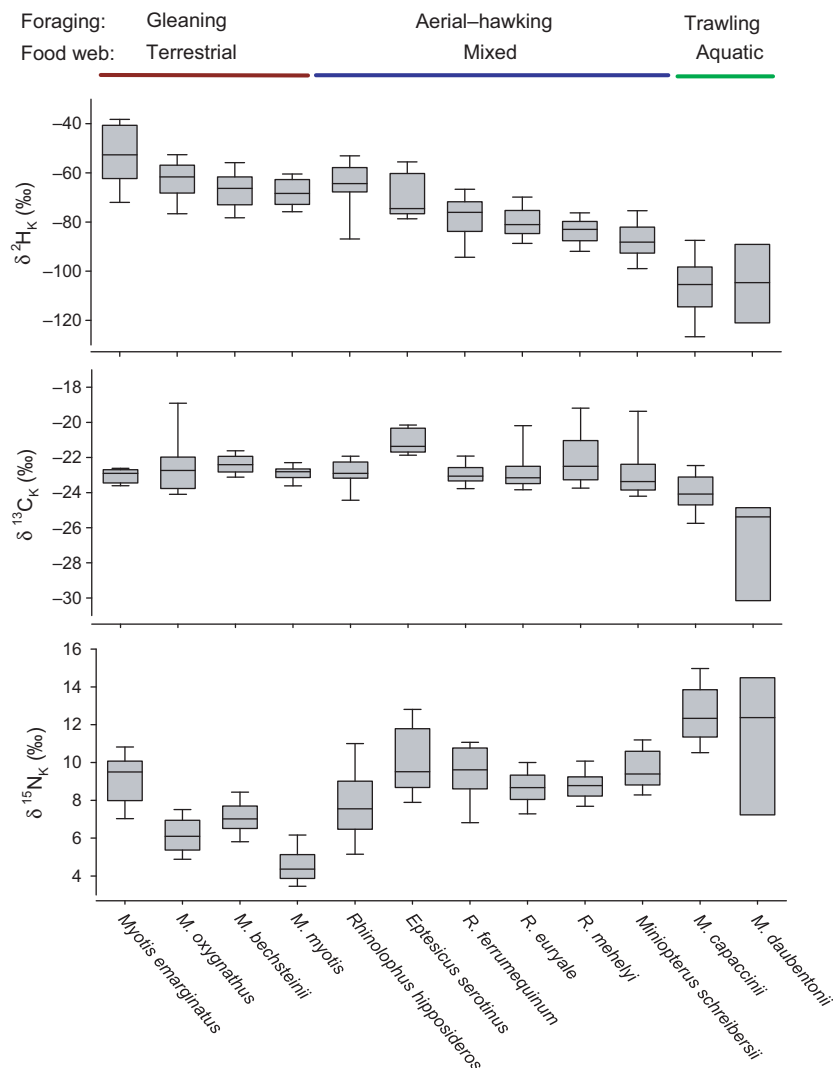**Fig. 2.** δ^2H_K, $\delta^{13}C_K$ and $\delta^{15}N_K$ values in 12 sympatric species (species within foraging category were sorted according to decreasing median δ^2H_K values). Box plots depict the 25 and 75 percentiles, and whiskers, the standard deviation. Solid lines inside the boxes indicate the median.

RELATIVE ASSOCIATION OF AERIAL-HAWKING BATS WITH TERRESTRIAL FOOD WEBS

Following our study in captive bats, we corrected for a trophic discrimination of 0·5‰ in ^{15}N between *Rhinolophus* and non-*Rhinolophus* species. Our Bayesian mixing model indicated that five out of six species of the aerial-hawking bat ensemble were primarily associated with terrestrial food webs (Table S2), with *R. hipposideros* exhibiting the strongest association with terrestrial food webs (mean 89%) followed by *E. serotinus* (71%), *R. euryale* (62%), *R. ferrumequinum* (59%) and *R. mehelyi* (57%). Isotopic data suggested that *M. schreibersii* was only associated by 43% with terrestrial food webs. The pattern across species was robust towards assumptions of diet-consumer discriminations (Table S2).

ISOTOPIC NICHE DIMENSIONS OF BATS

Within the ensemble of gleaning bats, species-specific ellipse dimensions (SEA$_B$; Table S3) varied between 51·2‰^{2} for *M. emarginatus* and 14·5‰^{2} for *M. bechsteinii*, indicating that *M. emarginatus* exhibited the largest isotopic niches and *M. bechsteinii* the smallest. The two sibling species *M. myotis* and *M. oxygnathus* showed intermediate isotopic niche dimensions (Table S3, Fig. 3). Range of isotopic niche dimensions was similar for species of the aerial-hawking bat ensemble, with *R. hipposideros* showing the largest isotopic niche (57·3‰^{2}) and *R. mehelyi* the smallest (16·9‰^{2}; Table S3, Fig. 3). Trawling bats had largely variable isotopic niche dimensions with *M. daubentonii* showing the largest value of all species (190·0‰^{2}). *Myotis capaccinii* had an isotopic niche dimension of 77·4‰^{2}.

When comparing SEA$_B$ dimensions across species of the gleaning bat ensemble, we found that only two out of six possible pairs overlapped in their isotopic niches (Table 1, Fig. 3), that is SEA$_B$ dimensions of *M. oxygnathus* and *M. bechsteinii* overlapped as well as those of *M. myotis* and *M. oxygnathus*. We observed the largest overlap between *M. myotis* and *M. oxygnathus*, that is 43·5% of SEA$_B$ of *M. oxygnathus* was

overlapped by SEA$_B$ of *M. myotis*, and 51·9% of SEA$_B$ of *M. myotis* was overlapped by that of *M. oxygnathus*. SEA$_B$ of gleaning bat species overlapped with SEA$_B$ of *R. euryale* (*M. myotis*) and *R. ferrumequinum* (*M. oxygnathus*, *M. emarginatus* and *M. myotis*). The two sibling species *M. myotis* and *M. oxygnathus* exhibited the largest overlap with *R. ferrumequinum*, that is 61·2 and 80·6%, respectively. Within the ensemble of aerial-hawking bats, 13 out of 15 possible pairwise comparisons revealed an overlap of SEA$_B$ (Table 1, Fig. 3). SEA$_B$ dimensions of only two species did not overlap: *E. serotinus* and *M. schreibersii*, and *E. serotinus* and *R. mehelyi*. SEA$_B$ dimensions of all *Rhinolophus* species overlapped, yet to varying degrees, ranging from 2·7% (*R. ferrumequinum* in relation to *R. mehelyi*) to 76·5% (*R. mehelyi* in relation to *R. hipposideros*). SEA$_B$ dimensions of three species of the aerial-hawking bat ensemble overlapped with SEA$_B$ dimensions of trawling bats, namely *M. schreibersii*, *R. euryale* and *R. mehelyi*. Lastly, within the trawling bat ensemble, SEA$_B$ of *M. daubentonii* encompassed completely that of *M. capaccinii*, yet only 28·3% of SEA$_B$ of *M. daubentonii* was overlapping with SEA$_B$ of *M. capaccinii* (Table 1, Fig. 3).

Discussion

In our study, we asked if stable isotope ratios of non-exchangeable hydrogen in keratin (δ^2H$_K$) are related to a consumer's association with an aquatic or terrestrial food web. Specifically, we predicted that δ^2H$_K$ values differ between bat species that glean terrestrial insects and those that trawl insects from water surfaces or feeding largely on insects with aquatic larval stages. Indeed, we observed that two species of the genus *Myotis* that are known to be closely associated with aquatic food webs, such as ponds and streams, exhibit considerably lower δ^2H$_K$ values than four congeneric species hunting insects in terrestrial habitats. Isotopic differences in δ^2H$_K$ values between members of these two ecological ensembles ranged between 37–53‰, which were in the range of differences observed before for syntopic bats and birds (Fraser *et al.* 2011; Voigt, Schneeberger & Luckner 2013). To improve our
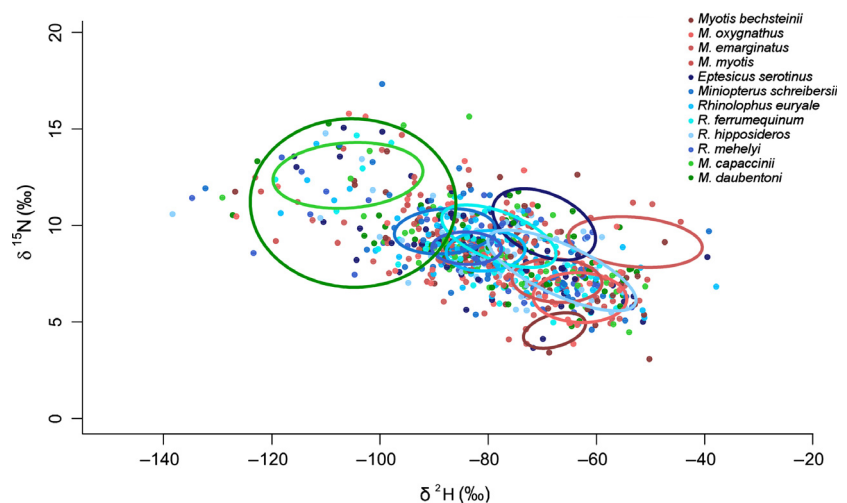

Fig. 3. Nitrogen isotope ratio (δ^{15}N$_K$) in relation to stable isotope ratios of non-exchangeable hydrogen in fur keratin (δ^2H$_K$) of 12 sympatric bat species. Dots indicate individual data points, and species are coded by colour. Ellipsoids mark the relative isotopic niche width for the two elements. Colours code for species according to the legend.

Table 1. Proportional overlap of ellipse areas (SEA_B) for the 12 study species. Data in the upper right triangle code for the relative overlap of SEA_B from the first column species by SEA_B of the species from the top row, and that of the lower left triangle code for the relative overlap of species from the top row by SEA_B of the species from the first column. This means, for example, that Mdau encompasses Mcap 100%, but the whole SEA_B of Mcap only covers 28·3% of Mdaub. Abbreviations code for *Myotis bechsteinii* (Mbec), *Myotis oxygnathus* (Moxy), *M. emarginatus* (Mema), *M. myotis* (Mmyo), *Eptesicus serotinus* (Eser), *Miniopterus schreibersii* (Msch), *Rhinolophus hipposiderus* (Rhip), *Rhinolophus euryale* (Reur), *Rhinolophus ferrumequinum* (Rfer), *Rhinolophus mehelyi* (Rmeh), *Myotis capaccinii* (Mcap), *Myotis daubentonii* (Mdau)

Foraging	Gleaning				Aerial-hawking						Trawling	
	Terrestrial				Mixed						Aquatic	
Food web	Mbech	Moxy	Mema	Mmyo	Eser	Msch	Rhip	Reur	Rfer	Rmeh	Mcap	Mdau
Mbech		14·2	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Moxy	6·2		0·0	43·5	0·0	0·0	0·0	0·0	61·2	0·0	0·0	0·0
Mema	0·0	0·0		0·0	12·4	0·0	0·0	0·0	0·2	0·0	0·0	0·0
Mmyo	0·0	51·9	0·0		0·2	0·0	0·0	5·8	80·6	0·0	0·0	0·0
Eser	0·0	0·0	12·3	0·2		0·0	2·3	26·7	23·2	0·0	0·0	0·0
Msch	0·0	0·0	0·0	0·0	0·0		17·4	42·6	0·5	25·2	0·0	55·1
Rhip	0·0	0·0	0·0	0·0	4·9	24·8		61·6	36·0	54·3	0·0	0·0
Reur	0·0	0·0	0·0	3·9	30·6	33·1	33·5		43·2	17·6	0·0	5·3
Rfer	0·0	35·2	0·2	38·2	19·2	0·3	14·2	31·2		2·7	0·0	0·0
Rmeh	0·0	0·0	0·0	0·0	0·0	50·8	76·5	45·5	9·7		0·0	8·7
Mcap	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0		100
Mdau	0·0	0·0	0·0	0·0	0·0	7·2	0·0	0·9	0·0	0·6	28·3	

understanding of possible differences between δ^2H_K and δ^2H values of consumed water, we performed experiments with captive bats, which showed that bat δ^2H_K values were closer to δ^2H values of food source than to those of water sources. Unfortunately, we were not able to deduce if δ^2H_K values were close to δ^2H values of the organic matrix. Hydrogen of the dietary organic matrix may contribute to δ^2H_K values as well (Hobson, Atewell & Wassenaar 1999).

We then used δ^2H_K values of bats from the aquatic and terrestrial food web to estimate the relative association of aerial-hawking insectivorous bats to these two food webs. We found that all species except *M. schreibersii* were mostly associated with terrestrial food webs, yet to varying degrees. Overlap of isotopic niches was relatively large in the ensemble of the aquatic food web, intermediate in the ensemble of aerial-hawking insectivorous bats and relatively low in the ensemble of gleaning insectivorous bats.

DIFFERENCES IN δ^2H_K BETWEEN BATS OF AQUATIC AND TERRESTRIAL ECOSYSTEMS

Following earlier food web studies (Doucett *et al.* 2009; Soto, Garcia & Catalan 2013; Yang *et al.* 2014), we assumed that differences in δ^2H values between aquatic and terrestrial food webs would translate into corresponding differences of δ^2H_K values in terrestrial vertebrate consumers. Our data reveal differences in δ^2H_K values between members of aquatic and terrestrial food webs, that is fur keratin of gleaning insectivorous bats was more enriched in ^2H compared to aerial-hawking bats and to trawling bats. Comparison of stable isotope ratios between feeding ensembles suggested that differences were strongest between species-rich ensembles, that is we only found a trend for a significant difference between the species poor

gleaning ($n = 4$ species) and trawling ($n = 2$ species) bat ensembles. Further, our experiment with captive bats supports the idea that δ^2H_K values of bats feeding on a terrestrial diet, that is mealworms, are more similar to the body water δ^2H values of their insect diet than to that of offered drinking water. We infer from this pattern that δ^2H_K values of bats with a predominant or exclusive diet of terrestrial insects may show large deviations from δ^2H values of local water. This is consistent with observations of cross-continental surveys that demonstrate an offset between bat δ^2H_K values and local δ^2H values of precipitation water (Cryan *et al.* 2004; Popa-Lisseanu *et al.* 2012; Voigt *et al.* 2012, 2014; Cryan, Stricker & Wunder 2014) and also consistent with previous studies on syntopic, tropical bats (Voigt, Schneeberger & Luckner 2013).

RELATIVE ASSOCIATION OF AERIAL-HAWKING BATS WITH TERRESTRIAL FOOD WEBS

We estimated the proportional association of aerial-hawking bats to terrestrial food webs by relating their δ^2H_K values to reference points from trawling and gleaning bats. The outcome of our mixing model was relatively robust towards varying assumptions about isotopic discrimination. To interpret our results, it is important to consider the natural habitats in which bats of the aerial-hawking ensemble foraged. Our study site consisted of a network of rivers embedded in a system of canyons. Along these rivers, we find gallery forests that stretch up the slopes and are interspersed in parts with open grassland areas, offering a great variety of habitats on a relatively small area. We found that most of the species of the aerial-hawking bat ensemble were more associated with terrestrial than with aquatic food webs. Only *M. schreibersii* obtained about half of its H from aquatic food webs, as indicated by their relatively

low δ^2H_K values. Previous radiotracking studies highlighted the large foraging area of this species (Vincent, Nemoz & Aulagnier 2011) and also the use of rivers for foraging and landmark orientation (Serra-Cobo *et al.* 2000; Russo & Jones 2003). The latter studies imply that *M. schreibersii* may hunt for insects with aquatic life stages, which is consistent with our isotopic data from Bulgaria. Indeed, the colony from which we collected most samples was adjacent to a river and several lakes which are likely to be prime foraging grounds.

Three species of *Rhinolophus* had relatively similar levels of associations of about 60% with terrestrial food webs. Earlier radiotracking studies highlighted that most European rhinolophid species forage in deciduous woodland, which may contradict our isotopic data at first glance (Bontadina, Schofield & Naef-Daenzer 2002; Goiti *et al.* 2003; Russo *et al.* 2005). Yet according to data from our Bulgarian study site, these species of *Rhinolophus* may consume a diet that is partially composed of insect prey from aquatic food webs when foraging adjacent to rivers and lakes (Dietz, Nill & von Helversen 2009). This might be facilitated when insects from aquatic food webs disperse from their primary aquatic habitats into adjacent forests, where they are hunted and consumed by rhinolophid bats. *Rhinolophus mehelyi* seems to prefer open areas, which can also be found along rivers of our study site (Dietz, Nill & von Helversen 2009). Therefore, we consider the isotope data to be congruent with behavioural data from earlier studies. Small, but biologically relevant differences in wing morphology might determine the specific mode and site of foraging for these ecologically similar species (Voigt *et al.* 2010). The fourth and smallest rhinolophid, *R. hipposideros*, showed the strongest association with terrestrial food webs, separating it ecologically from the other three congeneric species. Possibly, the dense population of larger *Rhinolophus* bats may force the smallest species to move to less contested foraging grounds with a lower biomass production (Arlettaz, Godat & Meyer 1994). Presumably, *E. serotinus* foraged opportunistically at relatively high height above ground. Our data shows that most of its diet consists of terrestrial prey, supplemented by aquatic insects that it might catch, for example over lakes.

When interpreting the outcome of our mixing models, it is important to keep in mind that the results depend on the isotopic signature of fur keratin that has grown over the limited period of fur moult, which is usually after the reproductive period for temperate bat species (e.g. *M. myotis*: reviewed by Fraser, Longstaffe & Fenton 2013). Here, we assumed that the period of moult is similar in the studied species because the reproductive period is also similar across the studied species (Dietz, Nill & von Helversen 2009). Yet, we recognize that our proxies for dietary niche, that is the relative association to terrestrial food webs, and the size and overlap of isotopic niches may be different before and after the time of moult.

ISOTOPIC NICHE DIMENSIONS OF BATS

The comparison of standardized ellipse areas as a proxy for the isotopic niche of a bat species revealed interesting patterns within and across feeding ensembles. The isotopic niches of the two trawling bat species overlapped largely. The strong overlap in isotopic niches of *M. capaccinii* and *M. daubentonii* suggests a strong competition of the two trawling species in our study area. This competition might be mitigated by a spatial segregation between a set of small aquatic habitats. Previous studies suggested that *M. daubentonii* is a flexible, generalist species and *M. capaccinii* to be more specialized (Dietz, Nill & von Helversen 2009; Krüger *et al.* 2014). However, sample size for *M. daubentonii* was low and even though the analysis incorporated a correction for low sample size, inferences from the analysis should be done with caution. Isotopic niches of three aerial-hawking insectivorous bats (*R. mehelyi*, *R. euryale*, and *M. schreibersii*) overlapped partially with that of trawling bats, suggesting some sharing of food items from aquatic food webs.

The ensemble of aerial-hawking insectivorous bats included the largest number of species in our study. Two species pairs showed exclusive isotopic niches, that is *E. serotinus* neither overlapped with *M. schreibersii* nor with *R. mehelyi*. Eighty-seven per cent of dyadic tests (13 out of 15 possible combinations) revealed some overlap of isotopic niches, which suggests that most aerial-hawking bats may feed opportunistically on available insects and may therefore overlap in their dietary niche. In the ensemble of gleaning bats, 33% of pairwise comparisons suggested an isotopic overlap. The strongest overlap was observed between the sibling species *M. oxygnathus* and *M. myotis*. Although hunting in similar terrestrial habitats, earlier studies suggested that these two species differ in their trophic position. *Myotis oxygnathus*, a predominant forager of phytophagous orthopterans, feeds on a lower trophic level than *M. myotis*, a predominant forager of omnivorous carabid beetles (Arlettaz 1999; Siemers *et al.* 2011). These two gleaning bat species also showed a relatively large overlap with the aerial-hawking bat *R. ferrumequinum*, the heaviest of all local *Rhinolophus* species, which may also feed on large bodied insects such as beetles. Although *R. ferrumequinum* shows similar habitat associations like *R. mehelyi* and *R. euryale*, its larger isotopic niche dimensions support the wider variety of consumed prey in contrast to the two middle-sized horseshoe bats which are more specialized on moths (Dietz, Nill & von Helversen 2009).

IMPLICATIONS FOR ECOLOGICAL STUDIES

Our study demonstrates a difference in δ^2H_K values between bats of aquatic and terrestrial food webs. We argue that such differences could as well be found in other mammals and possibly also in other taxonomic groups such as birds. Our study also highlights a novel approach in using δ^2H_K values for assessing the relative association of mammals to aquatic and terrestrial food webs. Possibly, δ^2H values of a non-keratin matrix may also be suitable as a proxy for assessing the relative association of consumers to aquatic or terrestrial food webs. However, the potential of this element may not be fully understood before technical solutions have been established to control for the effect of exchangeable hydrogen in non-keratinous material (Meier-Augenstein, Hobson & Wassenaar 2013).

In previous studies that used an isotopic approach in predicting the breeding origin of migratory animals, authors reported variations in δ^2H_K values of birds and bats that were not explained by δ^2H values of local precipitation water (Fraser *et al.* 2011; Voigt, Schneeberger & Luckner 2013). This noise seemed to be intrinsic to the isotopic approach and was previously explained by small-scale variations in δ^2H values of open water. Our study highlights that individuals of the same species may vary in their relative association with aquatic or terrestrial food webs, which may contribute significantly to the overall variation of δ^2H_K values in local bird or bat populations. We speculate that the slope of the regressional equation of the bivariate relationship between δ^2H_K and δ^2H values of precipitation water may deviate from 1, if the relative association of bird or bat consumers with aquatic and terrestrial food webs may vary systematically across continents (e.g. from shorelines to inland habitats), latitudes or season. Lastly, we emphasize that the application of δ^2H_K in non-migratory mammals and birds will provide a powerful addition to the isotopic toolbox of ecologists to better understand the ecology of animals, nutrient fluxes, trophic interactions and ecological networks in ecotones of aquatic and terrestrial ecosystems.

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Data accessibility

Data deposited in the Dryad repository http://datadryad.org/resource/doi:10.5061/dryad.43dt0 (Voigt, Lehmann & Greif 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Methods.

Table S1. Mean stable hydrogen ($\delta^2 H_K$), carbon ($\delta^{13}C_K$) and nitrogen isotope ratios ($\delta^{15}N_K$; mean $\pm$ 1 standard deviation = SD) of gleaning, aerial-hawking and trawling bat species.

Table S2. Relative association of species in the feeding ensemble of aerial-hawking bats with terrestrial food webs calculated using a Bayesian mixing model according to Parnell and Jackson (2013).

Table S3. Metrics of standard ellipse areas ($\permil^2$) of bats corrected for sample size (SEA$_B$) and uncorrected median SEA for isotopic data of species belonging to the three different feeding ensembles.