



## Quantifying niche partitioning and multichannel feeding among tree squirrels

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### ARTICLE INFO

#### Article history:

Received 16 September 2019

Received in revised form 30 September 2019

Accepted 30 September 2019

Available online xxxx

#### Keywords:

Autotroph

Carbon

Detritus

Isotopic fingerprinting

Nitrogen

Sciuridae

### ABSTRACT

Quantifying resource partitioning is central to community and food web ecology and of increasing interest in an era of rapid global change disrupting biotic interactions. Multichannel feeding – consuming resources from both green and brown food webs – can be a stabilizing force in communities. While multichannel feeding has been well-documented in invertebrate and aquatic systems, it has been relatively under-studied in terrestrial vertebrate populations. Applied ecologists are seeking approaches to assess niche partitioning and cryptic trophic pathways, like multichannel feeding, which have been difficult to quantify, especially among vertebrates. Using both bulk ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and compound specific stable isotope ratios ( $\delta^{15}\text{N}$  glutamic acid and phenylalanine), we tested how three common and competing tree squirrel partition resources. Our complementary analyses revealed that squirrels partitioned niche space and, because of differences in multichannel foraging, possessed different trophic identities. While all squirrels consumed food items from green and brown food webs, their dependence on each differed, revealing an important, yet cryptic, mechanism behind apparent stable co-occurrence of these competitors. Our work supports multichannel feeding as a potential mechanism promoting coexistence in this guild of terrestrial vertebrates, and provides a framework to quantify resource partitioning in other ecological communities.

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### 1. Introduction

Competition is one of the greatest forces structuring ecological communities and driving evolutionary change (Hardin, 1960). Interspecific competition reduces niche overlap among competing species, drives ecological divergence, and structures food webs (MacArthur and Levins, 1967). Rapid loss of biodiversity is now disrupting vertebrate assemblages globally (Estes et al., 2011); resultant novel communities can be characterized by reshaped biotic interactions with important consequences for overall trophic structure and ecosystem processes (Galetti and Dirzo, 2013; Radeloff et al., 2015). Consequently, quantifying how community members partition dietary niche space and trophic position (TP) is of interest, not only to ecologists and evolutionary biologists, but also to conservation biologists, in predicting how ecosystems will respond to rapid global changes (Van der Putten et al., 2010; Diaz et al.,

2013). In particular, there is growing interest in consumers that utilize both green food web pathways (based on primary producers) and brown web pathways (based on decomposers), and especially how this “multichannel feeding” affects community and ecosystem stability (Wolkovich et al., 2014; Zou et al., 2016). It is predicted that multichannel feeding should be common (Polis and Strong, 1996), but empirical evidence is largely drawn from aquatic (e.g., Lau et al., 2014) and invertebrate (e.g., Perkins et al., 2017) communities.

Sciurids are one of the most diversified (292 extant species) and widely distributed (across 5 continents) families of mammals (Kaprowski et al., 2016). Understanding this group’s rapid diversification (Mercer and Roth, 2003), especially via ecological factors like competition and niche partitioning (Rocha et al., 2016), has garnered much attention. In Holarctic forests, three common and synoptic competitors – grey (*Sciurus carolinensis*), red (*Tamiasciurus hudsonicus*) and flying (*Glaucomys sabrinus*) squirrels (Ivan and Swihart, 2000) – occupy unique ecological roles. They are one of the few mammals to harvest, hoard, consume and disperse both seeds from trees and spores from

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fungi, thereby exerting strong ecological and evolutionary effects on forest ecosystems (e.g., Parchman and Benkman, 2007; Johnson, 1996; Goheen and Swihart, 2003). These competing tree squirrels also appear to occupy distinct niches, at least in terms of fine-scale habitat selection (Riege, 1991). Studies on the feeding habitats of tree squirrels have focused on stomach or fecal contents, or direct observation, which only provides a brief snapshot of resource consumption and can miss important, but cryptic, resources. In general, grey squirrels principally depend on the reproductive tissues from deciduous trees, especially calorically rich mast, which they scatter-hoard and consume throughout the year (Korschgen, 1981). Flying squirrels are primarily fungivores, specializing on the fruiting bodies of hypogeous fungi, but will also consume mast, lichen and animal matter (Flaherty et al., 2010). Red squirrels consume mast, especially from conifers, and larder-hoard thousands of cones in middens (Vernes et al., 2004). Red squirrels also supplement their diet with animal matter, especially birds, and will switch to consuming, and even hoarding, fungi when cones are scarce (Fletcher et al., 2010). Thus, to some degree, it appears these three species partitioning resources, although direct quantification of TP and assessment of dietary partitioning do not exist.

Stable isotope analysis is often used in foraging ecology to estimate dietary niche characteristics and TP (Boecklen et al., 2011). In particular, the simultaneous analysis of bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measures the assimilation of resources in consumer tissues and enables the estimation of proportional dietary inputs and niche overlap (Newsome et al., 2007; Manlick et al., 2019). Further, due to the enrichment of  $^{15}\text{N}$  up the food-chain, bulk  $\delta^{15}\text{N}$  values are frequently used to predict the TP of consumers and construct food webs. However, variation in isotopic baselines and the discrimination of nitrogen during trophic transfers can lead to flawed estimates of TP (Vander Zanden and Rasmussen, 1999; Post, 2002). Compound specific isotope analysis of individual amino acids, especially glutamic acid (i.e., a “trophic” amino acid) and phenylalanine (i.e., a “source” amino acid; Chikaraishi et al., 2007, 2009), can provide more accurate TP estimates compared to bulk methods (Steffan et al., 2013; Bowes and Thorp, 2015; Blanke et al., 2018). Given that heterotrophic microbes are trophically indistinguishable from animals (Steffan et al., 2015), the consumption of microbe-rich detrital complexes increases the TP of the consumer predictably (Steffan et al., 2017); consequently, compound specific approaches also enable the quantification of microbivory within a food chain. While compound specific TP estimates have been validated via controlled feeding trials (Steffan et al., 2013, 2015), and applied to invertebrates (Steffan et al., 2017) and marine systems (Chikaraishi et al., 2009), they rarely have been used for free-ranging terrestrial vertebrates (although see Campbell et al., 2017).

Herein, we analyzed bulk and compound specific stable isotopes for a guild of free-ranging vertebrates to test how grey, red and flying squirrels partition trophic niche space in forests of North America. Specifically, we explored how the role of multi-channel feeding could lead to dietary and trophic partitioning and act as a potential mechanism for their stable co-occurrence. We hypothesized that one mechanism in which these species limit competitive overlap is along a trophic axis. Based on previous work, we predicted that grey squirrels would primarily be mast eaters (Korschgen, 1981), and thus occupy  $\text{TP} = 2$  (i.e., herbivorous); we predicted that flying squirrels consume mast and fungi, while red squirrels rely upon mast, fungi, and birds (Layne, 1954; Vernes et al., 2004). Because fungi and animal matter should be trophically elevated above autotrophs (i.e.,  $\text{TP} = 2$ ), we predicted both flying and red squirrels would feed at  $\text{TP} > 2$ , but that the source of this TP enrichment would differ, with flying squirrels more reliant on fungi, and red squirrels more dependent on animal protein.

## 2. Methods

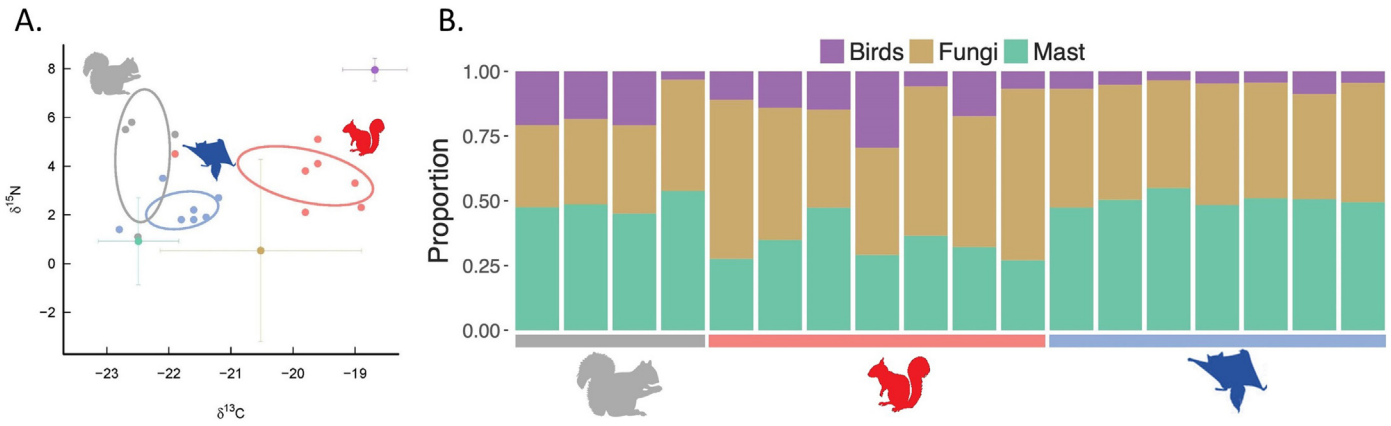
We collected samples from the Chequamegon-Nicolet National Forest of northern Wisconsin, USA in May 2017. The region is characterized

by mixed hardwood and coniferous forests. We captured and anesthetized squirrels, biopsied a small tissue sample (<5 mg) from the ear, and collected a hair sample, before releasing the animals (grey squirrels,  $n = 4$ ; red squirrels,  $n = 7$ ; flying squirrels,  $n = 7$ ). To characterize potential diet sources, we collected mast from five conifer species (white pine [*Pinus strobus*,  $n = 3$ ], red pine [*Pinus resinosa*,  $n = 2$ ], eastern hemlock [*Tsuga canadensis*,  $n = 3$ ], balsam fir [*Abies balsamea*,  $n = 1$ ], and white spruce [*Picea glauca*,  $n = 5$ ]), and acorns from oak trees (*Quercus* spp.,  $n = 12$ ), as well as common fungi in and around squirrel caches of mast: artist's fungus (*Ganoderma applanatum*,  $n = 2$ ); common earthball (*Scleroderma citrinum*,  $n = 1$ ); mossy maze polypore (*Cerrena unicolor*,  $n = 2$ ); red belt conk (*Fomitopsis pinicola*,  $n = 1$ ); violet-toothed polypore (*Trichaptum bifforme*,  $n = 4$ ). Because squirrels, especially red squirrels, are known predators of bird eggs, nestlings and even adults (Layne, 1954), and can be important predators of birds' nests in the spring and summer (Wilson et al., 2003) we collected feathers (Passeriformes,  $n = 4$ ) to represent the consumption of this animal matter.

We prepared all samples for bulk analyses following standard techniques (Pauli et al., 2009; Kirby et al., 2017) and analyzed  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values using a Costech 4010 and Carlo Erba 1110 Elemental Analyzer (Costech, Valencia, CA) attached to a Thermo Finnigan Delta PLUS XP Continuous Flow Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). We analyzed a subset of samples with compound specific approaches of white spruce seeds ( $n = 2$ ), oak acorns ( $n = 2$ ), and *Ganoderma applanatum* fungi ( $n = 2$ ), in addition to the three focal consumer species, grey squirrels ( $n = 4$ ), red squirrels ( $n = 6$ ), and flying squirrels ( $n = 5$ ). The  $\delta^{15}\text{N}$  values of glutamic acid and phenylalanine were analyzed using established protocols (Chikaraishi et al., 2007, 2009). Briefly, samples were hydrolyzed, and the hydrolysate was washed with n-hexane/dichloromethane. Derivatization of amino acids was performed sequentially with thionyl chloride/2-propanol (1/4, v/v) and pivaloyl chloride/dichloromethane (1/4, v/v). Gas chromatography (GC) using a 6890N GC connected to a flame ionization detector and nitrogen-phosphorus detector was used to determine amino acid abundance. The Pv/iPr derivatives of amino acids were injected using a programmable temperature vaporizing injector (Gerstel) into a HP-5 ms capillary column (Agilent Technologies). Stable nitrogen isotopic composition of amino acids was determined by GC-combustion-isotope ratio mass spectrometry (GC/C/IRMS) using a 6890N GC (Agilent Technologies) instrument coupled to a DeltaplusXP IRMS instrument through combustion (950 °C) and reduction (550 °C) furnaces, countercurrent dryer (Permeable membrane, Nafion™), and liquid nitrogen CO<sub>2</sub> trap via a GC-C/TC III interface (Thermo Fisher Scientific).

We used bulk isotope compositions for each squirrel species to estimate niche breadth and pairwise niche overlap via standard ellipses corrected for small sample size (SEA<sub>c</sub>; Jackson et al., 2011), and used a multi-response permutation procedure with 1000 iterations to test for significant differences among squirrels in  $\delta$ -space (Manlick et al., 2019). To estimate the proportional diets of squirrels, we identified three isotopically distinct dietary groups (all pairwise  $P < 0.05$ ) – birds, mast, and fungi – via a K nearest-neighbor randomization test (Rosing et al., 1998). We estimated dietary contributions of each prey group to individuals (function siarsolomcmcv4) and populations (function siarmcmcdirichletv4) using Bayesian-based isotopic mixing models in SIAR (Parnell et al., 2010). All models incorporated uniform prior distributions, concentration dependence using the mean elemental concentrations for each prey group, and were corrected for trophic enrichment of squirrels ( $\delta^{13}\text{C} = 3.3 \pm 1.0\%$ ;  $\delta^{15}\text{N} = 1.9 \pm 1.0\%$ ; Hobbie et al., 2017). Each model ran 200,000 iterations, with an additional 25% burn-in, and was sampled 10,000 times.

We estimated TP for white spruce seeds, oak acorns, fungi, and the three squirrel species based on the  $^{15}\text{N}$  values of glutamic acid



**Fig. 1.** A. Bulk tissue isotope signatures ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ), standard ellipses (SEAC; solid lines) and, B. proportional diets, for individual grey (*Sciurus carolinensis*; grey;  $n = 4$ ), flying (*Glaucomys sabrinus*; blue;  $n = 7$ ), and red squirrels (*Tamiasciurus hudsonicus*; red;  $n = 7$ ) from Wisconsin, USA. Potential squirrel diet items ( $\pm$ SD), birds (purple), mast (green), and fungi (brown), are also shown.

( $\delta^{15}\text{N}_{\text{glu}}$ ) and phenylalanine ( $\delta^{15}\text{N}_{\text{phe}}$ ) using the equation (Chikaraishi et al., 2009):

$$TP = \frac{\delta^{15}\text{N}_{\text{glu}} - \delta^{15}\text{N}_{\text{phe}} + \beta}{\Delta_{\text{glu-phe}}} + \lambda \quad (1)$$

where  $\beta$  (~8.4‰) corrects for the difference in  $^{15}\text{N}$  values between glutamic acid and phenylalanine in  $\text{C}_3$  plants;  $\Delta_{\text{glu-phe}}$  represents the net trophic discrimination between glutamic acid and phenylalanine ( $=7.2$ ; Steffan et al., 2015, 2017); and  $\lambda$  represents the basal trophic level ( $=1$ ).

### 3. Results

Isotopic niche sizes, as based on bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , were comparable between grey ( $\text{SEA}_C = 3.8$ ) and red squirrels ( $\text{SEA}_C = 3.7$ ), but smaller for flying squirrels ( $\text{SEA}_C = 1.4$ ; Fig. 1A). We observed differences between red and grey squirrels ( $P = 0.008$ ), red and flying squirrels ( $P = 0.001$ ) and little overlap ( $\text{SEA}_C$  overlap = 0.1;  $P = 0.02$ ) between grey and flying squirrels (Fig. 1A). Population- (Table 1) and individual-level (Fig. 1B) dietary estimates suggested that, while all squirrel species consumed all three sources, their proportional dependence on each appeared to differ: in general grey squirrels primarily consumed mast and secondarily fungi and animal matter, flying squirrels split their diet between mast and fungi, while red squirrels foraged on mast, fungi and animal matter (Table 1; Fig. 1B).

Compound specific analyses of  $\delta^{15}\text{N}_{\text{glu}}$  and  $\delta^{15}\text{N}_{\text{phe}}$ , revealed relatively discrete trophic partitioning across consumer groups ( $F_{4, 16} = 60.6, P < 0.001$ ; Fig. 2). Regressing individual values of  $\delta^{15}\text{N}_{\text{glu}}$  and  $\delta^{15}\text{N}_{\text{phe}}$  (Eq. (2); Fig. S1), we estimated the average TP ( $\pm$  1 SD) for oak acorns and pine nuts of  $TP = 1.1 (\pm 0.12)$ , fungi  $TP = 2.0 (\pm 0.04)$ , grey squirrels  $TP = 2.1 (\pm 0.01)$ , and red and flying squirrels with most elevated at  $TP = 2.5 (\pm 0.19)$  and  $TP = 2.5 (\pm 0.20)$ , respectively (Fig. 2).

### 4. Discussion

Our complementary analyses of bulk and compound specific stable isotopes revealed that three common squirrels of forests across North America appeared to partition dietary resources and, especially trophic position, leading to niche partitioning. In particular, bulk isotopic estimates of stable isotopes showed that the relative dependence of tree squirrels on broad categories of food items differed. Grey squirrels predominantly consumed mast but augmented their diet with fungi and animal protein, red squirrels consumed three dietary pools more evenly, and flying squirrels were primarily mycophagous and granivorous. It is worth noting, however, that the credibility intervals around our bulk analyses of consumption at the population level were broad, and overlapping. The fact that our isotopically distinct food items were coarse in nature, each dietary group encompassed multiple species of potential prey, and limited in sample size likely contributed to this observed overlap. Future research exploring the importance of species specific resource consumption and fine-scale resource partitioning might reveal greater dietary partitioning among these three competing species. Previous work on this guild shows a high degree of niche overlap, which has led to questions on the mechanism behind their apparent stable co-occurrence throughout much of their distributional ranges (Ivan and Swihart, 2000). Our work reveals that while niche overlap exists, differences in foraging strategies, especially relative reliance on green and brown food webs, may contribute to sustaining these competing species.

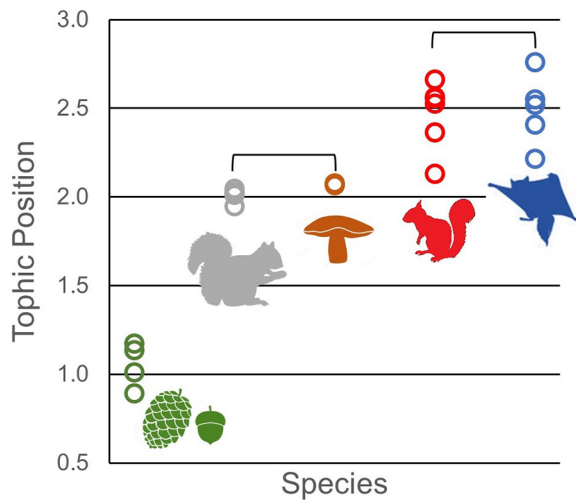
Differences in TP, as suggested by isotope data, also suggested resource partitioning. Estimates derived from compound specific analysis of isotopes fell predictably and with basic understanding of their ecology. Flying squirrels and red squirrels exhibited the highest TP, consistently  $>2$ . Flying squirrels are well known mycophages, but also consume mast (Flaherty et al., 2010; Vernes et al., 2004). Red squirrels are larder-hoarders, especially of spruce and pine seeds, and cache cones in “middens” piles which create a warm and humid microenvironment underground. In years of mast failure, red squirrels consume cones hoarded within middens and even stockpile fungi (Fletcher et al., 2010). Their consumption of fungi is not only

**Table 1**

Population-level dietary estimates (mean, 95% credibility interval) of grey squirrels (*Sciurus carolinensis*), red squirrels (*Tamiasciurus hudsonicus*) and flying squirrels (*Glaucomys sabrinus*) on mast (oak acorns and pine nuts), fungi and birds using bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

Species	Mast	Fungi	Bird
Grey squirrel	0.576 (0.260–0.910)	0.300 (0–0.589)	0.124 (<0.001–0.280)
Red squirrel	0.285 (<0.001–0.579)	0.608 (0.305–0.902)	0.106 (0.014–0.235)
Flying squirrel	0.608 (0.357–0.872)	0.357 (0.098–0.603)	0.035 (<0.001–0.062)





**Fig. 2.** Trophic position estimated via the analysis of  $\delta^{15}\text{N}$  in glutamic acid and phenylalanine and (using Eq. (1)) of white spruce seeds (*Picea glauca*), red oak acorns (*Quercus rubra*), fungi (*Ganoderma applanatum*), grey squirrels (*Sciurus carolinensis*), red squirrels (*Tamiasciurus hudsonicus*) and flying squirrels (*Glaucomys sabrinus*) from Wisconsin, USA. All pairwise comparisons were significant ( $P < 0.05$ ), except between grey squirrels and fungi, and between red and flying squirrels (denoted with brackets).

from mushrooms, but also likely through the consumption of cached cones that support fungal growth (Smith, 1968; Vernes et al., 2004). Thus, traditional approaches may underestimate the degree of mycophagy of red squirrels, as these techniques fail to account for squirrels' consumption – incidental or intentional – of fungi growing on cached cones within middens.

Our data suggests that multichannel feeding through consumption of resources from both green and brown food webs enables partitioning of resources for co-occurrence and suggests that these three species likely possess different functional roles – e.g., as seed predators and spore dispersers – in their forest ecosystem. Further, these results reinforce the growing body of literature utilizing  $\delta^{15}\text{N}_{\text{glu}}$  and  $\delta^{15}\text{N}_{\text{phe}}$  for the estimation of TP across diverse food webs, and exploring the interdigitization of brown and green food webs (Steffan et al., 2015, 2017). Fungi, in particular, represent exceedingly common heterotrophic organisms within terrestrial ecosystems (Moore and de Ruiter, 2012), and register predictably within trophic hierarchies (Steffan et al., 2015; Steffan and Dharampal, 2018). When animals feed on fungal 'meat' (Steffan et al., 2015) the consumers exhibit trophic inflation (elevation of trophic position), given that their diet contains multiple trophic groups (Steffan et al., 2017). This phenomenon appears to be exemplified in the red and flying squirrels.

While the analysis of bulk isotopes was suitable in the estimation of population- and individual-level diets, it was not for the quantification of TP. Indeed, relying solely on bulk  $\delta^{15}\text{N}$  would have led us to the incorrect conclusion that grey squirrels possessed the highest TP and flying squirrels the lowest (Fig. S2). It is also notable that, we observed a strong negative correlation between mast and fungi, as the dietary mixing models struggled to partition the importance of mast versus fungi consumption. This is likely why our dietary models assigned more mast consumption to flying squirrels, while compound specific placed their TP higher, suggesting a greater dependence on fungi. Although variation with compound specific discrimination factors, especially around protein content and routing of nitrogenous excretion have been noted, these are more apparent in aquatic organisms than in terrestrial ones (Ohkouchi et al., 2017). A combination of bulk and compound specific stable isotope analyses provides a useful framework for researchers to simultaneously quantify the diets, niches, trophic partitioning, and degree of multichannel feeding of organisms – from microbes to vertebrates – across ecological communities.

## Declaration of competing interest

None.

## Acknowledgements

We thank S. Petersen for assistance in the laboratory and the U.S. Forest Service for access to conduct fieldwork. This work was supported by the Graduate School at the University of Wisconsin-Madison. Our study was approved by the University of Wisconsin Institutional Care and Use Committee (A005524).

## Appendix A. Supplementary data and analyses

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodweb.2019.e00124>.

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