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The role of seed attributes in eastern gray squirrel foraging

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THE ROLE OF SEED ATTRIBUTES IN EASTERN GRAY SQUIRREL FORAGING

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by

Mekala Sundaram

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of

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For my parents, ‘a Pretty Heavy Dissertation’

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ABSTRACT

Sundaram, Mekala. PhD, Purdue University, December 2016. The Role of Seed Attributes in Eastern Gray Squirrel Foraging. Major Professor: Robert Swihart.

Seed attributes are important predictors of rodent foraging behaviors. I examined the role of seed attributes in eastern gray squirrel (*Sciurus carolinensis*) foraging behavior from an evolutionary, economic, ecological and biochemical perspective. From an evolutionary perspective (chapter 2), I found that squirrel foraging behaviors are influenced by a combination of phylogenetically conserved and evolutionarily labile seed traits, which supports a diffuse coevolutionary relationship between hardwood trees and squirrels and provides indirect evidence supporting the Janzen-Connell and handling time hypotheses. From an economic perspective (chapter 3), I found that eastern gray squirrels are homogenous with respect to their preferences for seed attributes, which is likely due to natural selection favoring caching of specific seeds in the fall. I also provide evidence that squirrels trade between 3 attributes when selecting seeds for caching, which results in a variety of seed types being cached. In contrast, squirrels trade between 2 attributes when selecting seeds for consumption, which leads to fewer seed types being consumed in the fall. From an ecological context perspective (chapter 4), I provide evidence of seed traits interacting with relative seed availability to predict caching. Specifically, when

seeds of different caching value (i.e., utility) were paired, relative frequency of availability played a minimal role in predicting seed caching. In contrast when seeds of similar caching utility were paired, relative frequency of availability significantly influenced probability of seed selection for caching. From a biochemical perspective (chapter 5), I identified biochemical and anatomical changes at the cellular level associated with radicle dormancy that serve as a signal of lack of dormancy to eastern gray squirrels. In combination, my dissertation chapters support the existence of complex reciprocal evolutionary effects between hardwood trees and eastern gray squirrels.

CHAPTER 1. OVERVIEW OF DISSERTATION RESEARCH

1.1 Introduction

Animals such as the eastern gray squirrel (*Sciurus carolinensis*) influence tree regeneration processes in two ways that have opposing effects on a tree's fitness (Vander Wall 1990). Firstly, by consuming large proportions of seeds, granivores can reduce numbers of seeds available for recruitment (Chambers and Macmahon 1994). Alternatively, scatter hoarding rodents may disperse seeds horizontally away from the parent tree and bury seeds in caches, thereby enhancing chances of germination for unrecovered seeds (Chambers and Macmahon 1994). Both predation and dispersal behaviors of scatter hoarding rodents such as eastern gray squirrels are influenced by traits of seeds (Wang and Chen 2008; Wang et al. 2012; Sundaram et al. 2015). Therefore, understanding seed-trait-based preferences of granivores is important because of potential ramifications on seed mortality and dispersal (Wang and Smith 2002; Wang et al. 2012).

Both seed removal and seed caching are processes influenced by intrinsic traits of seeds (Xiao and Zhang 2006; Wang et al. 2012). On encountering a seed, scatter hoarding rodents likely evaluate quality and condition (Preston and Jacobs 2009; Delgado et al. 2014). Seeds germinating immediately or seeds with a lower mass are of a low storage value and tend to be consumed immediately and carried shorter distances (Smallwood et al. 2001; Sundaram et al. 2015). In contrast, seeds of higher storage value are dispersed and cached at greater distances (Sundaram et al. 2015). Although the role of seed traits in rodent foraging behaviors has been examined in several systems (Xiao and Zhang 2006; Wang and Chen 2008; Gong et al. 2014), some challenges to understanding the complex interactions still exist.

One challenge stems from the fact that seed traits estimated across different tree species are not independent of one another; common ancestry likely results in related species that possess suites of similar seed traits. In chapter two, I quantitatively combined seed trait estimates, phylogenetic relatedness of hardwood tree species and squirrel foraging behaviors to address two questions: 1. Do groups of hardwood tree species exhibit similar suites of traits? 2. Is squirrel behavior predicted by phylogenetically conserved seed traits and thus consistent with coevolution? I found that groups of hardwood tree species do exhibit similar suites of traits and specific strategies in assembling seeds (Sundaram et al. 2015). Further, covarying suites of phylogenetically conserved and evolutionary labile traits predict as much as 70% of squirrel consumption and dispersal behaviors suggesting coevolution. Studies of herbivory have suggested that plants often evolve suites of defensive traits that covary across species due to shared ancestry and adaptive convergence (Agarwal and Fishbein 2006). These covarying suites of traits evolve as a result of plant ecological interactions with herbivores and are referred to as ‘plant defense syndromes’ (Agarwal and Fishbein 2006). My first dissertation chapter offers evidence of the existence of ‘seed dispersal syndromes’ where species groups have evolved suites of seed traits that play a role in influencing rodent foraging behaviors and, thus seed dispersal.

Strong correlations between seed traits results in a second challenge of parsing out the effects of individual traits on seed selection by granivores (Xiao and Zhang 2006; Wang et al. 2012). In chapter three, I demonstrated a method to design and conduct ‘choice experiments’ wherein squirrels are presented with pre-determined seed pairs selected for their ability to parse out the effects of individual correlated seed traits on seed selection (Sundaram et al., in prep). More specifically, I created a design consisting of 10 seed pairs that could segregate the effects of protein concentration, tannin content, kernel mass and toughness of shell. After analyzing the resulting seed selection events with discrete choice models, I found that kernel mass, shell toughness and tannin concentration predicted selection for caching whereas dormancy period and toughness predicted selection for consumption. Further, I found that selection of seed traits by squirrels was consistent across locations and individual squirrels. This result is likely due to strong selective forces associated with maintaining cached resources. Moreover, the number of different seed types cached in the fall was high, probably due to the fact that squirrels traded between 3 different seed traits when caching. In contrast, squirrels traded between 2 traits when selecting seeds for consumption, which likely results in fewer numbers of seed types being consumed in the fall. The methods I developed provide a means of linking correlated traits to resource selection by animals; in this case, seed traits to desirability for caching or desirability for consumption by squirrels. Therefore, seeds can be ranked in order of caching or consumption preference. These comparisons, however, only hold when one of each seed type is available to a squirrel.

In contrast to seed traits, the role of relative frequency of seed types on seed selection by rodents is less well studied. In chapter four, I conducted controlled captive experiments to test whether seed traits and relative frequency of seed types interact to influence seed selections by squirrels (Sundaram et al., in press). Using rankings of caching preference developed in my second chapter, I presented the following seed pairs at varying frequencies to captive squirrels: 1. Seeds of the highly preferred black walnut (*Juglans nigra*) and seeds of the less preferred American hazelnut (*Corylus americana*), 2. Seeds of the similarly preferred Chinese chestnut (*Castanea mollissima*) and English walnut (*Juglans regia*). Consistent with predictions, I found that at all frequencies of presentation, black walnut was selected over American hazelnut for caching by squirrels. This is due to the strong preference for caching seeds such as walnuts that are characterized by large kernel mass and a tough shell. In contrast, when similarly preferred Chinese chestnut and English walnut were paired, relative frequency of availability predicted seed selection by squirrels in a negative frequency-dependent manner such that the rarer seed type was selected at a higher rate than would be expected based on frequency of availability. I also found evidence that pre-existing cached resources influence seed selection when seeds of equal preference were paired. The results can be extended to predict primary seed dispersal in different ecological conditions. Specifically, probability of selection for caching by squirrels and survival of a seed type can be predicted depending on which seed types (and seed traits) exist within an area of interest, and the relative abundance (masting patterns) of each type. Finally, the negative frequency-dependent caching likely stabilizes prey or tree diversity and supports assertions of eastern gray squirrel being a keystone seed disperser (Steele et al. 2004).

The role of odors, although important in many predator-prey systems (Hegab et al. 2015; Sentis et al. 2015) has been studied to a limited extent in rodent foraging (Vander Wall 2010). Eastern gray squirrels are sensitive to chemical cues in the pericarp of acorns (Steele et al. 2001). Specifically, squirrels tend to consume dormant acorns with pericarps washed in acetone, dormant acorns modified by replacing pericarps with germinating seeds' pericarps and germinating acorns (Steele et al. 2001). Because dormant seeds are typically cached, these results suggest that breaking of dormancy is accompanied by a chemical change in the pericarp, which signals perishability of a seed and leads to seed consumption. From comparisons of Gas Chromatography-Mass Spectrometry (GC-MS) chromatograms of dormant and germinating oak and chestnuts, I determined that pericarps of dormant oaks have a wax coating composed of long chain fatty acids and fatty acid esters that are broken down during the process of stratification. The loss of waxes was confirmed by visualizing shell structure under a scanning electron microscope. Additionally, compounds found in the kernel were detected on shells of germinating chestnut and acorns but not dormant seeds. Finally, the field trials suggest that occurrence of kernel compounds on the shell induces seed consumption behavior in eastern gray squirrels. In addition, loss of wax on scraped seeds and occurrence of metabolic byproducts such as acetaldehyde significantly influenced squirrel foraging behaviors. From these results, I concluded that germinating seeds have degraded waxes occurring on the shell, which potentially leads to seeds emitting lower molecular weight kernel compounds and byproducts. Squirrels likely cue in on the physical loss of wax and release of chemical odorous compounds to detect lack of dormancy.

In combination, my dissertation chapters suggest that reciprocal evolutionary interactions exist between eastern gray squirrels and hardwood trees species. The hardwood tree species examined in my second chapter appear to have evolved specific suites of traits (or dispersal syndromes) that influence squirrel foraging behaviors. I examined the proximate mechanisms involved in the influence of a particular seed trait (dormancy period) on squirrel foraging in my fifth chapter. In my third and fourth chapters, I examined the reciprocal effect; i.e. how squirrels influence seeds and seed traits. I found that squirrels consistently select specific seed traits when caching seeds in the fall. Further, the willingness to trade between multiple traits allows a large variety of seeds to be cached. If relative frequencies of seeds are altered and seeds available are of equal caching value, then negative frequency-dependent caching by squirrels can stabilize tree diversity. These reciprocal interactions between squirrels and hardwood trees probably have important fitness consequences and suggest coevolution. However, at most 50% of the variation in squirrel behavior is predicted by hardwood tree phylogeny (Sundaram et al. 2015). Therefore, this evolutionary relationship is likely to be of a weak or diffuse nature.

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CHAPTER 2. SEGREGATING THE EFFECTS OF SEED TRAITS AND COMMON ANCESTRY OF HARDWOOD TREES ON EASTERN GRAY SQUIRREL FORAGING DECISIONS

Mekala Sundaram, Janna R. Willoughby, Nathanael I. Lichti, Michael A. Steele, and Robert K. Swihart. 2015. Plos One 10:e0130942.

2.1 Abstract

The evolution of specific seed traits in scatter-hoarded tree species often has been attributed to granivore foraging behavior. However, the degree to which foraging investments and seed traits correlate with phylogenetic relationships among trees remains unexplored. I presented seeds of 23 different hardwood tree species (families Betulaceae, Fagaceae, Juglandaceae) to eastern gray squirrels (*Sciurus carolinensis*), and measured the time and distance travelled by squirrels that consumed or cached each seed. I estimated 11 physical and chemical seed traits for each species, and the phylogenetic relationships between the 23 hardwood trees. Variance partitioning revealed that considerable variation in foraging investment was attributable to seed traits alone (27–73%), and combined effects of seed traits and phylogeny of hardwood trees (5–55%). A phylogenetic PCA (pPCA) on seed traits and tree phylogeny resulted in 2 "global" axes of traits that were phylogenetically autocorrelated at the family and genus level and a third "local" axis in which traits were not phylogenetically autocorrelated. Collectively, these axes explained 30–76% of the variation in squirrel foraging investments. The first

global pPCA axis, which produced large scores for seed species with thin shells, low lipid and high carbohydrate content, was negatively related to time to consume and cache seeds and travel distance to cache. The second global pPCA axis, which produced large scores for seeds with high protein, low tannin and low dormancy levels, was an important predictor of consumption time only. The local pPCA axis primarily reflected kernel mass. Although it explained only 12% of the variation in trait space and was not autocorrelated among phylogenetic clades, the local axis was related to all four squirrel foraging investments. Squirrel foraging behaviors are influenced by a combination of phylogenetically conserved and more evolutionarily labile seed traits that is consistent with a weak or more diffuse coevolutionary relationship between rodents and hardwood trees rather than a direct coevolutionary relationship.

2.2 Introduction

Scatter-hoarding rodents influence seedling establishment in many communities by acting as seed dispersal agents and seed predators (Vander Wall 1990; Theimer 2005). Whereas most seeds harvested by rodents are ultimately consumed (Vander Wall 1994; Theimer 2005), many rodents enhance the probability of germination and establishment by caching in suitable microsites (Wenny 2001) and then subsequently failing to recover a portion of these seeds (Theimer 2005; Vander Wall et al. 2005). Most rodents can therefore, easily shift from mutualism to seed predation, and this conditional nature of mutualism between seed-bearing trees and scatterhoarders is a delicate one (Theimer 2005) that often follows from the rodents' responses to seed characteristics (Vander Wall 1990; Forget and Vander Wall 2001).

From the perspective of a rodent, seed preference and seed handling involve a sequence of behavioral decisions. At each step in the process, scatter-hoarders presumably evaluate costs and benefits associated with alternatives to maximize use of resources (Lichti 2012; Wang et al. 2012). Experiments with artificial and natural seeds suggest that seed dispersal and handling behavior is related to the perceived value of a seed, or the benefits of attractive traits discounted by the costs levied by defensive seed traits (Xiao and Zhang 2006; Wang et al. 2012). While numerous studies have focused on the relationship between seed traits and fate, fewer have examined the influence of seed traits on the specific behavioral decisions of the scatter hoarder (Vander Wall 2010; Delgado et al. 2014). Fine-scale assessments of the seed handling process provide evidence that rodents evaluate the condition of seeds (e.g. by paw manipulation in *Sciurus niger*), which may be predicted by traits indicating seed quality or condition (Delgado et al. 2014).

From the perspective of a tree, some seed traits may improve the probability of being cached or consumed, thereby influencing fitness (Chen et al. 2012). Creating an attractive seed (e.g., with a high caloric value) in comparison to that of competitors is beneficial because such seeds may be dispersed longer distances (Jansen et al. 2004; Xiao et al. 2005; Wang and Chen 2012) reducing density-dependent mortality from seedling competitors or seed predation (Janzen 1970, 1971; Jansen et al. 2008). On the other hand, a seed with defensive traits such as a hard shell may be beneficial because such seeds increase handling costs and induce seed caching behaviors thereby increasing seed survival (Smith and Follmer 1972; Lewis 1982; Jacobs 1992; Vander Wall 2010; Lichti et al. 2014). Seeds of hardwood trees in China, for example, show trade-offs with respect to investment in seed traits that likely influence handling costs and seed dispersal (Chen et al. 2012).

Observations of seed handling and seed chemistry provide abundant evidence for reciprocal evolutionary effects between rodents and woody plants (Steele 2008). In tree squirrels, suites of behavioral adaptations and morphological adaptations have resulted from selective pressures associated with seed morphology and chemistry (Steele 2008). For example, gradients of tannin concentrations have been observed in oak kernels, which impart physiological and metabolic costs to rodents and result in portions of kernels being rejected (Robins et al. 1991; Steele et al. 1993; Shimada and Saitoh 2003; Salminen and Karonen 2011). In response, some rodents have adapted to detoxify tannins (Chung-MacCoubrey et al. 1997; Shimada et al. 2006), and many cache acorns with high tannin concentrations more frequently, possibly to avoid or delay the costs associated with ingesting tannins (Steele et al. 2006; Wang and Chen 2008). In trees, changes in seed chemistry and morphology have evolved in response to predation and dispersal pressures exerted by tree squirrels (Steele et al. 2001). For instance, white oak seeds show no dormancy and multiple-seeded acorns possibly to minimize length of exposure to predation and escape mortality from embryo-excision behaviors by eastern gray squirrels (Steele et al. 2001; Steele et al. 2006). Thus far all approaches to rodent-tree coevolution have collected some combination of detailed morphological, physiological, behavioral, and biogeographic data (Steele 2008). While these studies suggest that coevolutionary interactions exist between granivorous rodents and hardwood trees, the strength of this interaction is variable (Steele 2008).

Coevolutionary relationships between plants and seed dispersers have been described across a continuum, ranging from strong, pairwise interactions to weak, diffuse interactions. Pairwise coevolution occurs between specific species and leads to strong selective pressures on traits (Janzen 1980). Recent observations of acorn (*Quercus* spp.) embryo excision behavior shown by naïve squirrels (Sciuridae spp.; Steele et al. 2001; Steele et al. 2006) is an example of a behavioral adaptation and suggests a strong and perhaps pairwise coevolutionary relationship between oaks and squirrels (Steele et al. 2006; Xiao et al. 2010). In contrast, weak selective pressures due to disparities in evolutionary rates of woody species and animal dispersers, unpredictability of conditions for seed germination, and a plethora of other factors leads to diffuse or weak coevolution (Howe and Smallwood 1982; Herrera 1985).

To better understand the nature of coevolutionary interactions between hardwood tree seeds and squirrels, I used a combination of phylogenetic and ecological methods to determine how seed caching and seed consumption behaviors of eastern gray squirrels (*Sciurus carolinensis*) varied as a function of seed traits and phylogenetic relationships of 23 hardwood trees species. Specifically, I hypothesized that gray squirrels would invest more time and travel longer distances to consume and cache seeds with strong physical defenses and high nutrient values. In addition, I predicted that conspecific interference would be related to physical defenses of a seed and nutrient value of a seed. I incorporated phylogenetic relationships of hardwood trees into analyses to evaluate the relative proportion of gray squirrel seed handling behaviors that is influenced by common ancestry of hardwood tree species and to determine which phylogenetically related seed traits influence handling. I hypothesized that if strong coevolutionary relationships occur between gray squirrels and hardwood tree species, most of the variation in gray squirrel behavior would be explained by seed traits with strong phylogenetic signals. However, weaker, more diffuse coevolutionary relationships would be explained by a mix of seed traits with a strong phylogenetic signal and those traits with no phylogenetic signal. Finally, if no coevolutionary relationships occur, phylogenetically conserved traits should explain a small portion of the variation in gray squirrel behavior.

2.3 Materials and Methods

2.3.1 Seed handling metrics

Free-ranging eastern gray squirrels on the campus of Purdue University were presented with a sequence of seed types in pseudo-random order between 1 October 2011 and 15 February 2012. Seeds were obtained from commercial seed companies (F. W. Schumacher Co., East Sandwich, MA and Sheffield' s seed Co., Locke, NY) and from beneath trees on campus. Seeds were stored at 4°C, separated by species in plastic containers. Seed presentations were conducted by tossing a seed to a squirrel from 1–4 m. If the squirrel retrieved the seed, the observer video recorded the event until the seed was consumed or cached. When the seed was consumed, the observer recovered the remains of the seed kernel and shell by sifting through the litter and soil. The distance a squirrel moved to cache or consume a seed was determined by retracing the path of the squirrel, marking the final destination, and then measuring the straight-line distance between the point of encounter of the seed and the final location. If the distance travelled was too great to be accurately estimated with a tape measure, a GPS (Garmin Model No. 72) was used to obtain coordinates. Straight-line distance was then calculated in ArcGIS 10 (Environmental Systems Research Institute 2010). A second seed was presented to a squirrel only after the first seed was handled, and the process repeated until squirrels no longer recovered seeds. Presentations of the same seed types were spatially separated by selecting locations that were > 100 m apart to increase the likelihood that data were collected from different squirrels. Exceptions were permitted only if two different squirrels could be recorded simultaneously. The protocol included non-intrusive observation with no hindrance to the animals, and therefore, I did not seek additional approval from Purdue Animal Care and Use Committee (PACUC).

Foraging trial videos were collected for 23 seed types from the tree families Betulaceae, Fagaceae,and Juglandaceae (summarized in Supplementary information of Sundaram et al. 2015). The time taken to consume or cache each seed was calculated from the videos. Time required to consume and cache a seed was defined as the time needed to consume and completely bury a seed respectively, and did not include travel time. I quantified the number of recorded trials per seed type in which the focal squirrel was chased by a conspecific while handling a seed, which I define as conspecific interference (Parker and Nilon 2008).

2.3.2 Seed traits

The value of each seed trait was measured for 3 seeds for each of the 23 seed types unless specified otherwise (summarized in Supplementary information of Sundaram et al. 2015). Percentage of moisture in seeds was calculated by drying samples at 103°C for 3 days. Percentage of tannic acid equivalents in nutmeat was determined by performing radial diffusion assays (Hagerman 1987; Johnson et al. 1993). The value was expressed as tannic acid equivalents occurring in 100 grams of dry nutmeat. Energy content of the nutmeat was obtained in a bomb calorimeter (PARR 1262 bomb calorimeter, Parr Instrument Co., Moline, IL) using benzoic acid as a calibration standard. The value was adjusted to obtain calories per gram of dry weight. Seed hardness was estimated as the peak load (kilograms) required to break a seed' s shell and pierce the nutmeat. Testing was done with a MTS/Sintech computerized testing machine (MTS Corporation, Eden Prairie, MN) using a crosshead fashioned after the skull of an eastern gray squirrel and designed to mimic a squirrel' s incisor action (see Supplementary information of Sundaram et al. 2015). Only the upper incisors were used to obtain hardness estimates. Peak load within 5 mm crosshead displacement was sufficient to break the seed shell of most seeds and was used as an estimate of seed hardness. Only butternut (*Juglans cinerea*) seed shells were not pierced with the 5 mm crosshead; therefore, 10 mm displacement was employed for this seed. Seed shell thickness was determined by visualizing the cross section of seed shells with a stereoscopic microscope attached to a Nikon Imaging System at 4.91 micrometers per pixel. Proximate analysis was performed following Association of Official Analytical Chemists (AOAC) protocols to estimate percentage of crude proteins (Kjeldahl, AOAC Official Method 984.13 A-D, 2006), fats (ether extraction, AOAC Official Method 920.39 A, 2006) and carbohydrates (difference method, FAO 2003) in nutmeat. I combined kernels from multiple seeds to obtain at least 10g of dried material, and duplicate samples were used in proximate analyses for each seed type. Finally, I used number of days of cold stratification required before germination in a chamber as an index of dormancy period of seed types. Estimates of cold stratification days were obtained from the literature (Carpenter and Guard 1954; Bonner and Maisenhelder 1974; Bonner and Karrfalt 2008), and averages were computed and used whenever a range of days was provided.

2.3.3 Molecular and phylogenetic methods

I conducted a phylogenetic analysis to estimate and incorporate evolutionary relationships between hardwood tree species into analyses in this study. To achieve this goal, I first extracted DNA from plant tissue using two methods. For the majority of species, I extracted DNA from leaf tissue using a phenol-chloroform protocol (Sambrook and Russell 2001), modified by grinding fresh leaf tissue with 500mg PVPP in liquid nitrogen (Angeles et al. 2005). I used the PowerPlant Pro DNA Isolation Kit (MoBio Laboratories, Inc; Carlsbad, CA) to extract DNA from *Carya tomentosa* leaf tissue and embryos removed from the seeds of *Quercus prinus* and *Quercus bicolor*.

To create a phylogeny for the 23 hardwood tree species in this study, I used sequence data from two chloroplast genes- ribulose-bisphosphate carboxylase oxygenase large subunit (rbcL) and maturase K (matK) as well a portion of the nuclear genome that included the internal transcribed spacer 1, 5.8S rRNA gene, and the internal transcribed spacer 2 (ITS). When possible, I obtained relevant sequences from Genbank (see Supplementary information of Sundaram et al. 2015). For the remaining species, I sequenced each sample using polymerase chain reaction (PCR) on an Eppendorf Mastercycler (Eppendorf, Westbury, New York). Initial amplification was performed in 20 µL reactions containing approximately 40 ng template DNA, 1 unit of NEB Taq polymerase, 0.3 lM of each primer, 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.5 mg/ml BSA, and 0.2 mM of each dNTP. PCR parameters included an initial denaturing temperature of 95°C for 30 seconds followed by 30 cycles of 95°C for 30 seconds denaturing, annealing temperature of 53°C for rbcL and matK (Burgess et al. 2011) and 58°C for the ITS gene (primers ITS5 and ITS4; White et al. 1990), and 72°C for 1 minute elongation, with a final elongation temperature of 72°C for 5 minutes. I cleaned the resulting PCR product with the MinElute PCR Purification Kit (Qiagen, Valencia, California). Sanger sequencing reactions were performed at 10 µL and contained 50 ng of template and Big Dye 3.1. I used an ABI Prism 3730XL sequencer (Applied Biosystems, Foster City, CA, USA) and trimmed sequences in Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI).

I aligned the sequences for each gene using M-coffee web server hosted by the Centre for Genomic Regulation (Wallace et al. 2006; Moretti et al. 2007). I tested for congruence of species pairwise distance matrices derived for the 3 aligned genes in this study by calculating Kendall’s W statistic of concordance as implemented by the function ‘CADM’ within the package ‘ape’ in R (Campbell et al. 2011). After rejecting the null hypothesis of incongruence between distance matrices, I concatenated sequences of the 3 genes for further analyses. The function ‘modelTest’ in the package ‘ phangorn’ was used to select the best nucleotide substitution model for the concatenated sequences (Schliep 2011). Model SYM (symmetrical model; Zharkikh 1994) + G (gamma distributed rate variation) was selected based on BIC values.

I generated a phylogeny for the 23 species using the program BEAST (Drummond and Rambaut 2007), and prepared an input file using BEAuti (Drummond et al. 2012). I used a relaxed lognormal molecular clock (Drummond et al. 2006), the Yule speciation model (Yule 1925; Gernhard 2008), and *Rubus occidentalis* as an outgroup. I ran 5 independent runs of 50 million steps, thinning to every 1000 trees. I confirmed convergence of each run with Tracer, and filtered the first 10% each group of runs using TreeAnnotator.

2.3.4 Statistical analyses

I estimated pairwise Spearman rank-correlations between the seed handling metrics and all seed-trait values in this study. I also computed Moran's I metric of phylogenetic signal (Gittleman and Kot 1990) for all seed traits and evaluated significance by permuting seed trait values across tips of the phylogeny 1000 times. For all further statistical analyses, I included the following seed traits in the seed trait matrix: percent lipids, percent carbohydrates, percent proteins, caloric concentration, dormancy period, log hardness, log shell thickness, log interaction of hardness and thickness, log kernel mass, log shell mass, and log tannin concentration. Natural log transformation was used for seed traits with skewed distributions. Before log-transforming tannin concentrations, a low value of 0.35% TAE was assigned to the 3 seeds with 0% TAE to avoid undefined numbers.

I incorporated the phylogeny of hardwood trees into statistical analyses using two methods— variance partitioning analyses and phylogenetic PCA. To partition the variation in each seed handling metric between seed traits and phylogenetic relationships, I used phylogenetic eigenvectors and a partial regression analysis implemented in the R package 'PVR' (Santos et al. 2012). The PVR method converts a matrix of double-centered phylogenetic distances into eigenvectors, which are then used as predictors to explain variation in a trait of interest. The first few eigenvectors, which capture most of the variation in the distance matrix, represent differences between clades at the root of the phylogeny. Subsequent eigenvectors capture variation among taxa or groups closer to the tips. Selection of all eigenvectors as predictors in regressions is not necessary and may result in large or inflated R^2 values. In contrast, selection of too few eigenvectors can result in residual autocorrelation because all of the phylogenetic dependence is not captured. For this analyses, I selected phylogenetic eigenvectors that minimize residual autocorrelation as estimated by Moran’ s I (Desdevises et al. 2003; Diniz Filho et al. 2012). The PVR method then uses the phylogenetic eigenvectors and environmental variables (i.e. seed traits) as predictors in a partial regression analyses to partition the variation in a trait. I used the PVR method to obtain the variation in each seed handling metric (handling time and distances travelled prior to handling for cached and consumed seeds) explained by seed traits alone, selected phylogenetic eigenvectors alone, information shared between seed traits and selected phylogenetic eigenvectors, and finally unexplained factors.

Next, I identified linear combinations of seed traits that exhibit autocorrelations with the hardwood tree clades observed in the phylogeny. I performed a phylogenetic principal components analysis (pPCA) using the Abouheif method (equivalent to Moran’s I metric) of computing phylogenetic signals (Jombart et al. 2010a). The pPCA groups seed traits into possible ‘global’ and ‘local’ structures given a candidate phylogeny and traits of interest. The ‘global’ structures constitute pPC axes that are positively correlated to clades at the root of the phylogeny. Large positive eigenvalues represent axes showing large variance and positive phylogenetic autocorrelation or Moran’s I. In contrast, ‘local’ structures constitute pPC axes and traits with negative phylogenetic autocorrelation indicative of traits that are different between related taxa. Large negative eigenvalues represent axes explaining a large variance and axes with a negative Moran’s I. Together, the global and local pPC axes can suggest specific life history strategies adopted by the taxa. I performed a pPCA on the seed trait matrix using function ‘ppca’ in the package ‘adephylo’ (Jombart et al. 2010b). To ensure that inconsistencies in the phylogeny were not driving the observed relationships, I collapsed subclades into groups and performed the pPCA a second time. The collapsed clades in the phylogeny included-walnuts (*Juglans*), hickories (*Carya*), white oak group (*Quercus* section Quercus), red oak group (*Quercus* section Lobatae), and chestnuts (*Castanea*). In addition, the tree contained the following singleton taxa— hazelnut (*Corylus americana*), beechnut (*Fagus grandifolia*) and tanoak (*Notholithocarpus densiflorus*). I retained the first three pPC axes (2 global and 1 local as determined by the sign of the eigenvalue) explaining the highest proportion of variation. I tested selected pPC axes for significant positive and negative phylogenetic autocorrelation by performing Abouheif’s test on the PC scores using the ‘abouheif.moran’ function. Finally, I regressed the 4 squirrel seed handling metrics in 4 different multiple linear regression models against the 2 global and 1 local pPC axes. Plots of residuals indicated unequal variances and departures from normality, so I evaluated significance of predictors by permuting the response variable 1000 times and estimating the null distribution of coefficients. Finally, I performed a post-hoc Poisson regression to predict number of trials where a conspecific interfered with seed handling using the 3 pPC axes as predictors. To evaluate if interference caused an increase in handling time, I performed a paired t-test comparing average times spent handling a seed type when interference was observed to when interference was not observed pooling across seed types. All analyses were performed in R 3.1.0.

2.4 Results

I recorded 272 foraging trials, which included 5–7 seed-consumption and 4–6 seed-caching trials per seed type (mean of 5.04 seed-consumption and 5.52 seed-caching trials/seed type). For tanoak (*N. densiflorus*) 4 caching trials were recorded. For black walnut (*J. nigra*), the distance travelled to consume a seed was computed for 4 of the 5 consumption trials. Kernels were typically consumed or cached.

Pairwise Spearman rank correlations between seed traits of the 23 seed types revealed several patterns of correlation between groups of seed traits (Supplementary table in Sundaram et al. 2015). Nutrient and perishability related variables such as percentage of proteins, carbohydrates and lipids in the kernel, caloric concentration, and dormancy period were positively correlated. Physical defensive traits including hardness, shell thickness, hardness and thickness interaction, and dry mass of shell were also positively correlated. In addition, lipid concentration was positively correlated to shell thickness and hardness. Tannin concentration of kernels was correlated to protein concentration and dormancy period only (corrrelations in Supplementary information of Sundaram et al. 2015).

I successfully extracted DNA from plant tissue for 12 taxa for which sequences were not available in GenBank (see Supplementary information in Sundaram et al. 2015). The concatenated dataset contained 1864 sites, out of which 332 were parsimony informative. Topology of the Bayesian tree (Fig. 1) is supported by other existing phylogenies and current taxonomic classification of the hardwood trees species (Manos and Stone 2001; Li et al. 2004; Manos et al. 2008; Oh and Manos 2008; Zhang et al. 2013). In addition, posterior probabilities associated with the clades were usually > 0.9. Weak node support occurred only at the tips of the phylogeny within the white oak group (*Quercus* section Quercus), and the split of shagbark and pignut hickory (*Carya glabra* and *Carya ovata*, Fig. 1).

After deconstructing the phylogeny into eigenvectors, I performed variance partitioning to estimate the proportion of squirrel seed handling investments explained by seed traits and phylogeny (Table 1). Seed trait variation alone explained between 27 and 73% of the variation in the seed handling behavior of squirrels. Phylogeny independent of the measured traits explained < 6% of all seed handling behaviors. For time required to consume a seed and distance travelled to cache a seed, the variation explained by the combination of seed traits with a phylogenetic structure was 44% and 56%, respectively. Unexplained variation was relatively high (21% and 32%) for distance moved to consume a seed and time required to cache a seed.

The 11 seed attributes of 23 seed types (Fig. 2, Supplementary table in Sundaram et al. 2015) were reduced to three phylogenetic principal components (pPC), cumulatively explaining 83.3% of the total variation. I interpreted the first global pPC axis as family-level differences between seeds. This axis accounted for 63.5% of the total variation. Percentage of carbohydrates loaded highly and contrasted with percentage of lipids and shell thickness. Scores from pPC1 were phylogenetically autocorrelated (I = 0.83, p = 0.002), and differentiated lipid rich, thick shell seeds of Juglandaceae and Betulaceae from carbohydrate-rich Fagaceae (Fig. 2). The second global pPC axis explained 8.4% of the variation, and reflected genus and section-level differences in seeds. The axis positively loaded protein concentration, and negatively loaded tannin concentration and dormancy period. Scores from pPC2 axis were also phylogenetically autocorrelated (I = 0.36, p = 0.004) and separated high protein *Juglans* seeds from *Carya* seeds and high protein *Castanea* seeds from low dormancy *Quercus* section Quercus seeds (Fig. 2). Finally, the third pPC axis reflected kernel size of seeds and explained 12.4% of the total variation. This axis positively loaded kernel and shell mass and negatively loaded energy value of seed. Scores from pPC3 were not significantly phylogenetically autocorrelated (I = -0.12, p = 0.694, Fig. 2). The pPC axes did not change substantially after reducing the phylogeny to only well-established clades.

Time required to consume a seed was predicted by the two global pPC axes and the local pPC axis ($R^2 = 0.76$, Table 2). The distance moved to consume a seed was positively predicted predominantly by the local pPC axis and marginally by the first global pPC axis ($R^2 = 0.30$, Table 2). Time required to cache a seed was negatively predicted by the first global pPC axis and positively to the local pPC axis ($R^2 = 0.43$, Table 2). Distance moved to cache a seed was correlated negatively with the first global pPC axis and positively with the local pPC axis ($R^2 = 0.76$, Table 2).

Competitive interference was observed in 10 seed types (range 0– 3 trials per seed type) and was observed in 3 seed caching videos and 11 seed-consumption videos. Number of trials in which conspecific interference was observed was not predicted by any of the pPC axes. The mean handling (consumption and/or caching) time did not differ within a seed type between trials in which interference was observed and trials where interference was not observed (Paired t test t = 0.22, df = 10, P = 0.95).

2.5 Discussion

The results of this study provide evidence that foraging investments are influenced by a mixture of seed traits that are phylogenetically autocorrelated (mass of shell, hardness of shell, shell thickness, lipid concentrations, and carbohydrate concentrations) and those that are not (kernel size and tannin concentrations, see Table 2). I suggest that these results support the existence of a diffuse coevolutionary relationship between eastern gray squirrels and hardwood tree seeds.

By incorporating phylogenetic information, I found that gray squirrel foraging investments are influenced to different degrees by seed trait information, phylogenetic relatedness of hardwood trees and unexplained sources of variation. Distance moved to consume a seed and time to cache a seed showed a relatively high degree of unexplained variation in the variance partitioning analyses, suggesting that these metrics may be influenced to a relatively greater degree by variables not measured in this study. A different pattern of variance partitioning was observed for time required to consume a seed and distance to cache a seed, both of which are behaviors related to caching decisions and seed survival. Specifically, seeds with large handling times may be cached more often, increasing the possibility of germination and establishment, and seeds cached at long distances may escape density-dependent sources of mortality (Janzen 1970, 1971; Jansen et al. 2008; Vander Wall 2001). These behaviors were explained to a large extent by the shared information in seed traits and phylogeny (Table 1), suggesting possible coevolution between hardwood trees and eastern gray squirrels.

Coevolution results from interactions that occur over long time scales and should extend to ancestral states existing prior to differentiation of genera (Howe and Smallwood 1982). The pPCA methods allowed me to determine which seed trait variants correspond to family and genus-level differentiations among the 23 hardwood tree species of this study. By regressing squirrel foraging investments against pPC axes, I indirectly determined if gray squirrels are sensitive to family or genus-level seed trait variations.

Family-level seed trait differences were more important in predicting gray squirrel behavior than genus- or section-level differences. The earliest split observed in the hardwood tree phylogeny corresponds to the separation of Juglandaceae and Betulaceae from Fagaceae (Fig. 1), which coincides with two different strategies of making seeds (as suggested by the global pPC1 axis)— lipid-rich and thick shelled seeds in Juglandaceae and Betulaceae, as opposed to thin shelled but carbohydrate-rich Fagaceae seeds (Fig. 2). Specifically, squirrels travel farther to cache and invest more time caching/consuming lipid-rich and thick-shelled Juglandaceae and Betulaceae seeds (Table 2), potentially improving seed survival and fitness of these trees. The second pPC axis, describing differences in seed traits between genera and section clades, was also included as a predictor in regression analyses but positively explained only time to consume a seed (Table 2). This finding supports the existence of coevolutionary interactions between squirrels and hardwood trees.

If coevolutionary interactions exist between gray squirrels and hardwood trees, either a weak diffuse coevolution or a strong pairwise coevolution could exist. The local axis, with no phylogenetic signal, was an important predictor of squirrel behavior and thus supports a diffuse coevolutionary interaction. Specifically, large loadings on the local pPC3 axis reflected high kernel mass, which was one of two seed traits showing no phylogenetic signal (Supplementary tables in Sundaram et al. 2015). This local axis explained only 12% of seed trait variation, but it was a significant predictor of distance travelled to consume or cache a seed and time to cache a seed. If kernel size is an evolutionarily more labile trait, then trees from all the three families can improve chances of recruitment by producing seeds with larger kernels to attract dispersers and increase handling costs.

The adaptive strategies of trees to influence seed handling are not limited to attributes of a single seed. Trees with smaller kernels and smaller seed mass may adopt strategies not examined in this study such as production of large numbers of seeds and synchronized masting (Kelly 1994; Schnurr et al. 2014). Environmental variables not considered here could also influence foraging behaviors, particularly behaviors with a relatively high proportion of unexplained variation including number of conspecific interferences per seed type, time to cache a seed and distance to consume a seed. These foraging behaviors may be influenced by environmental metrics such as density of conspecifics at a location and perceived predation risk. However, seed traits alone accounted for 73% of distance travelled to consume a seed and 41% of time to cache a seed in variance partitioning analyses, which were larger than the R^2 values associated with the multiple regression analyses involving 2 global and 1 local pPC axes. Thus, additional local pPC axes showing no significant phylogenetic signal, and not considered here, might play an important role in explaining seed handling behaviors.

Although the small sample (n = 3) of seeds could lead to imprecise estimates for attributes, the seed trait values match published values (Vander Wall 2001), which also indicate that seeds of Juglandaceae have higher energetic values and lipid content as opposed to seeds of Fagaceae. Given the large differences in seed traits among species, trait variation within species is unlikely to substantially alter the phylogenetic signals computed here. That said, the effects of seed traits on the fitness of individual trees will depend on their specific environmental and competitive contexts. Therefore, future studies that directly address intraspecific trait variation will be needed to completely understand the coevolutionary dynamics of trees and rodents.

From a gray squirrel' s perspective, the degree to which seed traits influenced foraging investments differed depending on whether the seed was consumed or cached. For consumed seeds, degree of physical protection, caloric potential of seed and net protein availability influenced handling behaviors (Table 2). Consistent with other studies (Lewis 1982), consumption time increased for large seeds with thick, hard shells. Consumption time was also negatively related to tannin concentration of the kernel, which may result from squirrels rejecting portions of kernels with high tannins (~0.25–0.5g), potentially because high tannin concentrations render a seed unpalatable (Steele et al. 1993). Distances to consume and cache seeds were influenced by lipid concentration, shell thickness, and kernel size, which is consistent with other studies (Jansen et al. 2004; Moore et al. 2007). Moreover, I observed a significant positive correlation between handling time and distance moved to consume a seed ($r = 0.674$, see Supplementary information of Sundaram et al. 2015) which suggests that squirrels may carry seeds farther to sites where predation risk or conspecific competition is low before consuming seeds that require more time and energy to handle (Vander Wall 2010).

I found no evidence of interference from conspecifics varying with respect to seed traits. Paired t-test results suggest that conspecific interference does not influence time required to consume a seed. However, I evaluated interference post-hoc, and the results were based on only 14 instances. Therefore, studies designed to examine effects of interference from conspecifics are needed to confirm results. For seeds that were cached, I found increased cache time for seeds with a thick shell and high lipid concentration (pPC1), and large kernel size (pPC3). These results indicate that large seeds require a deeper site to be excavated for caching. Alternatively, this result could also arise if squirrels perceive large seeds to be valuable and consequently invest longer amounts of time in caching them (Hopewell and Leaver 2008).

The results reinforce the notion that different seed traits can influence foraging investments related to caching versus consumption. Moreover, these results suggest that it is unwise to discount the behavioral importance of seed traits that show no phylogenetic structure or explain little variation in trait-phylogeny space. Future research using phylogenetic methods in concert with trait measurements and behavioral observations will be useful to compare the effects of selection pressures imposed by rodents on seed-bearing trees in ecosystems that presumably vary in their coevolutionary histories.

2.6 Figures

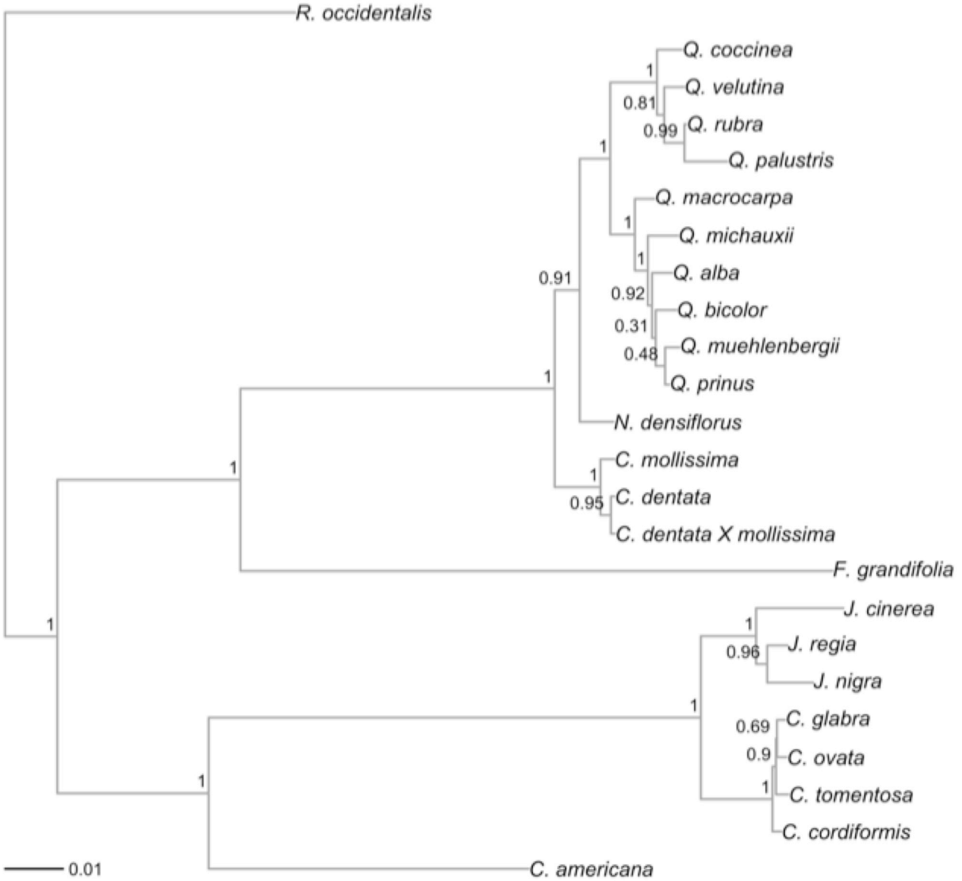

Figure 1. Bayesian maximum clade credibility tree for 23 hardwood tree species from the families Fagaceae, Juglandaceae and Betulaceae, using *Rubus occidentalis* as an outgroup. Tree inferred from rbcL, matK and ITS gene sequences. Posterior node support indicated by node labels.

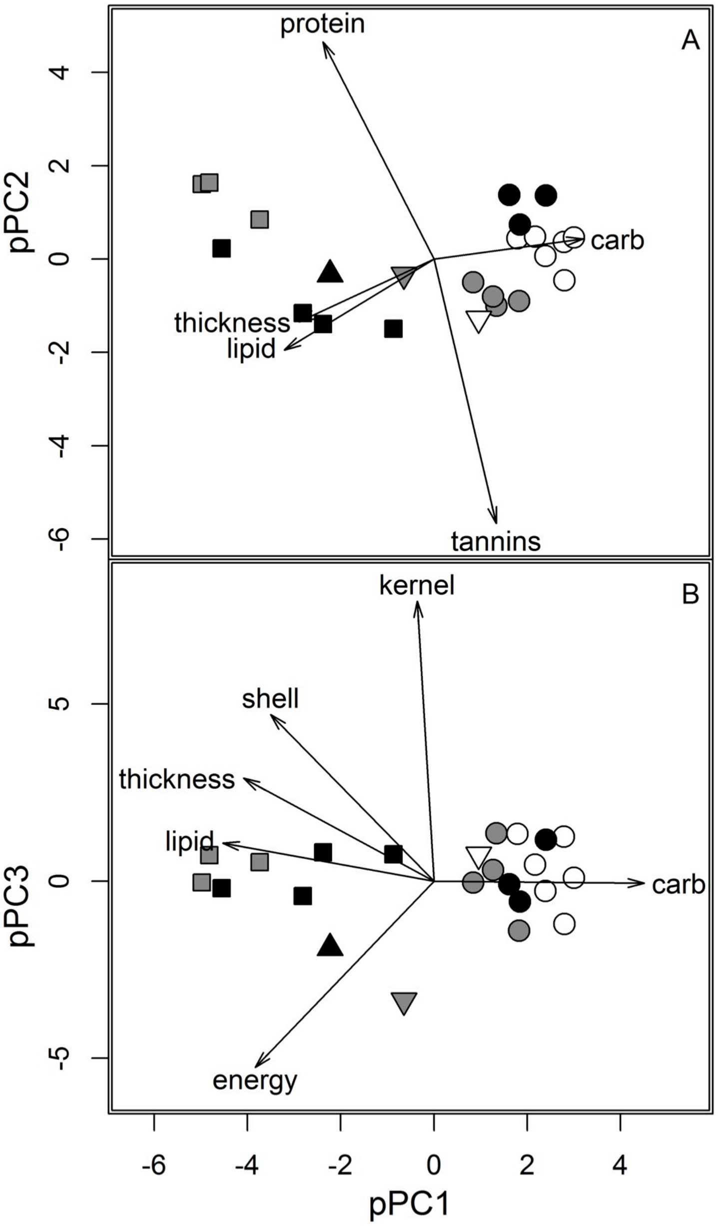

Figure 2. Biplots of seed traits and scores of 23 hardwood tree species obtained from a phylogenetic principal components analysis (pPCA). Distribution of hardwood tree species across the first 2 'global' pPC axes (A). Distribution of tree species across the first 'global' and third 'local' pPC axes (B). Separation of species belonging to families Juglandaceae and Betulaceae (symbols: square and upright triangle) from Fagaceae

(symbols: circles and inverted triangle) is observed across phylogenetically autocorrelated pPC1. Separation of *Juglans* (gray filled square) from *Carya* (black filled square) and separation of *Castanea* (black filled circle) from *Quercus* section Quercus (open circle) and *Quercus* section Lobatae (gray filled circle) is seen across pPC2. The third axis is not significantly phylogenetically autocorrelated and species are not differentiated by taxonomic clades across pPC3. Biplots also include *Notholithocarpus densiflorus* (inverted gray triangle) and *Fagus grandifolia* (inverted open triangle). Biplot arrows plotted only for seed traits with loadings greater than 75th percentile of absolute loadings (pPC loadings and 75th percentile cutoff in Supplementary information of Sundaram et al. 2015).

2.7 Tables

Table 1. Variance partitioning results for each squirrel seed handling behavior, including time to consume, distance travelled to consume, time to cache, and distance travelled to cache a seed. Values are proportion of variation (R^2) of each behavior explained by seed trait information alone, combined information between seed trait and phylogeny, phylogenetic information alone, and unexplained sources of variation.

Response variable	Seed traits alone	Combined traits & phylogeny	Phylogeny alone	Unexplained
Time to consume	0.502	0.440	0.006	0.052
Distance travelled to consume	0.731	0.055	0.000	0.214
Time to cache	0.409	0.213	0.058	0.320
Distance travelled to cache	0.274	0.556	0.004	0.156

Table 2. Regressions of squirrel foraging behavior (time to consume, distance travelled to consume, time to cache, and distance travelled to cache a seed) against 3 phylogenetic PC axes (pPC1, pPC2, pPC3). Estimates of slope, t-statistic and p-value are provided for each predictor**.** Boldface depicts values of $p < 0.05$; italics depict $0.05 \leq p < 0.10$.

Response variable	Predictor	Slope	t-statistic	p-value
Time to consume a seed	Intercept	9.216	7.039	**0.000**
	pPC1	-3.112	-6.208	**0.000**
	pPC2	4.196	3.039	**0.006**
	pPC3	2.843	2.388	**0.033**
Distance moved to consume a seed	Intercept	6.048	5.341	0.072
	pPC1	-0.736	-1.699	0.090
	pPC2	-0.007	-0.006	0.994
	pPC3	2.460	2.389	**0.039**
Time to cache a seed	Intercept	2.090	10.044	**0.039**
	pPC1	-0.220	-2.864	**0.016**
	pPC2	0.130	0.614	0.544
	pPC3	0.507	2.133	**0.047**
Distance moved to cache a seed	Intercept	26.283	12.400	**0.000**
	pPC1	-5.263	-6.757	**0.000**
	pPC2	0.437	0.204	0.840
	pPC3	8.742	3.621	**0.002**

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CHAPTER 3. INSIGHTS FROM DISCRETE CHOICE EXPERIMENTS: EASTERN GRAY SQUIRRELS ARE CONSISTENT SHOPPERS OF SEED TRAITS

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3.1 Abstract

Trait-dependent trophic interactions are widespread, but covariance among prey traits can make it difficult to understand which traits predators respond to. I demonstrate a means of designing choice experiments to parse out and test the effects of correlated prey traits on behavioral decisions of free-ranging consumers with discrete choice modeling methods. I successfully applied a designed discrete choice experiment to differentiate eastern gray squirrel (*Sciurus carolinensis*) foraging responses to 5 correlated seed traits (protein content, tannin concentration, kernel mass, dormancy period and toughness of shell) using n=426 seed-pair presentations. Squirrels preferentially consumed seeds with short dormancy or tougher shells, and preferentially cached seeds with larger kernel mass, tougher shells and higher tannin concentrations. By incorporating random effects, I found that squirrels exhibited consistent preferences for seed traits, which is likely due to the fitness consequences associated with maintaining cached resources. Furthermore, I found that squirrels were willing to trade between multiple traits when caching seeds, which likely results in more seeds being cached in the fall. This approach allowed me to compute the relative values of different seed traits to

squirrels, despite covariance among studied traits across seed species. This method can reliably be used to separate the effects of moderately correlated traits (approximate $\rho<0.7$) on resource selection by animals. Although less effective for differentiating very highly correlated traits (approximate $\rho>0.7$), it offers significant improvements over traditional methods that correlate behaviors and traits post hoc and therefore represents a useful new tool for ecologists studying trait-based behaviors and resource selection.

3.2 Introduction

Trait-dependent trophic interactions occur in a wide variety of systems (Schmitz et al. 2004; Curtsdotter et al. 2011; Albert et al. 2015). Numerous studies corroborate observations that plasticity of traits leads to complex interactions and changes in ecosystem function (Schmitz et al. 2004; Berg and Ellers 2010). However, formal integration of trait-based behaviors into foraging ecology in a manner that allows predictions to be made under different environmental contexts has been lacking (Lichti et al. *In press*). Seed selection by granivorous rodents is seed trait-dependent (Wang and Chen 2008; Wang et al. 2012; Sundaram et al. 2015) and context-dependent (Lichti et al. 2014; Lichti et al. *In press*) and thus provides a good system within which to develop a trait-based model.

Traditional rodent seed selection studies use observational data involving seeds of multiple species presented in sequential or cafeteria-style presentations wherein order of selection is determined after some defined period of time (Blythe et al. 2015). Subsequently, ranks or proportions of seeds removed are correlated to various factors such as seed traits (mass of seed, tannin concentration, etc.) to evaluate whether selection by rodents was influenced by traits of seeds (Gong et al. 2014). Because such comparisons in selections of different seed types are made post hoc, differentiating between the effects of correlated seed traits on seed selection is difficult (Smallwood et al. 2001; Xiao and Zhang 2006). To overcome the problem of correlated traits, some studies have used artificial seeds wherein traits such as tannin concentrations, protein content and lipid content are manipulated to be uncorrelated (Wang and Chen 2008). This approach creates an orthogonal design that can segregate the effects of specific seed traits on seed selection, but the degree to which results from artificial seed studies can be extrapolated to real seed-rodent systems has not been determined.

Discrete choice experiments (DCEs) offer an unexplored alternative to studying trait-based selections in ecology even when the traits being studied covary (Fig. 1). Subjects in DCEs select between alternatives in a set of pre-determined, designed choice scenarios (Kuhfeld et al. 1994; Louviere et al. 2010). Individually, each scenario represents a choice between items, each of which contains a specific combination of traits. Provided that researchers are willing to discretize traits into levels, careful selection of the items presented in each scenario will allow them to construct a set of scenarios in which all possible combinations of traits are presented while maintaining a balanced and orthogonal statistical design (Kuhfeld et al. 1994). For example, consider a hypothetical experiment involving 2 traits (Fig. 1A). The two traits may represent the price and quantity of nutrients in several different brands of milk, where consumer preference for price relative to nutrients is unknown. After discretizing the 2 traits into high and low levels (akin to treatment levels in analysis of variance), all possible combinations of the trait levels are represented by 4 different alternatives (Fig. 1A, step 2). These combinations of traits, which are found in the different brands of milk, are then paired

(Fig. 1A, step 3), presented to consumers, consumer choices recorded, and the selections interpreted as a function of the milk-brand traits (price and nutrient content) using discrete choice models or DCMs (Louviere et al. 2010).

Importantly, discrete choice pairings (Fig. 1A, step 3) represent a balanced and orthogonal design. The design is balanced since the number of ‘lows’ in the design equals number of ‘highs’ represented for each trait. The design is also orthogonal since trait X and Y are not correlated to one another in the final design, which is important to decouple the effects of the traits on the choices made by subjects. In more complex experiments that involve multiple traits or trait levels (e.g., Fig. 1B), an orthogonal and balanced set of choice scenarios can be selected using design algorithms (Kuhfeld et al. 1994). While DCMs have been used to study selection of habitat or prey characteristics by free-ranging animals (McCracken et al. 1998; Cooper and Millspaugh 1999; McDonald et al. 2006; Cooper et al. 2007), DCEs have not been employed to design studies of trait-based selections in ecology.

In contrast to standard, observational DCM study designs, DCEs can more easily parse out the effects of correlated traits on preference by pairing items so that correlations among trait levels in the final design are minimized, ideally to zero (Hensher et al. 2005; Kuhfeld 2010). In addition, DCEs can take advantage of fractional factorial designs that maximize the proportion of information captured relative to the full factorial design while minimizing the number of unique scenarios presented (The proportion of information captured in a candidate design is typically measured using an optimality criterion such as D-efficiency, Hensher et al. 2005; Kuhfeld 2010). Consequently, the designed approach avoids logistical difficulties of using full factorial designs where every trait level combination must be paired and tested (Kuhfeld 2010).

In this paper, I employ a DCE to evaluate how preferences for seeds in eastern gray squirrels (*Sciurus carolinensis*) are related to seed traits. This is an ideal system for the development of designed trait-based methods in ecology for two key reasons. Firstly, seed traits are highly correlated with one another, which creates challenges when relating seed traits to seed preference (Gong et al. 2014; Sundaram et al. 2015). Further, seed choices and seed fate can be easily observed and recorded for squirrels in urban environments (Parker and Nilon 2008; Delgado et al. 2014). I use the seed-squirrel system to illustrate the design, execution, analysis, and interpretation of DCEs, including the quantification of animals' willingness to trade one trait against another. I then discuss the value of DCEs in ecology and the implications of results for the seed-squirrel system specifically.

3.3 Methods

3.3.1 Designing the choice experiment

For the squirrel study, I was interested in the effects of 4 seed traits on selection and use by squirrels. These traits were: (a) grams of dry seed kernel (measured by weighing kernels); (b) grams of protein contained in the seed kernel (protein concentrations obtained by performing proximate analysis); (c) grams of tannin contained in the seed kernel (tannin concentrations measured by radial diffusion assay); and (d) interaction of seed hardness and shell thickness (calculated as a product of force in Newtons to pierce the seed shell and shell thickness in meters, force was measured using a mechanical testing machine and shell thickness was measured by visualizing seed shells under a microscope) (Sundaram et al. 2015). I chose seed species to represent the traits of interest from a candidate set of 22 nut-bearing tree and shrubs in the families Betulaceae, Fagaceae, and Juglandaceae. In a previous study, 11 physical and chemical seed traits were quantified for these species and results showed that the species-level mean trait values significantly predicted differences in seed handling time and transport distance by eastern gray squirrels (Sundaram et al. 2015). All species in Sundaram et al. (2015) were used in this study with the exception of *Notholithocarpus densiflorus*, a seed species that is difficult to store and that does not occur within the eastern gray squirrel range (Bonner and Karrfalt 2008). I use the same species-level trait values here as in Sundaram et al. (2015) and refer readers to the earlier work for details of the trait quantification methods.

Seed trait values across the 22 tree species were correlated. Specifically, shell thickness and hardness have very large positive correlations with lipid concentrations (Spearman's ρ of approximately 0.7) with seeds of families Betulaceae and Juglandaceae showing thick shells and higher lipid concentrations over seeds of Fagaceae (Sundaram et al. 2015). Additionally, shell toughness can predict seed selection because tough shell seeds may be cached more often to avoid large time investments associated with consumption (Jacobs 1992). Therefore, I used only shell toughness to design seed pairs. However, I considered both shell toughness and lipid concentrations as predictors in models during data analyses. Protein content, tannin content and kernel mass show a range of correlations from no significant correlations (ρ = -0.30 or 0.17) to moderate degrees of correlations with one another (ρ = -0.66 or 0.42) and were included in this study because these traits explain significant proportions of rodent seed handling behaviors (Wang et al. 2012; Yang et al. 2012; Gong et al. 2014; Sundaram et al. 2015).

It should be noted that both kernel mass and shell toughness together reflect total seed mass. Because this design already included kernel mass and shell toughness, I did not consider total seed mass as an additional variable.

To design the DCE scenarios, I discretized trait values into categories of low and high (two levels), or low, medium and high (three levels) by identifying natural breaks in seed trait values across the candidate seed set (Table 1; Fig. 1B, step 1). While traits may be divided into more than 3 categories, increasing the number of discretized levels significantly increases the number of combinations to be tested (Hensher et al. 2005). Therefore, I limited the number of levels investigated to preserve tractability of the design. In addition, it is important to note that the discretization used here applied only to the selection of items for the experimental design; analysis is based on the actual, continuous trait values of the items presented.

Next, a list of all unique combinations of low or medium or high attribute levels of the 4 seed traits were computed (Fig. 1B, step 2). Since 3 traits had two levels and 1 trait had three levels (Table 1), this step produced 24 ($2^3 \times 3^1$) unique combinations of seed trait levels, each representing a particular type of seed. Many of these trait-level combinations can be represented by naturally occurring seed species (e.g. *Fagus grandifolia* seeds have low kernel mass, low protein content, low tannin content and low toughness levels (Fig. 1B, step 2). However, some combinations of trait levels generated in the list do not exist in real seeds. Therefore, I eliminated 14 unrealistic combinations from the design (represented as ‘No seed’ in Fig. 1B, step 2).

Finally, I used PROC PLAN and OPTEX in SAS 9.2 to identify an optimal set of choice scenarios using the remaining 10 combinations (Fig. 1B, step 3). PROC PLAN generates a full factorial design with all possible choice scenarios (Kuhfeld et al. 1994). In this study, a total of 45 (10 choose 2) choice scenarios were possible. PROC OPTEX uses the modified Federov algorithm to drop and add candidate choice scenarios and identify those that maximize an optimality criterion (Kuhfeld et al. 1994). The optimality metric I used was D-efficiency, a measure of the proportion of information captured relative to a fully orthogonal and balanced design, where 100% signifies a completely orthogonal and balanced design (Kuhfeld et al. 1994). The final design had a D-efficiency of 92% and consisted of only 10 choice scenarios, a 78% reduction in logistical requirements relative to the full factorial design (Table 2; Fig. 1B step 3).

3.3.2 Data collection and field methods

To implement the DCE, subjects are individually presented with choice scenarios and their selections are recorded. Each scenario is replicated multiple times. Ideally, scenarios are replicated across multiple independent subjects and each subject is presented with all scenarios in a randomized sequence. When scenarios cannot be presented to subjects in a completely controlled manner (a common situation in field studies), a random effects model may be used to search for evidence of non-independence post hoc (see **Random parameters logit model**).

I collected choice data for the 10 choice scenarios in the study during 2 phases. The first phase occurred between 1 September 2012 and 5 January 2013. Nine seed species were used to represent the combinations in the 10 scenarios (Table 2; Fig. 1B step 4). For each choice, a pair of seeds was presented to a free-ranging eastern gray squirrel on the Purdue University campus. Presentation locations were separated by minimum 150m to reduce the chance that data was collected from the same squirrel. Exceptions to this 150m rule were made only if seeds could be presented to different squirrels at the same time. For each presentation, an observer balanced a pair of seeds (Table 2) on golf tees spaced approximately 3cm apart at the base of a tree and waited for a squirrel to approach the seed pair. Observers noted the seed species selected first and its fate (cached or eaten). The next seed pair was then presented on the golf tees, until the squirrel stopped making selections or until data for all ten choice scenarios was collected from the squirrel. The order in which the ten paired trait levels were presented was randomized for each location.

The second phase of data collection occurred between 4 November 2012 and 5 January 2013. During this phase, I replaced seed species for those seed types in the design that could be represented by more than one species (Table 2). When more than 2 species could represent a particular seed type, I randomized the species assignments across presentations. The final design included the same 10 choice scenarios as phase 1, but a total of 20 seed species were used to represent the 10 seed types in the design (Table 2). Phase 2 thus enabled me to verify that choices truly were dependent on seed traits rather than on seed species alone. Presentations followed the same procedure as phase 1.

3.3.3 Data analysis

Seed selection by squirrels was analyzed using 2 different types of DCM. I initially fit simple multinomial logit models to the data. Then I fit a random parameters logit model to test for heterogeneity in preferences, a *post hoc* indicator of non-independence among the choice trials. Finally, I used the results from these models to calculate willingness to trade estimates. These estimates quantify the degree to which the squirrels in the study viewed different seed traits as tradable. The next three sections describe these models and metrics in greater detail.

Multinomial logit model

Choice experiments can be analyzed using DCMs based on random utility theory. In economics, utility is interpreted as satisfaction derived from consuming or using a product. Although utility cannot be measured directly, it can be inferred from choices made by individuals that select among a set of options. Random utility models assume a decision-maker (a squirrel in this study) always maximizes utility by selecting the alternative with the highest utility from all available options (i.e., one of the seed species in a presentation) (Manski 1977). Mathematically, utility for the ith selection event (i.e., seed pair presentation) and jth alternative (i.e., jth seed species) is a random variable (U_{ij}) expressed as

$$U_{ij} = V_{ij} + \varepsilon_{ij} \tag{1}$$

Here, V_{ij} is the systematic portion of utility and ε_{ij} is an independently and identically distributed error term with a Gumbel distribution. In this framework, each alternative represents a collection of traits, and selection of a particular seed species implies selection of the trait values associated with that species. To determine the preferences associated with specific traits, I defined the systematic portion of utility (V_{ij}) as

$$V_{ij} = \beta_{0j} + \beta_1 x_{ij1} + \beta_2 x_{ij2} + \cdots + \beta_n x_{ijn} \tag{2}$$

where x_{ij1}, x_{ij2}, …, x_{ijn} are the n trait values associated with the jth alternative in the ith choice scenario, β_{0j} is an intercept explaining utility of the jth alternative as a result of factors other than the n traits included in the model, and $\beta_1, \beta_2, \ldots, \beta_n$ are preference coefficients associated with each of the n traits.

The DCM assumes that decision-makers select alternative k if $U_{ik} > U_{ij}, \forall k \neq j$. Therefore, the probability of a decision-maker selecting the k^th^ alternative is given by (Louviere, Hensher, and Swait 2000):

$$P_{ik} = P\left(V_{ik} + \varepsilon_{ik} > V_{ij} + \varepsilon_{ij}\right), \forall k \neq j. \tag{3}$$

With a Gumbel error distribution, equation 3 reduces to the multinomial logit model (Boxall and Adamowicz 2002):

$$P_{ik} = \frac{e^{Vik}}{\sum_{j \in C} e^{Vij}} \tag{4}$$

The model may be fit using the method of maximum likelihood, provided that one of the potential alternatives in the overall experiment (i.e., one of the seed types) is defined as a reference case and assigned a fixed utility, typically 0. The odds of selecting alternative k over a reference alternative r are (Louviere et al. 2000):

$$\frac{P_{ik}}{P_{ir}} = \frac{e^{Vik}}{e^{Vir}} = e^{Vik-Vir} \tag{5}$$

and the logarithm of the odds of choosing k over r is (Louviere, Hensher, and Swait 2000):

$$log\left[\frac{P_{ik}}{P_{ir}}\right] = V_{ik} - V_{ir} = \sum_n \beta_n (X_{ikn} - X_{irn}) \tag{6}$$

Therefore, the model may be fit by setting the utility of the reference alternative r to 0 and solving j-1 simultaneous linear equations for the log odds of selecting j-1 alternatives over r in a choice scenario (Louviere, Hensher, and Swait 2000). Derivations and further mathematical details can be found elsewhere (McCracken et al. 1998; Louviere et al. 2000).

Seed caching and seed consumption involve different physical behaviors, time scales, and risks, and squirrels are therefore expected to adjust the value that they place on seed traits depending on how they use the seed (Lichti et al. 2015; Sundaram et al. 2015). To account for the outcome of seed selections, I used an indicator variable for caching (1 = cached, 0 = consumed) and interaction terms to estimate different coefficients for traits when seeds were cached vs. when seeds were consumed. Additionally, I created a third alternative of opting to ignore a seed pair in order to accurately model all possible alternatives available to a squirrel. This third alternative had a value of 0 for all seed traits and took a value of 1 for an indicator variable called 'ignore' if the squirrel investigated the presented seeds but left the area without taking either seed (if a seed was selected, 'ignore' = 0). I interpret the utility of 'ignore' as the value that the squirrel perceives in leaving to pursue other activities; in other words, the utility of ignore represents the missed opportunity cost associated with selecting a seed.

I fit three alternative nested models to squirrel choices using programs NLOGIT and LIMDEP. I used likelihood-ratio tests to compare models. The first model included continuous variables used to design the choice experiments namely kernel size (g), toughness (Nm), protein content of kernel (g) and tannin content of kernel (g). I also included the ignore variable and interactions of all traits with the indicator caching variable. Lipid concentration of kernel was also added to the model and evaluated for significance. Because protein content and lipid concentration were not significant in any model, these variables were removed. Additionally, tannin content of kernel (g) was found to be redundant with kernel size (g), and thus I replaced tannin content with tannin concentration (g per 100g of kernel) in all models. Therefore, model 1 can be given as:

$$V_{ij} = \beta_1 Kernel_j + \beta_2 Tannin_j + \beta_3 Tough_j + \beta_4 Kernel_j * cache_j + \beta_5 Tannin_j * cache_j + \beta_6 Tough_j * cache_j + \beta_7 Ignore_j \quad (7)$$

In the second model, I added dormancy period (measured by average number of cold stratification days required to germinate a given seed type, Sundaram et al. 2015) along with the interaction of dormancy period and caching as predictors. Although not used to design the choice experiments, I included dormancy period in model 2 because several studies have shown that squirrels can detect dormancy status of seeds and are more likely to consume rather than cache early germinating seeds (Smallwood et al. 2001; Steele et al. 2001; Steele et al. 2006). Model 2 can be expressed as:

$$V_{ij} = \beta_1 Kernel_j + \beta_2 Tannin_j + \beta_3 Tough_j + \beta_4 Dormancy_j + \beta_5 Kernel_j * cache_j + \beta_6 Tannin_j * cache_j + \beta_7 Tough_j * cache_j + \beta_8 Dormancy_j * cache_j + \beta_9 Ignore_j \quad (8)$$

Finally, in the third model I added an indicator variable to quantify how seed familiarity influenced utility of a seed (1=unfamiliar seeds which included chestnut species, 0=familiar seeds). A seed species was considered familiar if the tree species occurred within the geographic extent of the study area. This additional parameter resulted in the following model structure:

$$V_{ij} = \beta_1 Kernel_j + \beta_2 Tannin_j + \beta_3 Tough_j + \beta_4 Dormancy_j + \beta_5 Kernel_j * cache_j + \beta_6 Tannin_j * cache_j + \beta_7 Tough_j * cache_j + \beta_8 Dormancy_j * cache_j + \beta_9 Novel_j + \beta_{10} Ignore_j \quad (9)$$

I compared mean β ± 1SE intervals of coefficients for traits from multinomial logit models fit separately to the first and second phases of data collection to verify that choices were driven by seed traits rather than seed types. If intervals from the two models overlap for each trait, no significant differences between models can be concluded at α of 0.05 (Payton et al. 2003).

Random parameters logit model

If individuals in a population are heterogeneous in their preferences for modeled traits, then inferences made from the multinomial logit model can be incorrect (Olynk et al. 2010). Conversely, if no heterogeneity exists among individual choice events, then either a single squirrel was responsible for all choices (virtually impossible, given my sampling design) or squirrels behave consistently with one another and individuality can therefore be ignored from a statistical perspective. Heterogeneity is incorporated into the model by including a random utility term for each trait in a choice set. Therefore, equation 1 can be rewritten as (Tonsor et al. 2005):

$$U_{ijt} = V_{ij} + \left(u_{jt} + \varepsilon_{ij}\right) \tag{10}$$

where u_{jt} is the random utility estimate for the tth individual on the jth alternative distributed identically and independently across individuals and alternatives. The systematic portion of utility is still specified as a function of n traits as in equation 2. However, the addition of the random utility term across individuals allows for the estimation of variation around β coefficients due to individual differences in preferences for each trait (Louviere et al. 2000) (equivalently, $\mathbf{u}_{jt} = \boldsymbol{\gamma}_t\mathbf{X}_j$, so that V_{ij} are fixed effects and $\gamma_t \sim N(0, \Sigma)$ are random, individual effects on preference). If the estimated standard deviation of u_{jt} is close to 0, then variability across individuals is considered to be negligible and suggests homogeneity of preference for the trait. A standard deviation significantly different from 0 indicates heterogeneity of preferences for a specific trait (Hensher et al. 2005).

I fit a random parameters logit model for the traits in the best-fit model of the previous section, under the assumption that all selection events made at the same location were made by the same squirrel. Although I did not mark individual squirrels in this study, observers were able to visually track individuals between presentations at a given location. While I cannot be certain that the same individuals did not visit multiple locations, I believe this to be unlikely given my attempts to segregate locations geographically. Therefore, I estimated heterogeneity in squirrel preferences for traits across locations as a post hoc check for variability in individual preferences. The model was fit using programs NLOGIT and LIMDEP.

Willingness to trade (WTT)

An advantage of discrete choice models is the ability to estimate an animal's willingness to exchange value derived from different traits using willingness to trade, also referred to as the marginal rate of substitution (Cooper and Millspaugh 1999). The marginal utility of a continuous trait g is defined as the partial derivative of utility with respect to g, $\partial U/\partial g$. The exchange rate between g and another trait, h, is obtained by scaling the marginal utility of h so that it offsets a unit change in g and maintains the same constant utility value. That is, I need to know the value $W_{g,h}$ where $W_{g,h} = -(\frac{\partial U}{\partial h} \div$

$\frac{\partial U}{\partial g}$) and is interpreted as the amount of trait h that must be increased or decreased to offset a unit increase in trait g in order to maintain a constant utility. In a multinomial logit model that lacks interaction terms, $\partial U/\partial g = \beta_g$, so,

$$W_{g,h} = -\frac{\beta_h}{\beta_g} \tag{11}$$

For the multinomial logit model specified in equation (9), the WTT estimate of kernel for tannins can be given as

$$W_{kernel,tannins} = -\frac{(\beta_2+\beta_6*Cache)}{(\beta_1+\beta_5*Cache)} \tag{12}$$

where ‘cache’ is the indicator variable used to represent seed fate. Therefore, for a consumed seed the above equation becomes $-\beta_2/\beta_1$. It should be noted that these calculations are valid only if no heterogeneity exists in preferences and should be performed with coefficients from a multinomial logit model. I estimated the confidence intervals around mean marginal utilities for all seed traits from the best-fit multinomial logit model using the Krinsky and Robb method (Krinsky and Robb 1986). If the 95% CIs around the marginal utility of a trait overlapped 0, then the trait was considered to have a utility that is not significant. I did not compute WTT estimates for such traits because ratios involving 0 would either yield 0 or approach infinity and generate uninformative estimates.

3.4 Results

I presented a total of 426 seed pairs to eastern gray squirrels across 27 locations on the Purdue campus, out of which 307 (71.8%) trials resulted in seeds being cached, 119 (27.9%) resulted in seeds being consumed and 1 seed pair was ignored. The coefficient estimates were not significantly different between first and second phases of data collection (mean β ± 1SE intervals overlapped for all traits), providing verification that traits rather than seed species were determinants of selection. Therefore, I pooled data from the two phases of data collection and fit models 7-9. Of the 3 models, the most complex model (equation 9) was selected as the best model since this model significantly improved fit (Table 3).

Coefficients for interactions of seed traits with caching were significant. In particular, the utility of seeds chosen for consumption was positively associated with toughness of seed and negatively with species' dormancy periods (Table 4). On the other hand, the utility of seeds being cached was positively related to tannin concentration, kernel size, and dormancy period. Finally, a significant negative coefficient was found for the 'ignore' variable and a significant positive coefficient was associated with seed unfamiliarity (Table 4). The random parameters logit model showed no significant variation in preferences among locations with respect to any trait ($p>0.5$ for all trait standard deviations, suggesting standard errors of random trait coefficients are not significantly different from 0).

Willingness to trade estimates were calculated separately for consumed and cached seeds, and were different for different combinations of traits (Table 5). Mean WTT estimates were not computed for 26 of 36 seed trait combinations because at least one of the marginal utilities in the combination was not significantly different from 0 (Table 5). For consumed seeds, positive WTT estimates between toughness and dormancy period were positive, indicating that for selection of a seed for consumption to remain constant, any change in dormancy period would need to be accompanied by a corresponding, parallel change in toughness, or vice versa. For cached seeds, negative

WTT estimates were computed between kernel mass and toughness, kernel mass and tannins, and toughness and tannins. Therefore, for a seed to maintain a constant desirability for caching relative to other species, a decrease in kernel mass could be offset by increased toughness or increased tannin content, or a mixture of both. Similarly, an increase in kernel mass or tannin content could compensate for reduced toughness.

To aid in interpretation of WTT estimates, I plotted lines of indifference for consumed seeds using coefficient estimates from equation (9) (Fig. 2). Lines of indifference represent combinations of two traits for which utility remains constant. The slope of the line is equal to the WTT estimate for the two traits. For consumed seeds I created three lines of indifference to represent tradeoffs between toughness and dormancy period at utilities of 1, 0.02, and -1. For all three lines, I set tannin concentration at 0.5% TAE, kernel mass at 1g and assumed seed type is familiar. Overlaying the seed species with the highest estimated utility when consumed in Fig. 2, *Juglans cinerea*, *J. nigra*, *Quercus macrocarpa*, and *Q. prinus* fall close to the line of indifference at utility of 0.02, indicating that these seeds are approximately equivalent in terms of their desirability for consumption. All other seeds were of lower utility when selected for consumption and, if plotted, would occur below the -1 line of indifference (see e.g. *Castanea dentata* in Fig. 2).

More complex trading scenarios occurred for cached seeds. In this case, finite WTT estimates for three traits yield a 3-dimensional surface that represents combinations of kernel mass, toughness, and tannin concentrations with equivalent utility (Fig. 3; the dotted grid shows the surface at utility = 1). Slopes of the surface equation equal the WTT estimates and are interpreted for two tradable traits while maintaining the third trait a constant. For example, if tannin concentrations remain unchanged, then a constant utility is maintained if kernel mass increases by 1 g but toughness declines by 1.24 Nm (Table 5). I plotted observed values of toughness, tannins and kernel mass for *Quercus velutina*, *Q. macrocarpa*, *Carya ovata*, and all of the *Juglans* species used in this study. Three species, *Q. velutina*, *Q. macrocarpa*, and *C. ovata*, occur close to the plotted surface of indifference, suggesting these species have equivalent desirability for caching. In contrast, all *Juglans* species occur well above the surface and are therefore much more desirable than the other species as cached resources.

3.5 Discussion

3.5.1 Applying DCEs to an ecological system

I believe this study is the first to present discrete choice experiments to free-ranging animals based on a design informed by pre-determined traits. In theory, a main advantage of DCEs is the ability to create balanced and orthogonal fractional design with correlated traits. In practice, while I successfully parsed out the effects of moderately correlated traits, I faced some constraints in applying the approach to squirrel preferences for hardwood seeds. Developmental and evolutionary constraints have resulted in a high degree of correlation for some seed traits (Sundaram et al. 2015); consequently, some combinations of traits are not found in real seeds of any species. This issue of biologically missing trait combinations is likely to occur in many systems.

Missing traits may also arise if traits are inadequately sampled, although this is not likely for the seeds used here (Sundaram et al. 2015). If only a few trait combinations are missing, a choice experiment may still be designed with existing traits. D-efficiency of the resulting design, however, will be lower when compared to a design incorporating missing trait combinations.

The interpretation of coefficients and intercepts must be carefully considered when planning discrete choice experiments based on traits. The experiment used a design that was optimized over combinations of theoretical combinations of trait values, not combinations of seed species. During data collection, each combination of trait values could be represented by any of a number of actual seed species. Consequently, structuring models so that the intercept terms corresponded to one of the alternatives in a presentation (i.e., for alternative 1 or alternative 2) would not have yielded interpretable results since species representing the alternative changed across scenarios. Instead, I estimated an intercept for the third alternative available to squirrels, i.e., ignoring a seed pair. I interpret this coefficient as the perceived value of leaving to forage elsewhere or pursue other activities. It therefore provides an estimate of the missed opportunity cost associated with selecting seeds. Allowing for the option to ignore alternatives and not make a choice between available options is important in certain decision-making frameworks, but is often overlooked in traditional ecological resource selection models.

I designed the experiment with algorithms that maximize D-efficiency and used a multinomial logit modeling approach, but alternatives may be appropriate in some instances. D-efficiency is computed from the geometric mean of the determinants of candidate designs. Although D-efficiency is considered to be a standard metric, other metrics have been proposed, including A-efficiency and G-efficiency (Kuhfeld et al. 1994). Similarly, alternatives exist to the multinomial logit framework used here, including Bayesian choice models that offer additional flexibility when compared to traditional choice models. For example, Bayesian methods may include non-normal distributions for coefficients and allow for situations where the full choice set is unknown (Train 2003).

3.5.2 Seed selection by eastern gray squirrels

The methods and models outlined here allow general inferences to be made about seed selection by eastern gray squirrels and provide novel insights regarding the effects of seed traits on squirrel foraging and caching behaviors. Separate models for the first and second phases of this study showed a high degree of overlap between coefficient estimates despite changes in the seed species used to represent trait groups. This result strongly suggests that seed traits, and not species identities, drove selections made by the eastern gray squirrels in experiments in this study.

Further, the preferences for seed traits observed in this study corroborate previous findings. I found that the percentage of tannins in seed kernels significantly increased utility for caching and decreased utility for consumption, in agreement with other studies that show that eastern gray squirrels are sensitive to tannins and reject portions of kernels that contain relatively high concentrations of tannins when consuming seeds, but also preferentially cache high-tannin seeds (Steele et al. 1993). Mass of a seed may be increased by either increased shell mass and thus toughness, or by increased kernel mass and results show that both these factors positively influence utility for caching. Toughness and not percent lipid concentration in the kernel was a significant and positive predictor of seed selection for caching by eastern gray squirrels (Table 4), which supports previous assertions that selection by squirrels in the fall follows a time-minimization strategy rather than an energy-maximization strategy (Thompson and Thompson 1980).

Specifically, tougher shell seeds have a higher consumption time and are cached more often to reduce time spent handling these seeds and increase time available to cache additional seeds in the fall (Jacobs 1992; Sundaram et al. 2015; Thompson and Thompson 1980). It should be noted though that in the seed set of this study, toughness is highly correlated to lipid concentration and thus to energy content; seeds of Betulaceae and Juglandaceae (including walnuts and hazelnuts) have evolved both thicker shells and higher lipid concentrations when compared to seeds of Fagaceae (including oaks and chestnuts) (Sundaram et al. 2015). Thus, among consumed seeds, the selection of tough shell seeds by squirrels in conjunction with long dormancy periods results in the selection of the highly energetically valuable walnuts (*Juglans nigra* and *Juglans cinerea*) for consumption (Table 4, Fig. 2). Utility for caching was also influenced positively by kernel mass (Table 4). All else being equal, large kernels reflect high total caloric content thus making seeds more attractive to rodents and increasing probability of seeds being cached (Grubb and Burslem 1998; Yang et al. 2012).

Apart from corroborating existing findings, I tested and found additional seed trait trends that merit more attention. Total protein content of a seed was not a significant predictor of utility, in contrast to systems where rodents were found to maximize protein intake (Henderson 1990). Although several studies have found that seeds with higher protein content are selected more frequently (Lichti et al. 2015), these studies generally have relied on observational, post hoc correlations and could not unambiguously attribute selection to protein content. Finally, I found a significant positive effect of unfamiliar seeds on utility, implying that squirrels were more likely to choose unfamiliar chestnut seeds. While the addition of this variable significantly improved fit of the model (Table

3), I cannot determine from my results if squirrels are more likely to select chestnuts due to an unobserved trait or if squirrels are more likely to select chestnuts because they are unfamiliar. Studies directly comparing familiar and unfamiliar seeds are required to address this question.

Squirrels in this study were strikingly consistent in their preferences for seed traits. Such consistency in selection of seed traits across individuals, and extreme preferences for a trait, is not well documented in the ecology literature. Although these results could arise because I consistently presented seeds to the same set of squirrels, this is unlikely given that squirrels are abundant on Purdue University's >900ha campus, presentations were separated by a minimum of 150m, and presentations were spread across the campus. Consistency in seed preference of rodents has been noted in other study systems (Lobo et al. 2009) as well as reviews that have compared preferences among rodent consumers (Vander Wall 2010; Lichti et al. 2015).

Squirrels were consistent in preferences for seed traits. In contrast, consumer economic discrete choice models evaluating utility of a product with respect to product traits typically find heterogeneity in responses of human consumers (Ortega et al. 2011). Human heterogeneity is attributed to differences in taste of consumers (Olynk and Ortega 2013) and probably reflects the low fitness consequences of decision studied in consumer economics. When it occurs, heterogeneity in responses of free-ranging animals is sometimes attributed to differences in experience or age and differences in effort expended by individuals (Mauck et al. 2012). However, homogenous responses such as those I observed are likely a result of natural selection. For free-ranging squirrels, creation and maintenance of caches during fall is critical to ensure survival during winter when food is scarce (Nixon et al. 1975). In a separate study, I determined that significant proportions of foraging behaviors of eastern gray squirrels were explained by hardwood tree phylogeny, and that the seed traits explored in this study show different phylogenetic signals (Sundaram et al. 2015). The consistency reported here in selection of seed traits also indicates that squirrels may exert selective pressures on hardwood trees by consistently caching seeds with large kernel sizes, tougher shells and higher tannin concentrations.

Analysis of squirrels' marginal utilities and willingness to trade among different seed traits provides new insights into the interactions between trees and scatter-hoarding rodents. Previous theoretical and empirical work has shown that seed dispersal effectiveness in hoarded trees depends in part on the composition of seed crops available to rodents and the rodents' perception of different species values for consumption or caching (Lichti et al. 2014). Trees that increase the consumption utility of their seeds relative to their neighbors while decreasing their relative caching utility could face significant negative selective pressure. On the other hand, any tree that can decrease consumption utility and increase caching utility stands to benefit from hoarder behavior. Viewed from this perspective, it appears that hoarders do not exert directional selection on traits individually (e.g., selecting for larger seeds), but instead exercise directional selection on trait vectors, resulting in predictable patterns of trait co-occurrence. For example, either a decrease in the length of overwinter dormancy or an increase in toughness (and energy content) will make seeds more attractive for consumption (Table 4). However, assuming that neighbors remain static, simultaneous increases in both toughness and dormancy as seen in the shift from white oaks (*Quercus* section Quercus) to walnuts (*Juglans* sp) could maintain a tree's desirability for consumption at a constant level and avoid placing the tree at a disadvantage to its neighbors. The squirrels' behavioral response (expressed by its utility function) ties these two traits together and leaves some evolutionary trajectories more open than others. According to Table 4, desirability for consumption would be minimized in a low-toughness, high-dormancy seed. However, this phenotype may not be achievable in some species because the shell may also function to impede germination (Baskin and Baskin 2001).

In contrast to consumption, WTT estimates for cached seeds were negative (Table 5). As a result, increased kernel mass (e. g., *Q. macrocarpa*), increased toughness (e. g., *C. ovata*), increased tannin concentrations (e. g., *Q. velutina*), or increases in any combination of these traits would increase a seed's desirability for caching (Fig. 3). Given such conditions, squirrel behavior might be expected to drive diversification of seed types as various species develop along alternative evolutionary pathways. From the squirrel's perspective, the flexibility that results from trading off a larger number of traits may allow hoarders to reduce the time spent examining seeds in the fall when competition to cache is high and squirrels need to cache high numbers of seeds to ensure availability of food during winter (Thompson and Thompson 1980).

To further test the hypothesis that seed traits such as kernel size and tannin concentration are truly interchangeable from the squirrel's point of view at the estimated WTT values, an artificial seed experiment could be devised wherein these traits are manipulated. For example, an artificial seed with 2.9% TAE tannin concentration could be paired with a second seed having a 0% TAE but a larger kernel mass (larger by 1 g). If the two constructed seeds have the same total perceived value, as is suggested by the final model, then a squirrel should be equally likely to cache either of the constructed seeds, all else being equal. Further a discrete choice experimental framework would be useful to evaluate selection of traits in an artificial seed study to shed light on how squirrels perceive artificial seeds and assess whether results from artificial seed experiments can be extrapolated to hardwood tree seeds.

3.5.3 Conclusions

My study illustrates a designed approach to examine resource selection by animals. I suggest that the designed approach is useful for studying effects of correlated traits on resource selection, and particularly when testing in a full factorial design is not feasible. With my final model, I showed that eastern gray squirrels choosing between two seeds select for specific seed traits including dormancy period, tannin concentration, kernel mass and toughness of a seed. Moreover, I found that selection for traits is invariant across squirrels. This approach allowed me to compute tradeoffs between seed traits and show that squirrels may trade among multiple traits when caching seeds and between fewer traits when consuming seeds. The results suggest that tradeoffs by squirrels during fall may have substantial implications for the evolution and diversification of nut-bearing trees.

3.6 Figures

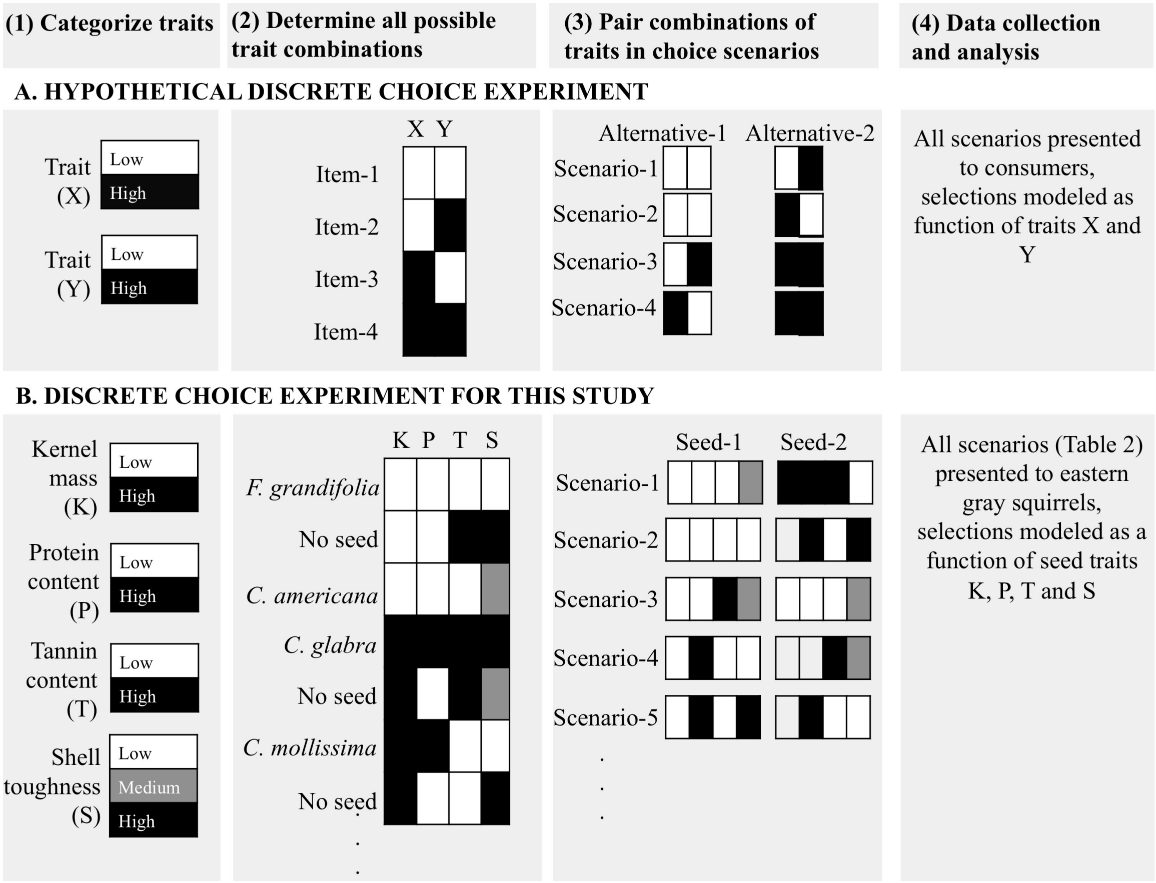

Figure 1. Four steps in designing and analysing data from discrete choice experiments (DCE). Steps outlined for (A) a hypothetical discrete choice experiment with 2 traits and (B) the discrete choice experiment in this study with 4 traits. The four steps include – 1. Categorize traits, 2. Determine all possible alternatives or combinations of traits, 3. Pair trait combinations in choice scenarios, and 4. Data collection and analysis. In A(1), traits-X and Y are categorized into low and high values. In A(2), all combinations of low and high for trait A and B are listed, such that items are represented across rows and traits across columns. In A(3), pairings of trait combinations are considered in a candidate design. Multiple choice scenarios are designed so that each scenario presents two

alternatives, and the combination of scenarios allows for all possible contrasts among trait levels In A(4), choice scenarios are presented to subjects and selections are analysed. In B(1), 4 seed traits are categorized into low and high or low, medium and high. In B(2), examples of all possible combinations of low, medium or high for the 4 traits are listed. Some combinations are represented by naturally occurring seeds, including *Fagus grandifolia*, *Corylus americana*, *Carya glabra*, and *Castanea mollissima*. Others do not occur in natural seeds and are eliminated from the list, shown as 'No seed'. In B(3), pairs of seeds are arranged in a design that attempts to capture all possible contrasts with the minimal number of scenarios. In B(4), choice scenarios are presented to squirrels and selections are analysed.

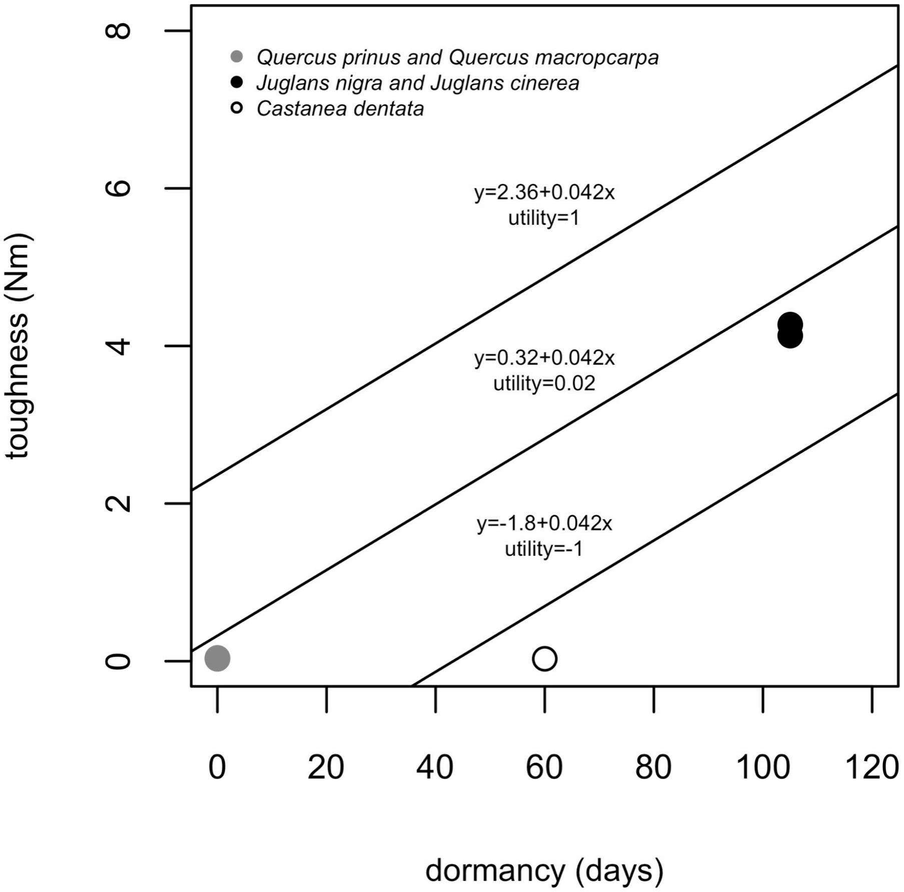

Figure 2. Utility contour lines plotted for varying seed toughness and dormancy period. Contours represent lines of indifference at utilities of 1, 0.02, -1, assuming a familiar seed with 0.5% TAE tannin concentration and 1g kernel mass that is consumed instead of cached. The lines of indifference represent combinations of toughness and dormancy viewed as tradable by eastern gray squirrels (*Sciurus carolinensis*) at the specified utility. Dormancy and toughness plotted for *Quercus macrocarpa*, *Quercus prinus*, *Juglans nigra*, *Juglans cinerea* and *Castanea dentata*. The two *Quercus* sp and two *Juglans* sp occur close to line of indifference at 0.02, indicating that these seeds are approximately equally likely to be consumed in pairwise trials. In contrast, *C. dentata* occurs below the -

1 line of indifference, indicating that this species is less likely to be consumed when presented with one of the *Quercus* or *Juglans* species.

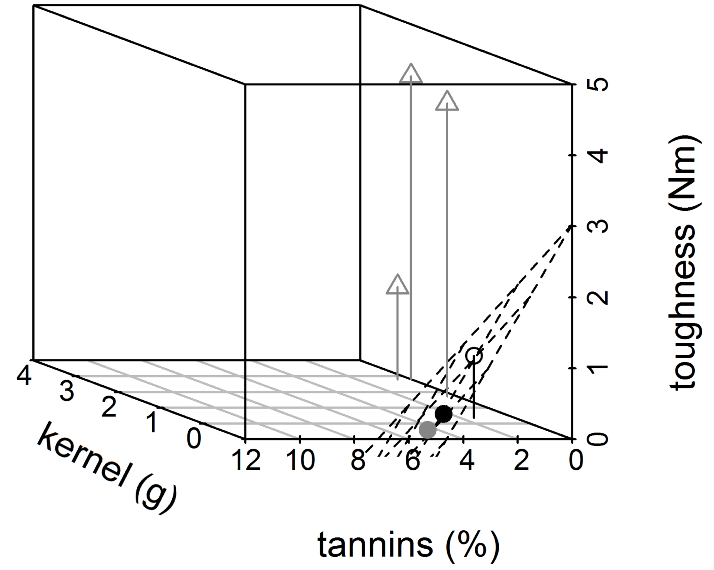

Figure 3. Utility contour surface plotted for varying seed toughness, kernel mass and tannin concentration. Surface of indifference (dotted grid, equation: toughness = 3.03 - 1.24*kernel - 4.24*tannin) plotted at a utility of 1 for a cached, familiar seed representing combinations of toughness, kernel mass and tannins viewed as tradable by eastern gray squirrels (*Sciurus carolinensis*). Toughness, kernel size and tannin concentration plotted for *Quercus macrocarpa*, *Quercus velutina*, *Carya ovata*, and *Juglans* species. *Q. macrocarpa*, *Q. velutina* and *C. ovata* occur on the same surface of indifference, indicating that these seeds are tradable with one another. In contrast, the three *Juglan*s sp occur well above the plotted surface of indifference, indicating that they are more likely to be cached when presented with *Q. macrocarpa*, *Q. velutina* and *C. ovata*.

3.7 Tables

Table 1. Breakpoints used to delineate attribute levels and create discretized seed traits in the experimental design.

Seed attribute	Attribute levels	Breakpoints
Kernel dry mass (g)	Low	< 2g
	High	> 2g
Protein in kernel (g)	Low	< 0.18g
	High	> 0.18g
Tannin in kernel (g)	Low	< 0.05g
	High	> 0.05g
Toughness (Newton-meter)	Low	< 0.088Nm
	Medium	> 0.088Nm, <0.88Nm
	High	> 0.88Nm

Table 2. Paired seeds (or choice scenarios) designed based on seed traits and presented to squirrels. Species 1 and 2 were presented to eastern gray squirrels (*Sciurus carolinensis*) by balancing seeds on golf tees. Seeds marked with * were used in the first phase of data collection. In the second phase of data collection, whenever multiple species could represent attribute combinations, one species was randomly selected from the list.

	Species 1					Species 2				
Seed pairs	Kernel mass	Protein mass	Tannin mass	Tough-ness	Seed type	Kernel mass	Protein mass	Tannin mass	Tough-ness	Seed type
1	Low	Low	Low	Medium	*Corylus americana*	High	High	High	Low	*Quercus rubra** or *Quercus prinus*
2	Low	Low	Low	Low	*Fagus grandifolia**, *Q. velutina*, *Q. muehlenbergii*, *Q. palustris*, chestnut	Low	High	Low	High	*Carya tomentosa*

					hybrid, *Q. macrocarpa*, *Q. alba* or *Q. coccinea*					
3	Low	Low	High	Medium	*Carya ovata* or *Carya cordiformis**	Low	Low	Low	Medium	*Corylus americana*
4	Low	High	Low	Low	*Castanea dentata*	Low	Low	High	Medium	*Carya ovata* or *Carya cordiformis**
5	Low	High	Low	High	*Carya tomentosa*	Low	High	Low	Low	*Castanea dentata*
6	Low	High	Low	High	*Carya tomentosa*	High	High	Low	High	*Juglans nigra**, *Juglans cinerea* or *Juglans regia*
7	High	High	Low	High	*J. nigra**, *J. cinerea*, or *J. regia*	Low	Low	Low	Low	*F. grandifolia**, *Q. velutina*, *Q. muehlenbergii*, *Q. palustris*, BC3 chestnut hybrid, *Q. macrocarpa*, *Q. alba* or *Q. coccinea*

8	High	High	Low	High	*J. nigra**, *J. cinerea*, or *J. regia*	High	High	High	Medium	*Q. michauxii*
9	High	High	High	Medium	*Q. michauxii*	Low	High	Low	High	*Carya tomentosa*
10	High	High	High	Low	*Q. rubra** or *Q. prinus*	High	High	Low	Low	*Castanea mollissima*

Table 3. Comparisons of models fit to seed selection events. The most complex model (equation 9) was selected as the best-fit model.

Model	Log Likelihood	No. of parameters	Likelihood ratio test statistic	p-value
Ignore + Unfamiliar + Tannin + Kernel + Tough + Dormancy + Tannin (*cache) + Kernel (*cache) + Tough (*cache) + Dormancy (*cache)	-253.74	10		
Ignore + Tannin + Kernel + Tough + Dormancy + Tannin (*cache) + Kernel (*cache) + Tough (*cache) + Dormancy (*cache)	-256.72	9	5.97	0.014
Ignore + Tannin + Kernel + Tough + Tannin (*cache) + Kernel (*cache) + Tough (*cache)	-261.56	7	9.69	0.008

Table 4. Best-fit model (equation 9) parameterized for choices made by eastern gray squirrels (*Sciurus carolinensis*). Bold, italicized rows represent variables significant at α of 0.05.

	Coefficient	Standard error	t - ratio	P - value	Lower C.I.	Upper C.I.
Tannin (%)	-0.071	0.058	-1.220	0.222	-0.184	0.043
Tannin*Caching	**0.212**	**0.067**	**3.164**	**0.002**	**0.081**	**0.344**
Kernel (g)	-0.104	0.139	-0.748	0.455	-0.377	0.169
Kernel*Caching	**0.514**	**0.169**	**3.051**	**0.002**	**0.184**	**0.845**
Toughess (Nm)	**0.477**	**0.160**	**2.983**	**0.003**	**0.163**	**0.790**
Toughess*Caching	-0.148	0.183	-0.809	0.419	-0.507	0.211
Ignore	**-5.616**	**1.172**	**-4.791**	**0.000**	**-7.913**	**-3.319**
Dormancy (days)	**-0.021**	**0.007**	**-3.133**	**0.002**	**-0.035**	**-0.008**
Dormancy*Caching	**0.023**	**0.008**	**2.848**	**0.004**	**0.007**	**0.040**
Unfamiliar	**0.660**	**0.272**	**2.423**	**0.015**	**0.126**	**1.194**

Table 5. Marginal utilities and Willingness to trade (WTT) estimates for all seed traits. Mean marginal utilities and 95% confidence intervals for seed traits under conditions of caching and consumption. Mean willingness to trade (WTT) estimates for all paired seed traits.

	Marginal utilities of consumed seeds			Marginal utilities of cached seeds		
Variable	Lower CI	Mean	Upper CI	Lower CI	Mean	Upper CI
Kernel mass (g)	-0.366	-0.100	0.165	0.212	0.412	0.607
Toughness (Nm)	0.152	0.472	0.804	0.106	0.328	0.548
Dormancy (days)	-0.035	-0.021	-0.009	-0.007	0.002	0.011
Tannin concentration (%)	-0.183	-0.072	0.041	0.034	0.142	0.249
	Willingness to trade column attribute (x) for row			Willingness to trade column attribute (x) for row		

	attribute (y) when seeds are consumed				attribute (y) when seeds are cached			
	Kernel mass (g)	Toughness (Nm)	Dormancy (days)	Tannin concentration (%)	Kernel mass (g)	Toughness (Nm)	Dormancy (days)	Tannin concentration (%)
Kernel mass (g)	NA	-	-	-	NA	-1.254	-	-2.899
Toughness (Nm)	-	NA	22.365	-	-0.798	NA	-	-2.313
Dormancy (days)	-	0.045	NA	-	-	-	NA	-
Tannin concentration (%)	-	-	-	NA	-0.345	-0.432	-	NA

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CHAPTER 4. FREQUENCY-DEPENDENT HOARDING BY *SCIURUS CAROLINENSIS* OCCURS WITH SEEDS OF SIMILAR PERCEIVED VALUE

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4.1 Abstract

Seed predation and rodent foraging behaviors depend on frequency of available seeds and seed traits. However, the interaction of frequency of seed availability and seed traits adds a new level of complexity to granivore-seed dynamics. I conducted experiments with eastern gray squirrels (*Sciurus carolinensis*) designed to elucidate the frequency × trait interaction. I hypothesized that frequency-dependent caching should occur only among pairs of seeds that are relatively similar in attributes that affect their perceived value as a storable food item. I also tested whether caching decisions were dependent on seeds already cached, a variable rarely considered in seed dispersal studies. Frequency-dependent seed caching occurred when seeds of relatively similar value (*Juglans regia* and *Castanea mollissima*) were paired. For this seed pair, caching decisions by *S. carolinensis* were dependent on seeds already cached such that squirrels tended to cache seeds that were either rare in the environment or in the cache. When seeds of very different caching values were paired (*Juglans nigra* and *Corylus americana*), a strong preference for the high-value seed was observed, but no frequency-dependent selection. I conclude that in pairings of seeds of differential caching value, the

highly preferred seed is cached regardless of frequency of availability. In contrast, when seeds of similar value are paired, rarer (more common) seeds are cached at a higher (lower) rate than expected, and this behavior potentially stabilizes seed survival across available seed types leading to increased tree diversity. The results indicate that caching of seeds by squirrels, and by implication recruitment of seedlings into plant populations, are likely driven by complex interactions between the relative frequency of seeds and their traits. I expect similar patterns to occur in any system in which foragers select among resource types that vary in perceived value.

4.2 Introduction

Plants can influence foraging by granivorous rodents through ‘seed-packaging’ strategies and masting (Vander Wall 2010). Physicochemical seed-trait measurements clearly show that hardwood trees produce seed packages that provide a balance of attractive and defensive traits (Chen et al. 2012; Sundaram et al. 2015). Attractive traits increase the probability that granivores will handle and cache seeds; they include large kernel mass or high lipid concentrations that make more energetic rewards available to consumers (Wang and Chen 2012; Yang et al. 2012). Defensive traits such as presence of hard pericarps and high tannin concentrations increase handling costs associated with seeds, and induce caching behaviors (Smallwood et al. 2001; Wang and Chen 2008; Vander Wall 2010). Several studies have examined the role of seed traits on selection of seeds by rodents, but few have examined the interaction between traits and relative seed abundance (but see Xiao et al. 2010).

Relative seed and granivore abundance can result in complex tree regeneration dynamics (Hoshizaki and Miguchi 2005; Xiao et al. 2013a). The differential abundance of multiple types of seeds is influenced by temporal patterns of local plant species composition and seed production (Hoshizaki and Miguchi 2005). Masting, the synchronized production of seeds by trees (Sork 1993; Kelly 1994), modifies both absolute and relative abundance of naturally available seeds and can affect seed selection by granivores (Hoshizaki and Hulme 2002; Klinger and Rejmánek 2009; Lichti et al. 2014). The ‘predator dispersal hypothesis’ posits that an increase in absolute seed abundance results in granivore satiation, which in turn leads to increased probability that seeds will be cached but not recovered by granivores, and hence survive to germinate (Kelly and Sork 2002; Zwolak and Crone 2011; Xiao et al. 2013b). By also considering consumer abundance, Theimer (2005) hypothesized that seed escape and seedling recruitment can be modeled as a function of the ratio of seeds to scatterhoarders, with seed predation predicted to dominate at low seed:scatterhoarder ratios and survival of cached seeds at high seed:scatterhoarder ratios. Although there is evidence that the presence of alternative seed types can significantly influence caching decisions (Lichti et al. 2014), the relationship between frequency of seed types and seed-caching behaviors of granivores remains unclear.

Frequency-dependent selection occurs when selection of prey items by the predator is not exactly proportional to prey availability (Greenwood 1985). Two types of frequency dependence may occur. Positive frequency-dependent selection occurs when a common prey item is selected at higher proportions than predicted by its frequency (Greenwood 1985). One mechanism by which such disproportionate selection of common prey occurs is when there is a high cost to hunting multiple prey items simultaneously (see Greenwood 1984 for additional mechanisms). Therefore, foraging efficiency of the predator is improved by focusing on the more common prey item (Greenwood 1984). An example of positive frequency dependence was observed in experiments where damselfly naiads selected between consuming mobile *Daphnia* prey and sessile Simocephalus prey (Akre and Johnson 1979; Sherratt and Harvey 1993). Naiads adopted an ambush strategy to catch *Daphnia*, whereas a more active search strategy was required to catch sessile Simocephalus. Positive frequency dependence was observed because naiads engaged in the search strategy required to catch the more abundant prey item leading to higher predation rates on the common prey (Akre and Johnson 1979; Sherratt and Harvey 1993). In comparison, negative frequency-dependent selection occurs when a rare prey item is selected disproportionately often (Greenwood and Elton 1979; Allen and Greenwood 1988). Consistent negative frequency-dependent estimates are observed in seed- and bait-selection experiments (Greenwood 1985; Celis-Diez et al. 2004; Celis-Diez and Bustamante 2005). The disproportionate selection of rare prey can occur when prey are crowded or at a high density, and may be a result of rare prey appearing conspicuous when grouped with common prey (Greenwood 1985; see Greenwood 1984 for additional mechanisms). Even in granivore systems where positive frequency dependence occurs, selection may switch to negative frequency dependence if moderate abundances of seed or bait are made available (Greenwood 1985). In rodent-seed experiments, to my knowledge, only 1 weak estimate of positive frequency dependence has been computed (Blythe et al. 2015), which may be attributed to low seed densities occurring in spring when the study was conducted. In contrast, experiments performed during seasons of seed production suggest that negative frequency dependence occurs even at very low seed densities (Celis-Diez and Bustamante 2005).

Rodent seed-selection studies performed thus far suggest that relative seed abundance interacts with seed traits to influence perceived value of seeds to rodents and therefore seed selection (Greenwood 1985; Celis-Diez et al. 2004; Celis-Diez and Bustamante 2005; Xiao et al. 2010). Therefore, rodents may select a less-preferred seed type if it occurs at a moderately high relative abundance (Hoshizaki and Hulme 2002; Xiao et al. 2010). Frequency-independent selection or preference also has been documented, where 1 seed type (*Ulmus glabra*) was consistently preferred by wood mice (*Apodemus*) over another seed type (*Fraxinus excelsior*), leading to the hypothesis that frequency-dependent seed selection occurs more commonly when seeds have low and equal palatability (Hulme and Hunt 1999).

Here, I design a study based on previous work to test whether seed caching by eastern gray squirrels (*Sciurus carolinensis*) results from an interaction between seed traits and relative frequency of seeds available. In previous work (Sundaram et al. 2015, Lichti et al. in press), I identified that: 1) the foraging behaviors of eastern gray squirrels are influenced by the traits of prey (or seeds), as opposed to species affiliation or phylogeny of prey alone; and 2) traits influence the prey selection process by rodents in a predictable fashion. Explicit consideration of these 2 features allows me to rank species based on traits (e.g., shell toughness, tannin concentration, mass of the kernel, etc.) that presumably affect perceived caching/storage value and hence selection. After ranking seeds, in the current study, I selected and presented a highly valued seed type with a less-valued seed type at varying frequencies and compared results to presentations of 2 nearly equally valued and highly palatable seed types at varying frequencies (throughout this paper, “highly valued” refers to the seed’s value for caching, based on seed trait estimates).

The primary goal was to evaluate when frequency dependence occurs. I predicted that:

1. Highly valued seeds would be preferentially cached at all frequencies of presentations in trials in which highly valued and less-valued seed types are paired.
2. Frequency-dependent seed caching would occur when 2 comparably valued seeds are presented.

To test the above predictions, I presented seeds at varying frequencies to captive squirrels under controlled conditions. To evaluate whether caching decisions made in captivity accurately represented those made by free-ranging squirrels, I presented the same seed pairs and frequencies to free-ranging squirrels and compared selections made to captive squirrels.

In addition to the primary goal, I had 2 secondary goals. One was to evaluate the type of frequency dependence. Due to the high abundance and density of seeds available to squirrels in this study, I predicted that, when frequency-dependent caching occurred, negative frequency-dependent caching would occur, i.e., rare seeds would be selected by squirrels at a higher rate than expected based on relative frequency while common seeds would be selected at a lower rate. The last goal was to evaluate if squirrels accounted for cache content when selecting seeds. I predicted that seed caching would vary based on frequencies of seeds in existing caches and not just based on the frequencies of seeds presented. Although most study designs do not explicitly account for the frequency of seeds in caches, squirrels likely remember cache contents and may vary seed selection accordingly. To further evaluate whether cache contents might affect seed selections, I performed a cache-raiding experiment with captive squirrels where I tested whether seed selections by squirrels changed after raiding caches.

4.3 Materials and methods

4.3.1 Experiments with captive squirrels

I live-trapped 12 adult eastern gray squirrels (5 female, 7 male) between 1 July and 10 Aug 2013 in Purdue University's Horticulture Park and Purdue's West Lafayette, Indiana, campus. All squirrels were transported 13 km to Martell Experimental Forest, Purdue University. Each individual was housed in an enclosure (1.8 m × 1.8 m × 2.4 m) provisioned with 2 nest boxes (35.5 cm × 22.8 cm × 45.7 cm), a seed tray, a motion-activated camera (PC900 HyperFire Professional, Reconyx Inc., Holmen, WI) focused on the seed tray, a water bowl, tree limbs for climbing, and a plastic box (63.5 cm × 44.5 cm × 22.8 cm) containing 10 cm of soil to cache seeds. Cages were built of wooden frames with hardware cloth sides and were arranged adjacent to one another separated by a minimum distance of approximately 0.3 m. A black plastic sheet was attached to 1 side of each cage so that squirrels were unable to observe one another.

While in captivity, squirrels were provided a daily diet of rodent block (Teklad 2014 global 14% rodent maintenance block, Harlan Teklad, Madison, WI), hardwood seeds (walnuts, hickories, acorns, and chestnuts), sunflower seeds, fresh apple pieces, and a filled water bowl. All individuals were allowed to habituate to captive environments and acclimate to seeds used in this study for at least 4 weeks, after which seed presentation trials were conducted between 10 Aug and 1 Nov 2013. All methods conformed to ASM guidelines (Sikes et al. 2011) and regulations for animal care and use (PACUC protocol No. 1303000832, Indiana Department of Natural Resources Scientific Purposes License 13-067).

I performed frequency-dependent seed selection trials with 4 seed types. Seed-caching experiments show that eastern gray squirrels preferentially cache high-energy seeds with tough shells (toughness is defined as the product of shell thickness and hardness, see Sundaram et al. (2015) for additional information on seed trait estimation), seeds with high tannin content, long dormancy periods and large kernel mass. The relationship of these seed traits to hoarding has been noted by numerous studies (see Lichti et al. in press for comprehensive review). Using the seed-trait estimates for 23 hardwood tree species from Sundaram et al. (2015), I computed standardized trait values by subtracting the mean and dividing by the standard deviation. I then computed pairwise 4-dimensional Euclidean distances between seed types, which represent differences in seed traits and thus caching value of all seed types. I used this information in 1 trial to pair a seed type of high value (black walnut, *Juglans nigra*) with a seed type of low value (American hazelnut, *Corylus americana*, Euclidean seed-pair distance of 3.95 was greater than 87.7% of other seed-pair distances). In a second trial, I paired 2 seed types with similar value, English walnut (*Juglans regia*) and Chinese chestnut (*Castanea mollissima*, distance of 1.26 was less than 88.2% of other seed-pair distances). The choice of seed pairs was verified with hierarchical agglomerative cluster analysis using Ward's minimum variance criterion implemented by 'hclust' in R 3.0.2; black walnut and hazelnut resided in separate groups until the final cluster was formed, whereas English walnut and Chinese chestnut were among the first pairs of seeds to be clustered together.

Each of the selected seed pairs was presented at 1 of 4 ratios (2:8, 4:6, 6:4, 8:2) to each squirrel for 10 consecutive days. Each seed pair × frequency combination was randomly assigned and presented to 5 different individuals (Table 1). All seeds presented were numbered and labeled with the date of presentation. Between 1600 and 1800 h, 10 numbered seeds in the assigned ratio were placed on seed trays, and motion-activated cameras were switched on. From the cameras, I determined order of seed selection and thus frequency of seed types left on the board after each selection event. Between 0800 and 1000 h the following day, researchers switched off cameras, collected data cards, counted numbers of seeds cached of each type from the previous day, counted numbers of seeds still cached from any previous trials, and replenished trays with rodent block, sunflower seeds, fresh fruit, and water. Cached seeds were found by sifting through dirt in cache boxes, checking corners of cages, and searching nest boxes. Cached seeds were returned unless the treatment explicitly involved raiding of caches. As each trial consisted of 10 seeds at the assigned ratio, and each trial was repeated for 10 consecutive days, a total of 100 seeds was presented to each squirrel for each seed pair × frequency combination (Table 1).

To further determine if selections made by squirrels were influenced by seeds already cached, I performed a separate experiment to compare seed selections made with and without raiding of caches. I randomly assigned 6 captive squirrels to 1 of the following seed ratios: 4:6, 6:4, 8:2 (i.e., relative frequencies of 0.4, 0.6, 0.8). I presented each of these squirrels with black walnut and hazelnut seeds paired at the assigned ratio for 10 consecutive days. All presented seeds were labeled with a seed number and date using a marker. Seed presentations occurred between 1600 and 1800 h, and researchers returned the following day to count cached seeds between 0800 and 1000 h. After 10 days, black walnut and hazelnut seeds were presented at the same ratios to the same squirrels, however, all cached seeds were raided and removed every day for another 10 consecutive days. I was unable to perform the cache-raiding experiment using Chinese chestnut and English walnut seed pairs because of difficulties in obtaining additional seeds within the time frame of this study.

4.3.2 Field trials

To determine if seed selections made by captive squirrels were similar to seed selections made by free-ranging squirrels, I presented free-ranging squirrels across 10 locations on the Purdue West Lafayette campus with the same seed pairs and ratios described for captive squirrels. Presentations occurred between 6 Oct and 20 Nov 2013. Each location was separated by at least 150 m to ensure that selections were made by different squirrels. Seed pairs at the pre-determined ratios were lined up in a row at the base of a tree. Observers waited for squirrels to handle seeds. The first seed type selected was recorded along with seed fate (consumed or cached).

4.3.3 Statistical model

I used the model of Greenwood and Elton (1979) to determine degree of frequency dependence and preference in selection. I modeled selection of each seed as a Bernoulli event (i.e., a binary response of 0 or 1),

$$y_i \sim Bernoulli(P_{i1}) \tag{1}$$

where y_i is 1 if black walnut is cached over hazelnut in the i^{th} selection event, or 1 if Chinese chestnut is cached over English walnut in the i^{th} selection event, and probability of caching seed type 1 in the i^th^ selection event (P_{i1}), conditional on caching of a seed, was given by the equation:

$$P_{i1} = \frac{(V \times n_{i1})^b / (n_{i2})^b}{(V \times n_{i1})^b / (n_{i2})^b + 1} \tag{2}$$

Here, V is preference for seed type 1 over seed type 2 (independent of frequency), b is the degree of frequency dependence, and n_{i1} and n_{i2} are numbers of seed types 1 and 2 available to the squirrel in the ith selection event. If V = 1, one seed type is not consistently preferred over the other. If V > 1, then seed type 1 is more likely to be selected irrespective of frequency. If V < 1, then seed type 2 is more likely to be selected irrespective of frequency. If b = 1, then selection of seeds is proportional to frequency of availability. If b > 1, then positive frequency-dependent selection exists with the common seed type selected disproportionately often relative to its availability. If b < 1, then negative frequency-dependent selection occurs with the rare seed type selected disproportionately often relative to its availability.

After I separated all seed-selection events that resulted in caching, pooled caching events across all squirrels for a particular seed pair, and removed events where only 1 seed type was available, I defined probability of caching seed type 1 (in equation-2) as the probability of selecting a black walnut in the black walnut-hazelnut seed pair and probability of selecting a Chinese chestnut in the Chinese chestnut-English walnut seed pair. I fitted weighted non-linear regressions to equations 1 and 2 with the function 'nls' in R 3.0.2, modified to minimize squared Pearson residuals (Nash 2014). For a Bernoulli event, the Pearson residual for the ith observation is defined as:

$$\frac{y_i - p_{i1}}{\sqrt{p_{i1}(1 - p_{i1})}} \tag{3}$$

where y_i is 1 if black walnut is cached over hazelnut in the i^{th} selection event, or 1 if Chinese chestnut is cached over English walnut in the i^{th} selection event, and p_{i1} is the predicted probability of black walnut or Chinese chestnut being cached in the i^{th} selection event as defined by equation 2 based on relative frequency of availability.

For Chinese chestnut and English walnut seed pairs, initial V and b values were computed for the regressions following methods described by Blythe et al. (2015). For black walnut and American hazelnut, I used multiple initial starting values to ensure convergence to a proper solution. The t-statistics as provided by 'nls' for V and b estimates were recalculated to test whether estimates were significantly different from 1 as opposed to the default comparison to 0. Despite randomly assigning frequency × seed pair combinations to individual squirrels, caching events by individual squirrels may not be independent. Therefore, I computed conservative critical t-values for each coefficient using 2 subtracted from the number of squirrels as degrees of freedom (Blythe et al. 2015). For free-ranging squirrels, selection events resulting in caches were pooled across all 10 locations. After recalculating t-statistics to test for significant difference from 1, critical t-values were computed using 2 less than the number of locations as degrees of freedom (Blythe et al. 2015).

4.3.4 For which seed pairs and in what form does frequency dependence occur?

Using the statistical model and methods described above, I computed frequency dependence (b) estimates for pairings of the highly preferred black walnut and less preferred American hazelnut and compared the value to estimates for pairings of the similarly preferred English walnut and Chinese chestnut. I tested for significant differences from 1 and determined if selection was positively ($b > 1$) or negatively ($b < 1$)

frequency-dependent. I evaluated if these findings were robust by comparing selections made by captive squirrels to selections made by free-ranging squirrels; specifically, I compared estimates of V and b for the 2 groups. I used the method of Payton et al. (2003) to conduct pairwise tests of V and b while adjusting for unequal standard errors. The method relies on computation of multipliers for the standard errors for a specified level of α while adjusting for unequal standard errors of the estimators being compared (Payton et al. 2003). Thus, I compared intervals of V ± 1.3 SE and b ± 1.23 SE across captive and free-ranging squirrels. Overlapping intervals suggest no significant difference at $\alpha = 0.05$.

4.3.5 Do squirrels account for cache content when making selections?

To evaluate if caching decisions by squirrels were dependent on seeds already cached, I compared models where availability was defined only by presented seeds to models where availability was defined by presented seeds as well as seeds already cached (i.e., I compared models where n_1 and n_2 were defined by numbers of seed type 1 and seed type 2 presented in cages, to models where n_1 and n_2 were defined by the numbers of seed type 1 and seed type 2 presented in cages and pre-existing in caches made by the squirrel). The best model was determined based on AIC values. I validated selection of the best model using a leave-one-out cross-validation method (LOO, Ugarte et al. 2016). Briefly, LOO involves sequentially dropping each row of data, fitting V and b coefficients to the remaining data using equations 1-3 and predicting which seed type is selected in the dropped row based on numbers of seeds available and computed V and b estimates. I estimated and compared the percentage of selection events predicted correctly in cross-validation for models in which availability was defined by seeds presented alone and in which availability was defined by seeds presented and cached. To further address whether cache contents affected seed selections, I used a paired t-test to compare numbers of black walnut seeds cached with and without cache raiding across squirrels.

4.3.6 Additional tests

I tested if cache decisions differed between sexes by comparing V and b estimates fit separately to selection events by male and female squirrels. I compared intervals of V ± 1 SE and b ± 1.22 SE to test for significant differences between sexes at $\alpha = 0.05$ and intervals of V ± 0.69 SE and b ± 0.9 SE for $\alpha = 0.1$ (multipliers based on Payton et al. 2003).

To fully understand the implications of estimated preference and frequency dependence terms, I plotted the predicted probabilities of selecting black walnut and Chinese chestnut by captive squirrels and predicted differences across sexes. Plots of American hazelnut and English walnut were not displayed because they were the inverses of the black walnut and Chinese chestnut plots, respectively. The 95% confidence bands were computed from variance-covariance matrices of V and b estimates using the delta-method, implemented by function ‘deltavar’ in package ‘emdbook’ (Bolker 2008, 2015).

4.4 Results

In experiments with captive squirrels, I successfully collected seed-caching data from 7 male and 5 female adult squirrels. I obtained an average of 9.1 days (median = 10, range = 4-10 days) of camera seed data per squirrel and frequency of seed-pair combinations in the captive trials (Table 1). Missing days of seed-selection data were due to logistical problems such as camera failures.

4.4.1 For which seed pairs and in what form does frequency dependence occur?

Across all 502 cached seeds in black walnut and American hazelnut presentations, only 2 hazelnuts were cached (by 1 squirrel, Table 1). The extreme preference for caching black walnuts over American hazelnuts resulted in a large preference term ($V = 8.9$) and no frequency dependence (b not significantly different from 1) in the best-fit model (next best model at Δ AIC of 45.85, Table 2). This result suggests strong preference for black walnut over hazelnut ($V > 1$) and no frequency dependence ($b = 1$) in 109 trials where at least 1 of each seed type was available and number of seeds available was known from camera data. Free-ranging squirrels cached black walnuts across all 27 presentations of black walnut and American hazelnut where caching was observed (Table 1). As a result of these extreme preferences, I experienced convergence problems in non-linear regression models fit to walnut-hazelnut caching events by free-ranging squirrels. In this seed pair, the final estimates of preference for black walnut approached infinity ($V \rightarrow \infty$) and frequency dependence approached 0 ($b \rightarrow 0$, Table 2). I did not use the LOO method to compute percentage correct predictions for this seed pair because captive squirrels almost always cached black walnuts and dropping the few rows of hazelnut selection resulted in final estimates of infinity for the preference term (V) and 0 for the frequency dependence term (b).

In contrast, squirrels cached both Chinese chestnuts and English walnuts (Table 1). The best model fit to 273 caching events of Chinese chestnut and English walnut seeds using equations 1-2 resulted in a preference term not significantly different from 1 ($V = 0.53$) and b significantly < 1 (next best model at Δ AIC of 24.8 and percent selection events predicted correctly declined by 6%, Table 2). This result suggests negative frequency-dependent selection ($b < 1$) in which squirrels selected rare seeds for caching at a higher rate than expected based on frequency, and no preference for either English walnut seeds or Chinese chestnuts ($V = 1$). Similar trends were observed in 32 caching events by free-ranging squirrels, with no preference for either English walnut seeds or Chinese chestnuts ($V = 1$) and negative frequency-dependence ($b < 1$, Table 2). Confidence intervals around the preference (V ± 1.3 SE) and frequency dependence (b ± 1.23 SE) terms from free-ranging and captive squirrels overlapped (i.e., $P >> 0.05$; Fig. 1).

4.4.2 Do squirrels account for cache content when making selections?

Incorporating the numbers of seeds already cached by squirrels into the measure of availability improved fit for Chinese chestnut and English walnut seed pairs but reduced fit for black walnut and hazelnut seed pairs (Table 2). Number of seeds presented was a better predictor of selections of black walnuts over the numbers of seeds cached (Table 2). In the cache-raiding experiment, no significant differences were found between numbers of black walnuts cached before and after cache raiding events ($t_5 = 1.48$, $P = 0.20$). I did not test for differences in V and b terms before and after caches were raided because all squirrels consistently selected black walnut.

4.4.3 Additional tests

Male and female squirrels showed differences in patterns of seed selection for caching. Comparisons of intervals of preferences for Chinese chestnut (V ± 1 SE) and frequency dependence (b ± 1.22 SE) showed minor overlap (Fig. 1). No overlap occurred in preference intervals computed at $\alpha = 0.1$ for preference (V ± 0.69 SE) and frequency dependence (b ± 0.9 SE), suggesting that the P-value for comparisons of V and b between male and female squirrels lies between 0.05 and 0.1. Female squirrels selected Chinese chestnuts and English walnuts in proportion to their frequency of availability ($b = 1$) and showed no preference for either seed type ($V = 1$). In contrast, males showed negative frequency dependence ($b < 1$) and consistent selection of English walnuts over Chinese chestnuts ($V < 1$). I did not test for sex differences in cache decisions when black walnut and hazelnut were paired, because all squirrels consistently selected black walnut.

To evaluate the combined effects of computed preference and frequency dependence terms, I plotted probability of selecting seeds for caching. Predicted probabilities of selecting black walnut in pairings with American hazelnut showed negligible differences when the frequency dependence term (b) was included in the model versus when the frequency dependence term was ignored by setting $b = 1$ (Fig. 2a). In contrast, predicted probabilities of selecting Chinese chestnut by captive squirrels differed when accounting for frequency dependence versus ignoring frequency dependence (Fig. 2b). In this seed pair, the effect of negative frequency dependence was moderate ($b = 0.298$, Table 2) leading to a lower proportion of Chinese chestnuts being cached when compared to the 1:1 line at high frequencies of availability (Fig. 2b). It is important to note that despite the negative frequency dependence, higher proportions of Chinese chestnuts ultimately were cached at higher frequencies of availability (Fig. 2b). Minor differences were observed across sexes in plots of predicted probabilities of selecting Chinese chestnut; predicted probabilities of selection by female squirrels overlapped the 1:1 line, whereas those of male squirrels did not overlap the 1:1 line (Fig. 3).

4.5 Discussion

I show that perceived value and relative availability of seeds interact to influence seed selection for caching. Previous studies have provided evidence of frequency-dependent seed predation and frequency-dependent seed removal (Celis-Diez et al. 2004; Celis-Diez and Bustamante 2005). My study provides additional evidence of seed caching as a frequency-dependent and trait-dependent process (see Xiao et al. 2010).

Preference rather than frequency dependence was important when a seed of high value was paired with one of lower value in experiments with both captive and free-ranging eastern gray squirrels. As expected per the first hypothesis, squirrels preferentially cached black walnuts across all frequencies of availability (Table 2 and gray line in Fig 2a), which is likely due to a specific combination of attractive and defensive attributes that makes them particularly valuable for hoarding. Although both walnuts and hazelnuts represent relatively non-perishable resources, dry kernel mass of a black walnut is 3.8 g, which has a higher total caloric value than a 0.4-g American hazelnut kernel. Further, walnuts have hard shells and long handling times in comparison to hazelnuts (Sundaram et al. 2015). Therefore, squirrels may have preferentially cached black walnuts over hazelnuts to maintain energetically valuable reserves and defer time and energetic investments of processing thick-shelled walnuts to some later time (Jacobs 1992).

Equal palatability of prey items leads to frequency dependence regardless of the absolute attractiveness of a prey. In the experiment, frequency-dependent seed caching occurred when comparatively equally valued Chinese chestnuts and English walnuts were paired. When ranked based on seed attributes, English walnuts are perceived by gray squirrels to be of marginally greater value than Chinese chestnuts (English walnut had higher standardized trait values than Chinese chestnuts), likely due to the relatively harder shells and higher lipid concentrations of English walnuts (Sundaram et al. 2015). Consistent with this observation, English walnut was marginally preferred over Chinese chestnut, but only by captive squirrels (Table 2). Consistent with my secondary hypothesis, a significant frequency-dependent term ($b < 1$) was observed when captive squirrels selected between English walnuts and Chinese chestnuts (gray line in Fig. 2b). Therefore, when seeds of relatively equal value and high palatability co-occurred, caching by squirrels was negatively frequency-dependent, which contradicts previous suggestions that frequency dependence occurs only when seeds of low and equal palatability are paired (Hulme and Hunt 1999).

Patterns of selection observed in captivity were replicated in a more natural environment. Specifically, the extreme preference to cache black walnuts was also observed in free-ranging squirrels, although meaningful V and b estimates were not obtained from models fit to extreme selections (Table 1). Further, captive squirrels showed negative frequency dependence ($b < 1$) and no significant preference ($V = 1$) between English walnut and Chinese chestnut (Table 2). However, standard errors for b and V were 2-4 times larger than standard errors for captive squirrels (Fig. 1, Table 2). The less precise estimates with free-ranging squirrels resulted from the lower number of selection events obtained in field trials compared with captive experiments ($n = 32$ versus $n = 273$). Captive squirrels may also have been more selective when caching seeds due to satiation from the large quantities of food provisioned in each cage. Additionally, seeds produced by trees on campus and already existing in caches can influence selections by free-ranging squirrels and were not measured here. Nonetheless, both free-ranging and captive squirrel selections showed the same qualitative trends of weak preference and negative frequency dependence in Chinese chestnut and English walnut seed pairs, lending support to the hypothesis that seeds of similar value evoke negative frequency-dependent selection.

Squirrels accounted for cached resources when deciding whether to cache seeds of similar value. When seeds of different value were paired, the best model was based only on frequency of seeds presented (Table 2). This result may have been due to the extreme preference that squirrels showed for black walnut. It suggests that, regardless of composition of cached resources, it is profitable for a squirrel to cache only the highly valued seed (black walnut) when paired with a seed of low value (American hazelnut). Accordingly, I did not find a significant effect in cache raiding experiments on seed selection in black walnut-hazelnut pairs. Designs seeking to study the influence of cache raiding on seed selection may find a significant effect within a 10-day time frame only if equally valued seeds are paired. When comparably valued seeds were paired, the frequency of cached resources and seeds presented predicted seed selection better than the frequency of presented seeds alone (Table 2). This result suggests that squirrels not only remember the placement of their caches (Jacobs and Liman 1991), but also account for cache content during foraging. Thus, studies aiming to quantify seed selection for caching of equally valued seeds by rodents should account for existing cached resources, which is a challenging task. Furthermore, the rarer seed type in caches was cached disproportionately more often whereas the common seed type in caches was selected to a lower degree. Such selection of rare seed types would allow the squirrel to maintain diverse cached resources containing multiple seed types. Maintaining mixed seed types in caches may provide benefits in the long-term for squirrels.

The sexes showed differences in their caching decisions within the sample size comparisons that were possible in this study. Estimates of preference and frequency dependence showed significant differences at an α of 0.1 between sexes when seeds of similar value were paired, with males selectively caching high-lipid English walnuts over high-carbohydrate Chinese chestnuts (Table 2, dotted gray line in Fig. 3). In contrast, female squirrels did not discriminate between these seed types and cached seeds in proportion to their availability ($V = 1$, $b = 1$, Table 2, solid gray line in Fig. 3). Further experiments are required to confirm whether such differences in seed selection actually exist. If confirmed, body size could play a role in the differences I observed. Eastern gray squirrels do not show sexual size dimorphism (Koprowski 1994), but average initial weight of males used in this study was greater than that of females (500 g versus 400 g, Table 1). Generally, larger individuals need to accumulate greater amounts of fat (Peig and Green 2009) and may explain why heavier individuals (males) selected English walnuts more often. Differences in foraging decisions by males and females could instead be neurologically based. Sex differences in the size of the hippocampus have been shown for rodents (Lavenex et al. 2000; Burger et al. 2013) and associated with differences in spatial ability and caching behavior of males and females (Jacobs et al. 1990; Burger et al. 2013). For eastern gray squirrels, though, Lavenex et al. (2000) noted sex differences in portions of the hippocampus but no variation corresponding to seasonal changes in caching behavior. Additional work is needed to test consistency in seed selection by sexes and to identify neurological, physiological, or other mechanisms that could explain sex-based differences in seed selection for caching.

In addition to the covariates examined in this study, other individual-level covariates likely play an important role in explaining seed caching behaviors. I designed the study specifically to examine how existing cached resources influence selection. I also found that sex (or body size) could play a role in influencing selection. Other studies have found that personality metrics can influence foraging decisions of consumers (Kurvers et al. 2010; Bergvall et al. 2011). Specifically, risk-taking traits can affect foraging (Dammhahn and Almeling 2012). Additionally, experiences of individuals including prior experiences handling specific prey items likely plays an important role during foraging (Pelech et al. 2010; Berger-Tal et al. 2014). While I tried to minimize the influence of prior experience by acclimating captive squirrels to the seeds used in this study, wild squirrels may still select prey items based on individual experiences. Future studies designed to specifically test the influence of these behavioral and cognitive traits on caching behaviors will be important. Based on the results of this study, I postulate that these processes should play an important role in predicting caching events only when seeds of relatively similar value are paired together in trials.

Negative frequency dependence was observed in selections by both captive and free-ranging squirrels, which matches findings of previous studies and is expected given the high density of seeds provided to squirrels (Greenwood 1985; Celis-Diez et al. 2004; Celis-Diez and Bustamante 2005). Studies of predation suggest that positive frequency dependence stabilizes prey diversity because the more common prey are predated upon heavily whereas the rare prey are consumed to a lower extent (Greenwood 1985). This study, however, estimates selection of seeds for caching. If caching and dispersal improve chances of seed survival and germination (as is predicted by Vander Wall 2010 and the Janzen-Connell hypothesis; Janzen 1970; Connell 1971), then caching as a negative frequency-dependent process is more likely to stabilize tree diversity because rare seeds are cached at a higher rate than expected while common seed types are cached to a lower extent. Thus, negative frequency-dependent caching has important implications for seed survival and forest composition.

My results suggest that masting patterns may combine with forest composition to influence caching behaviors and potentially recruitment patterns. If seed species of differing caching value occur in a forest stand and recruitment depends strongly on caching, then recruitment should depend primarily on the attributes of the available seeds. For example, in a stand of black walnut and American hazelnut, even at a low walnut frequency of 0.2, the probability of walnut being selected is approximately 0.8 (solid gray line in Fig. 2a). Additionally, this high probability of selecting walnut is preserved even if we ignore the frequency-dependent term (i.e., set b = 1, dotted gray line in Fig. 2a) because the preference term is large (V >> 1). This does not suggest that hazelnuts will not be cached, but that walnuts will be cached at a much higher rate.

On the other hand, if seeds of comparable caching value occur in a forest stand, then relative frequency of available seeds will likely influence caching patterns. For instance, in a stand of English walnut and Chinese chestnut with a low chestnut relative frequency of 0.2, probability that Chinese chestnut is selected is approximately 0.3 (solid gray line in Fig 2b), or about twice as likely as a situation without frequency dependence (i.e., set b = 1, dotted gray line in Fig 2b). Blight-resistant hybrids of American (*C.*

dentata) and Chinese chestnut are the focus of large-scale restoration planning in the eastern United States (Jacobs et al. 2013). If chestnuts from restoration plantings are relatively rare compared to seeds of similarly valued species in the forest, negative frequency dependence is predicted to elevate chestnut caching probability above what might otherwise be expected. Of course if financial resources allow, dense restoration plantings of chestnut mixed with other similarly valued species (e.g., several species of oak) should yield greater benefits from caching than sparse restoration plantings; the probability that Chinese chestnut is cached at high relative frequencies of chestnut availability, although lower than the 1:1 line, is still higher than the probability that chestnut is cached when available at low relative frequencies (Fig 2b). For either scenario, the addition of a frequency-dependent component can lead to more specific predictions about caching and thus potentially recruitment and success of restoration efforts for species of interest.

In summary, caching decisions made by gray squirrels, and perhaps other scatter-hoarders, appear to be a complex function of frequency of seed availability, perceived value of seed attributes, and cache contents. I have shown that relative frequency of availability may play no role in influencing decisions on which seed to cache when perceived values of seeds are very different. However, when differences in seed attributes translate to minor differences in caching value of seeds, frequency-dependent caching is likely to occur. More specifically, negative frequency-dependent caching is likely to occur with the rare seed type being cached disproportionately often. In such pairings of comparably valued seeds, gray squirrels take into account the frequency and types of seeds already cached when making selections such that the rare seed types in caches are selectively cached more often, thereby increasing diversity of seed types in caches. I expect that frequency-dependent selection of prey will occur in other predator-prey systems when low-contrast resource options are available. Although future experiments under controlled conditions will be needed to enhance understanding of seed selection, my findings demonstrate that ecological context drives the seed dispersal process (Theimer 2005; Lichti et al. 2014, Lichti et al. in press). Specifically, food availability, relative frequency, and pre-existing cached resources likely influence prey selection by foragers.

4.6. Figures

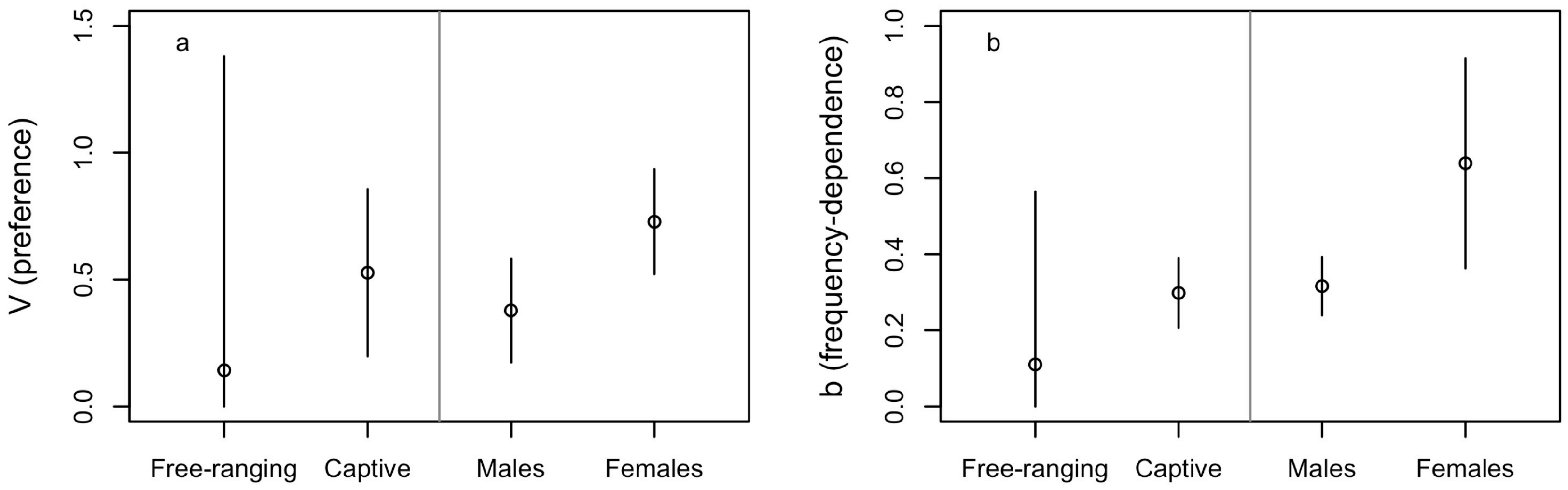

Figure 1. Plots of a) Preference term, V, and b) frequency-dependent term, b, from non-linear regression models fit to cache selections by free-ranging, captive, captive male, and captive female eastern gray squirrels (*Sciurus carolinensis*) presented with Chinese chestnut (*Castanea mollissima*) and English walnut (*Juglans regia*). Multipliers of 1.3, 1, 1.23, and 1.22 were applied to pairs of standard errors (beginning with left-most comparison in panel a of V across free-ranging and captive squirrels) to test for significant differences while correcting for unequal standard errors (Payton et al. 2003).

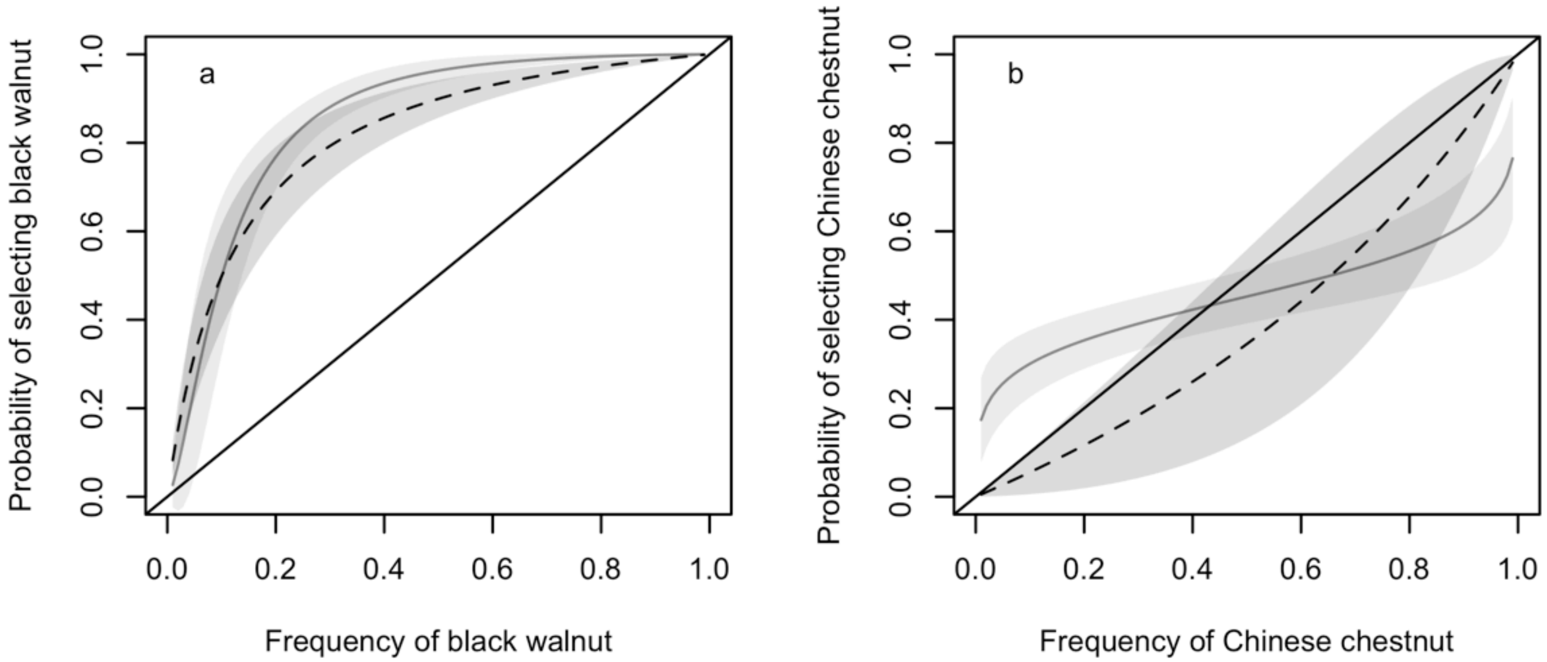

Figure 2. Plots of a) eastern gray squirrel (*Sciurus carolinensis*) probability of selecting black walnut (*Juglans nigra*) versus frequency of black walnut presented in black walnut (*J. nigra*)-American hazelnut (*Corylus americana*) pairs, and b) eastern gray squirrel probability of selecting Chinese chestnut (*Castanea mollissima*) versus frequency of Chinese chestnut presented in Chinese chestnut (*C. mollissima*)-English walnut (*Juglans regia*) pairs. Solid gray line represents predictions of the best-fit non-linear regression model, dotted–gray line represents predictions based only on preference term (V) from the best-fit model

(frequency dependence term b fixed at 1) and solid black line is the reference line when there is no preference for either seed in a seed pair (V = 1) and no frequency dependence (b = 1). Shaded regions represent 95% confidence bands (Bolker 2008, 2015).

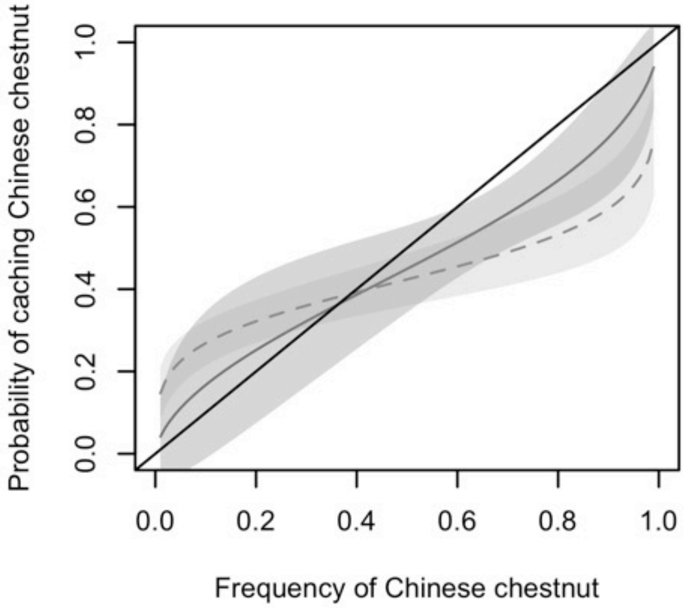

Figure 3. Plot of probability of selecting Chinese chestnut (*Castanea mollissima*) versus frequency of Chinese chestnut presented in Chinese chestnut (*C. mollissima*)-English walnut (*Juglans regia*) pairs for male versus female captive eastern gray squirrels (*Sciurus carolinensis*). Solid gray line represents predictions of the best-fit non-linear regression model for captive female eastern gray squirrels, dotted–gray line represents predictions for captive male eastern gray squirrels, solid black line is the reference line when there is no preference for either seed in a seed pair (V = 1) and no frequency dependence (b = 1). Shaded regions are 95% confidence bands (Bolker 2008, 2015).

4.7. Tables

Table 1. Summary across 12 captive eastern gray squirrels (*Sciurus carolinesis*) and free-ranging eastern gray squirrels of the ratios of black walnuts: American hazelnuts presented, the ratios of Chinese chestnut (*Castanea mollissima*): English walnuts (*Juglans regia*) presented, numbers of black walnuts (*Juglans nigra*) and American hazelnuts (*Corylus americana*) cached, numbers of Chinese chestnuts and English walnuts cached, total numbers of seeds presented, and average number of days of camera selection data available per frequency of presentation. Initial weight (in grams) and sex (M = Male, F = Female) of each of the 12 captive squirrels provided. Cache raiding treatment is indicated by (R) next to the frequency of presentation.

ID	Sex	Initial weight (g)	Total no. of seeds presented	Avg. days of camera selection data	Black walnut: American hazelnut frequencies	Total black walnuts cached	Total hazelnuts cached	Chinese chestnut: English walnut frequencies	Total Chinese chestnuts cached	Total English walnuts cached
1	M	500	400	10	2:8, 4:6	19	2	6:4, 8:2	85	9
2	M	375	400	9.5	4:6, 6:4	43	0	2:8, 4:6	37	123
3	M	500	400	8.75	6:4, 6:4(R)	14	0	4:6, 2:8	2	18
4	M	625	400	7.5	8:2, 8:2(R)	37	0	8:2, 6:4	3	4
5	F	500	400	9.25	8:2, 8:2(R), 6:4	106	0	6:4	28	23
6	F	375	400	9.75	6:4, 2:8, 8:2	99	0	8:2	32	1
7	M	500	300	9	2:8	1	0	2:8, 4:6	10	62
8	F	500	400	8.5	4:6, 4:6(R)	18	0	4:6, 2:8	1	22
9	F	250	400	9.25	2:8, 8:2	12	0	2:8, 8:2	1	5
10	F	375	300	9.33	4:6	20	0	8:2, 6:4	55	23

11	M	500	400	9.5	6:4, 6:4(R), 4:6	62	0	6:4	2	0
12	M	500	400	9.25	8:2, 8:2(R), 2:8	43	0	4:6	6	18
Free-ranging			80		2:8, 4:6, 6:4, 8:2	27	0	2:8, 4:6, 6:4, 8:2	9	17

Table 2. Results of weighted non-linear regressions predicting caching of black walnut (*Juglans nigra*) in black walnut (*J. nigra*)-American hazelnut (*Corylus americana*) seed pair and caching of Chinese chestnut (*Castanea mollissima*) in Chinese chestnut (*C. mollissima*)-English walnut (*Juglans regia*) seed pairs. Columns of the table provide estimates of preference (V), frequency dependence (b), standard errors (SE) for V and b, t-statistic and accompanying P-value testing for significant difference from 1, Δ AIC values computed for models within a seed pair and percent caching events correctly predicted in leave-one-out cross-validation. Rows of table represent different models: captive eastern gray squirrel (*Sciurus carolinensis*) caches predicted using frequency of presented seeds, captive squirrel caches predicted using frequency of cached and presented seeds, free-ranging squirrel caches predicted using frequency of presented seeds, captive male squirrel caches predicted using frequency of cached and presented seeds, and captive female squirrel caches predicted using frequency of cached and presented seeds.

Seed pairs	Model	b	SE (b)	t	P	V	SE (V)	t	P	Δ AIC (% correct
Black walnut: American hazelnut	Captive (seeds presented)	1.49	0.274	1.787	0.117	8.937	2.138	3.713	0.008	0 (NA)

	Captive (seeds presented and cached)	0.765	0.197	-1.19	0.273	7.256	5.325	1.175	0.279	45.85 (NA)
	Free-ranging	0					Infinity			
Chinese chestnut: English walnut	Captive (seeds presented)	0.195	0.125	-6.449	0.001	0.239	0.286	-2.665	0.026	24.8 (63%)
	Captive (seeds presented and cached)	0.298	0.075	-9.425	<0.001	0.527	0.254	-1.865	0.095	0 (69%)
	Free-ranging	0.11	0.37	-2.403	0.043	0.142	0.952	-0.902	0.394	
	Males	0.316	0.063	-10.827	<0.001	0.378	0.205	-3.037	0.029	
	Females	0.639	0.226	-1.601	0.251	0.728	0.207	-1.317	0.319	

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CHAPTER 5. A SHELL GAME: DETECTION OF SEED DORMANCY BY *SCIURUS CAROLINENSIS*

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5.1 Abstract

The mechanism underlying detection of seed dormancy by scatter-hoarding rodents such as eastern gray squirrels (*Sciurus carolinensis*) is unclear; although previous work suggests that the pericarp plays an important role in signaling dormancy status. Eastern gray squirrels consume non-dormant seeds and seeds with acetone-treated pericarps, while dormant seeds tend to be cached. I characterized chemical differences between germinating and dormant pericarps of northern red oak (*Quercus rubra*), American chestnut (*Castanea dentata*) and the hybrid of Chinese chestnut and American chestnut (*C. mollissima* x *C. dentata*, BC3 or third backcross) using Gas Chromatography-Mass Spectrometry (GCMS). I quantified structural differences in the pericarps using light and scanning electron microscopy. GCMS produced evidence of plant wax components on the shells of dormant red oak and chestnut seeds, which was further confirmed by staining red oak pericarps with Sudan-IV and visualizing under a light microscope. On the pericarp of germinating seeds, GCMS indicated evidence of compounds that reduce effectiveness of plant wax layers, including amyrins. Using SEM, I found evidence of peeling on the pericarp of germinating seeds. I tested sensitivity of free-ranging eastern

gray squirrels to different cues of seed dormancy. In field experiments, dormant seeds coated with a solution of compounds from the seed kernel or with cell metabolism byproducts such as acetaldehyde were more likely to be consumed instead of cached. Time squirrels spent manipulating seed in their paws was higher for seeds treated with acetaldehyde. Further, dormant seeds that were artificially treated to remove the wax layer were more likely to be consumed. The results suggest that, as seeds break dormancy, the wax layer on the pericarp degrades and is accompanied by the escape of lower molecular weight kernel compounds or cell metabolism byproducts (such as acetaldehyde). Squirrels appear to detect lack of dormancy by manipulating seeds with their paws and cue in on the physical loss of wax and escape of odorous chemicals. Detection of lack of dormancy ultimately results in an increased probability of a seed's consumption. The mechanisms underlying detection of seed dormancy by scatterhoarding rodents thus have important implications for seed fate and seed dispersal.

5.2 Introduction

Seed dormancy can be characterized as a block to germination or lack of germination even under favorable environmental conditions (Finch-Savage and Leubner-Metzger 2006). Several decades of research in multiple disciplines have furthered our understanding of physiological, biochemical, evolutionary and ecological changes associated with germination. The full body of literature is summarized across numerous reviews (Baskin et al. 2000; Baskin and Baskin 2001; Finch-Savage and Leubner-Metzger 2006; Yang et al. 2012; Nonogaki 2014). Despite these advances, integration across disciplines is lacking (Vleeshouwers et al. 1995; Thompson et al. 2003; Finch-Savage and Leubner-Metzger 2006). Progress has been made in integration of seed ecology and seed physiology to understand dormancy (Vleeshouwers et al. 1995). But little has been done to extend physiological and biochemical changes associated with dormancy to behaviors of seed predators.

Unlike physiological dormancy, the relationship of physical dormancy to behaviors of seed predators has received some attention. Physical dormancy arises due to the presence of water-repellent substances such as suberin, cutin, lignin and wax on sclerids of the pericarp (Roston 1978; Egley et al. 1983; Yan et al. 2009; Schreiber 2010; Vishwanath et al. 2013). This type of dormancy is posited to have evolved in response to seed-predation pressures (Paulsen et al. 2014). The coat of a physically dormant seed serves as a water-impermeable barrier, which is hypothesized to keep volatile compounds in and thus allow seeds to escape olfactory detection (Paulsen et al. 2013). The 'olfactory crypsis' of seeds has been examined by comparing volatile compounds released by hard seeds relative to soft seeds, and by noting hamster sensitivity to buried chemical 'cocktails' released by soft seeds (Paulsen et al. 2013).

Additional evidence that rodents discriminate between dormant and non-dormant seeds is observed in *Quercus* (oak) acorns that are consumed and hoarded by eastern gray squirrels (*Sciurus carolinensis*). Dormancy of seeds is a critical factor affecting cache management strategies of eastern gray squirrels (Steele et al. 2001b; Chapter-3). Once a seed germinates, growth of the taproot depletes nutrient reserves of the seed rapidly, reducing caloric value available to a granivore (Short 1976; Fox 1982). Accordingly, during fall squirrels consume high proportions of germinating seeds such as white oak (*Quercus alba*) and cache high proportions of dormant seeds such as red oak (*Q. rubra*) (Hadj-Chikh et al. 1996; Smallwood et al. 1998; Steele et al. 2001a). Further, squirrels are known to excise embryos and remove radicles prior to caching, which arrests germination and improves storage potential of germinating seeds (Steele et al. 2006). Squirrels also consume germinating or non-dormant red oak seeds just as often as germinating white oak seeds (Steele et al. 2001a).

Chemical cues in the pericarp of oak seeds likely signal dormancy (or lack of dormancy) to squirrels, thus allowing dormant seeds to be distinguished from non-dormant seeds (Steele et al. 2001a). Gray squirrels can be convinced to cache an early germinating white oak acorn by replacing the shell of the white oak acorn with the shell of a dormant red oak acorn (Steele et al. 2001a). Gray squirrels will also consume a dormant red oak kernel if the shell is washed in acetone (Steele et al. 2001a). These results suggest that squirrels respond to changes in the pericarp that indicate whether the seed is dormant or non-dormant (Steele et al. 2001a).

Some physiological and biochemical changes in oak acorns and pericarps have been noted during the process of stratification necessary to break dormancy. Specifically, red oak seeds show embryo dormancy induced by high concentrations of inhibitors like abscisic acid (Vogt 1974), which suggests that red oak seeds exhibit physiological dormancy rather than physical dormancy (Baskin and Baskin 2014). However, the pericarp also plays an important role in breaking of dormancy in this species; red oak seeds without a pericarp showed a higher rate of germination (Vogt 1974). Further, a thin layer has been observed on the pericarp of dormant red oak seeds, which likely obstructs exchange of water and gases between the seed and the environment (Bonner and Vozzo 1987). This layer is hypothesized to be lost as stratification proceeds, allowing inhibitors to leech out and germination to occur (Vogt 1974). If true, changes to the pericarp may represent a specific type of physiological dormancy (Baskin and Baskin 2014).

To identify the mechanism underlying detection of seed-radicle dormancy by eastern gray squirrels, I characterized chemical compositional differences between germinating (defined by the emergence of a radicle) and dormant (defined as seeds collected within 7 days of dropping from trees and without radicle emergence) hardwood tree seeds using gas chromatography-mass spectrometry (GCMS) methods, and differences in seed-shell structure using a combination of light and scanning electron microscopy methods. Specifically, I hypothesized that a wax layer exists on the pericarp of oak (*Quercus*) and chestnut (*Castanea*) seeds that is lost as the seed breaks dormancy and germinates. I then tested sensitivity of eastern gray squirrels (*Sciurus carolinensis*) to different cues of dormancy by performing field trials with artificially manipulated seeds. I hypothesized that one or more of the following cues signal to squirrels a lack of dormancy and result in consumption of a seed:

1. Physical loss of wax from the pericarp
2. Escape of kernel compounds to the pericarp surface as a result of loss of wax, or occurrence of specific compounds, such as pyrogallol, in the pericarp of germinating (but not dormant) seeds
3. Escape of cell metabolism byproducts, such as acetaldehyde, to the pericarp following breakdown of wax and kernel compounds

5.3 Materials and methods

5.3.1 Study site and species

I collected seeds of four hardwood tree species, northern red oak (*Q. rubra*), white oak (*Q. alba*), American chestnut (*C. dentata*), and BC3 hybrid of American and Chinese chestnut (*C. dentata* × *C. mollissima*). Seeds of white oak, American chestnut and the chestnut hybrids were obtained from trees on the Purdue campus and other Purdue properties in or near West Lafayette (Indiana, USA). Seeds of red oak were collected from trees on Purdue campus and were also procured from Sheffield's Seeds Co (New York, USA). All seeds were stored at 4C until chemical analyses or field presentations. Unlike the other seeds, white oak has essentially no dormancy; it germinates immediately even prior to dropping from trees in the fall (Young and Young 1992). Consequently, comparisons in surface chemical compositions between germinated and dormant white oak acorns were not possible.

5.3.2 Gas chromatography-Mass spectrometry

I used 3 different extraction solvents (methanol, acetone and hexane) to obtain polar and non-polar compounds occurring in pooled samples of 10 pericarps or 5 kernels (Because shells contain smaller quantities of compounds than kernels, shells from 10 seeds were used as starting material to extract sufficient quantities of detectable compounds) for each seed type. I added 50 ml of solvent to the pooled sample, vortexed for 1 min, and sonicated for 2 mins. I then separated the solid seed material from the solvent and concentrated samples under a gentle stream of nitrogen gas. For extracts of hexane, samples were concentrated to 1 mL. For extracts of acetone and methanol, samples were concentrated until no further reduction in quantity of samples was observed.

This resulted in 5 ± 2ml of sample for acetone extractions and 10± 3ml of sample for methanol extractions. I repeated extractions so that I obtained 3 replicates per kernel or shell sample per solvent per seed type; and thus, obtained a total of 3x2x3x4=72 samples.

I also germinated seeds of red oak, American chestnut and the chestnut hybrid in seed germination chambers at 20°C/27°C under 12h dark/12h light conditions. Seeds were monitored regularly. Once the radicle emerged, germinating seeds were removed from the chamber and stored at 4°C until processing (typically within 3 days). The above GCMS extraction steps were repeated for germinated seeds. Because 3 replicates were performed for kernels and shells separately, for each of 3 solvents and for each of 3 species germinated in the chamber, 54 sample chromatograms were obtained (3x2x3x3=54). A small fraction of samples (n=9) were also subjected to tri-methyl silyl (i.e., TMS) derivatization to improve detectability and separation of polar compounds.

The capillary GCMS analyses were carried out using an Agilent 5975C (Agilent Labs, Santa Clara, CA) mass spectrometer system. Typical electron energy was 70 eV with the ion source temperature maintained at 250°C. The individual components were separated using a 30m DB-5 capillary column (250 μm i.d. X 0.25 μm film thickness). The initial column temperature was set at 40°C (for 3 minutes) and programmed to 320°C at 10.0°C per minute. The flow rate was typically set at 1 ml/min. The injector temperature was set at 250°C (for negative ion methane chemical ionization the source temperature set at 150°C.)

Compounds in each chromatogram were scored using program Wsearch32 (www.wsearch.com.au, Ver 1.6.2005) and using the NIST standard reference database (National Institute of Standards and Technology, Gaithersburg, MD). Only compounds with high matches to reference chromatograms were considered for statistical analyses. A high match was defined as an R Match factor greater than 800 or probability of match, given unknown spectra available in the NIST database, greater than 0.75. I also compared retention times of compounds with published retention times and compared retention times across all samples and replicates to ensure I scored chromatograms correctly and consistently. Further, I deleted from consideration compounds occurring in only 1 replicate in further statistical analyses.

I assessed chemical differences across germinating and dormant kernels and shells using non-metric multidimensional scaling (NMDS) analyses (Oksanen 2008). Peak heights of compounds in each sample were summed to obtain sample total, and the proportion of each compound was computed by dividing its peak height by the sample total. This transformation was done so that relative concentrations of compounds could be compared across samples as opposed to comparing differences in raw peak abundances. Bray-Curtis distances were computed on proportions of chemical compounds in each replicate, thus creating a matrix of pairwise sample dissimilarities with respect to relative concentrations of chemical compounds. The R package ‘vegan’ and function ‘metaMDS’ were used to ordinate chemical compositional dissimilarities into 2 reduced NMDS axes. Chemical compounds weighted heavily (weights > 60% quantile of absolute weights of all chemicals in each NMDS axis) were displayed on the resulting biplots. Separate NMDS analyses were performed for each solvent and for kernel or shell samples of northern red oak. Due to lower numbers of replicates in chestnuts (resulting from lack of fresh seed material or fungal infestations), I combined American chestnut and BC3 chestnut hybrids prior to performing analyses. Because BC3 hybrids are >95% genetically similar to pure American chestnuts and are selected to be different only with respect to chestnut blight tolerance (Diskin et al. 2006), I suspect that the chemical compounds occurring in these seeds are likely to be similar enough to justify pooling for analysis.

5.3.3 Light and Scanning electron microscopy

I visualized shell samples of red oak using a light microscope to confirm the presence of waxes on the pericarp of seeds. For light microscopy, I used Sudan IV to stain lipids and waxes, phosphoglucinol-HCl to stain sclereid cells, and Toluidine blue (1%) to stain cells. Images were taken at a magnification of 7.5X to 75X.

To quantify loss of wax at a cellular level, I also visualized 3 shell samples each of red oak, germinating red oak, white oak and shell samples of red oak treated with acetone (for 5 minutes) using scanning electron microscopy (SEM) methods. Samples were sputter-coated with platinum for 120 secs and imaged with a FEI NOVA nanoSEM field emission SEM (FESEM) operating at an accelerated voltage of 5kV and at a working distance of approximately 5mm. Images were taken at 300X, 1500X and 3000X.

I observed damage to the shell of seeds, i.e. 'peeling' on the upper layer (Fig. 1). I quantified the amount of peeling on images of 1500X and 3000X using Fiji version 2.0.0 (Schindelin et al. 2012). A grid was applied to all images (using the grid plugin available for Fiji) such that each square had an area of 20000 pixels. Because all images were at a size of 1024 pixels x 943 pixels, this grid resulted in a total of 30 full squares within the image area. Each square was scored with a 0 or 1; 0 indicated no peeling and 1 indicated peeling on surface. Thus, for each image, I obtained a summed score of 0-30, which reflects total number of squares with visible peeling. I performed a permutation test to evaluate significant differences in mean number of squares of peeling between different treatments. I computed mean pairwise differences in numbers of squares of peeling between treatments. I randomized treatments across observations 1000 times and computed the pairwise differences in mean numbers of squares of peeling to obtain a null distribution of differences. I tested for significance by calculating the proportion of the null distribution that exceeded observed pairwise differences.

5.3.4 Field trials

From the results of GCMS, I tested the hypotheses that eastern gray squirrels detect kernel compounds on the pericarps of germinating chestnut released following breakdown of pericarp wax. I subjected pure American chestnut seeds to a flotation test (Gribko and Jones 1995) to ensure weevil-infested seeds were not used in field trials and weighed all seeds prior to treatment. I presented 35 dormant pure American chestnut seeds to squirrels across the Purdue campus between 15-Oct-2015 and 20-Oct-2015. Pericarps of 18 of the 35 seeds were treated with an artificial concoction of kernel compounds (see Appendix A1). Relative concentrations of compounds in the artificial concoction mimicked relative concentrations of compounds in kernels from one particular GCMS sample. Chemicals were obtained from Fisher Scientific and Sigma Aldrich and dissolved in distilled water. Dormant seeds were dipped in 5 ml of concoction for approximately 5 min and carried to the field in individual plastic bags. The remaining 17 ‘control’ seeds were dipped in 5 ml distilled water for 5 mins and stored in individual plastic bags. I presented one control and one treated seed sequentially and in a random order to squirrels at each of approximately 20 different locations. Identical treatment presentations were separated by >200 m to ensure that data were being collected from different squirrels. Exceptions to this 200m rule were made only if observers could present seeds to different squirrels at the same location simultaneously. Presentations involved an observer approaching a squirrel and tossing a seed in its general direction. The observer followed the squirrel until the seed was completely consumed or cached. Seed fate (consumed/cached/partially consumed) and ambient temperature during trials were recorded. Nitrile gloves were worn when handling seeds, and gloves were changed between seed presentations.

For acorns of northern red oak, I tested several potential cues of lack of dormancy. Similar to American chestnut seeds, I tested whether kernel compounds on the pericarp surface triggered consumption. Field trials followed the protocol described for American chestnut seeds. The same concoction described in Appendix A1 was used, given that kernel compounds in chestnuts and oaks were very similar. Trials were performed between 15-Oct-2014 and 20-Nov-2014 with 19 control and 18 treated seeds. Based on GCMS analyses for acorns of northern red oak, I determined that pyrogallol was the only kernel compound that occurred consistently on the pericarps of germinating seeds. Therefore, I tested specifically whether pyrogallol served as a cue of lack of dormancy. I also tested whether squirrels were sensitive to physical loss of wax from the pericarp and release of common odorous cell metabolism byproducts such as acetaldehyde. In order to test these hypotheses, I divided dormant red oak seeds randomly into 4 different treatment groups. After removing seeds with potential weevil infestation and weighing all seeds, I subjected the 4 groups to the following treatments: 1. ‘Scraped’ seeds were treated by placing dormant seeds in a water bath at 45°C for 5 min to loosen the wax, followed by removal of wax from the pericarp with sandpaper; 2. ‘Pyrogallol’ seeds were dipped in 0.02% pyrogallol solution (concentration determined from GCMS samples by addition of 100 μL 5-methylfurfural as internal standard in 600 μL solution) for 5 min; 3. 'Acetaldehyde' seeds were dipped in 40ppb acetaldehyde solution for 5 mins (concentration determined to be safe as per reference exposure limits); 4. 'Control' seeds were dipped in distilled water for 5 min. I presented 22 control, 22 scraped and 18 pyrogallol seeds to squirrels between 3-Nov-2015 and 7-Nov 2015 and then 16 control and 21 acetaldehyde seeds between 11-Nov-2015 and 20-Nov-2015. I followed the same presentation protocols described earlier and recorded fate and ambient temperature during presentation trials. I performed logistic regressions for American chestnut and northern red oak to test whether seed fate (outcome of 1 indicating seed was cached and 0 indicating seed was consumed) was predicted by type of treatment; seed weight, date, time and temperature were included in preliminary models but were not significant predictors and hence were dropped from final models.

I examined the behavioral mechanisms involved in the foraging decisions made by gray squirrels by video recording a subset of presentation trials. Studies with fox squirrels (*Sciurus niger*) identified two specific behaviors that are correlated with caching and consumption outcomes of a seed (Preston and Jacobs 2009; Delgado et al. 2014). Head-flicking by squirrels when handling a seed is correlated to caching, and experiments suggest that this behavior is a mechanism of evaluating nut weight and infestation (Preston and Jacobs 2009; Delgado et al. 2014). Time spent in paw manipulation behaviors is correlated to the likelihood that a seed is consumed. It has been proposed that this behavior is a means of evaluating weaknesses in the shell; however the function is still unclear (Delgado et al. 2014). I scored number of head flicks, time spent in paw manipulation (min) and travel time (min) in 4 trials of acetaldehyde treatment, 9 trials of control seeds, 7 of pyrogallol seeds and 10 of scraped seeds. I performed regressions to compare number of head flicks, travel time and time spent in paw manipulations across treatment groups for the entire dataset and for only those trials that resulted in caching. I predicted the number of head flicks as a function of treatment groups using a negative binomial regression implemented with the function ‘glm.nb’ in library MASS (Venables and Ripley 2002). I performed multiple linear regressions to predict the natural logarithms of time (in mins) spent in paw manipulation behaviors across treatments and travel time (in mins) across treatments. Seed weight, temperature and time were significant predictors only for travel time (natural logarithm). Accounting for these predictors did not alter qualitative results (i.e., sign and significance were unchanged) and were dropped from all final models.

5.4 Results

5.4.1 Gas chromatography-Mass spectrometry

Germinating and dormant chestnut pericarps showed differences in relative concentrations of chemical compounds, especially with respect to washings from hexane and acetone (Figs. 1A and B). Hexane washings of germinating chestnut pericarps showed reduced concentrations of long chain alkanes such as heptacosane, increased concentrations of long chain fatty acids (e.g. hexadecanoic acid), and the presence of amyrins. Kernel compounds (such as sucrose, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one) were observed on the pericarp of germinating chestnuts in washings of acetone and methanol (Figs. 1B and C). I also observed the presence of 6,7-dimethoxy coumarin on the shells of germinating chestnut (Fig. 1B, full list of compounds in Appendix A).

Northern red oak acorns showed similar changes in the pericarp in comparisons of germinating and dormant seeds. Germinating seeds showed reduced concentrations of long chain alcohols (tetracosanol), long chain aldehydes (tetracosanal and octadecanal) and long chain alkanes (heptacosane), whereas increases were observed in concentrations of long chain fatty acids (hexadecanoic acid) (Figs. 2A and B). Additionally, consistent changes in plant hormones were observed on the pericarp; 24-methylenecycloartanol found on shells of dormant red oak was replaced by 24-methylenecycloartan-3-one, Stigmast-4-en-3-one and Lup-20(29)-en-3-one on shells of germinating red oak. I consistently detected the presence of one kernel compound (namely, pyrogallol) on shells of germinating red oak (Fig. 2B). Finally, I observed 1-methylene-1H-indene on the shell of germinating oaks (Fig. 2A, see Appendix A).

Kernel compounds showed some differences in relative concentrations across washings. Primarily, I observed an increase in oxalic acid and acetic acid anhydride with formic acid in kernels of germinating chestnuts (Figs. 3B and C). This was accompanied by a reduction in concentrations of sugars such as sucrose. Further, I observed an increase in behenic alcohol in germinating kernels (Fig. 3A, see Appendix A).

5.4.2 Microscopy analyses

Light microscopy confirmed the presence of a wax layer on the surface of the pericarp. This layer stained orange with Sudan-IV (Fig. 4B). Beneath the wax, I observed sclereid cells stained pink by phosphoglucinol-HCl (Fig. 4A). The presence of cells was also confirmed by the general Toluidine (1%) stain (Fig. 4C). Although no detectable changes in wax layer were observed under light microscope magnifications, I observed corrosions or peeling on the shell surface (likely the wax layer) at 1500X and 3000X under a scanning electron microscope. Specifically, I observed a significant increase in peeling on the surface of germinating seeds, acetone treated shells, and white oak shells when compared to dormant and stratified red oaks (Figs. 4D-4F, Table 1). The amount of peeling was not significantly different for either germinating red oak seeds or early germinating white oak seeds and acetone-treated dormant shells (Figs. 4F-G, Table 1).

5.4.3 Field trials

Application of kernel compounds to pericarps of dormant seeds significantly influenced the decision to cache or consume a seed. Specifically, eastern gray squirrels were more likely to consume red oak and American chestnut seeds treated by dipping in an artificially created concoction of kernel compounds ($p=0.02$ and $p=0.03$, Table 2). Scraping of seeds and application of acetaldehyde to the pericarp resulted in seeds being consumed more often when compared to the control seeds ($p=0.06$, $p=0.02$, Table 2). The number of head flicks was unaffected by seed treatment (Table 3). However, marginally more time was spent in manipulating seeds with paws when the shell was treated with acetaldehyde ($p=0.07$, Table 3). Travel time was lower for scraped seeds and marginally larger for acetaldehyde-treated seeds when compared to control seeds ($p=0.05$, $p=0.07$, Table 3); however after incorporating consumed seeds, a marginally lower travel time was obtained only for scraped seeds and no significant differences were observed for acetaldehyde-treated seeds relative to control. For all other squirrel behaviors, I obtained the same qualitative results and significant p-values at an alpha of 0.05 after incorporating consumed seeds into analysis.

5.5 Discussion

This study provides a holistic view of the chemical and structural changes accompanying loss of radicle dormancy in oak and chestnut seeds, and permits evaluation of how these changes affect behavioral responses of an important predator and disperser of the seeds. Briefly, I found evidence of the existence of pericarp wax on dormant seeds, which likely degrades as seeds germinate and radicles emerge. Loss of wax is probably accompanied by the escape of chemicals from seeds that subsequently are detected via olfaction or gustation by squirrels. In chestnut, chemical cues to loss of dormancy may include release of lower molecular weight kernel compounds, which elicit in squirrels a greater tendency to consume the seed. In oaks, physical loss of wax and release of cell metabolism byproducts likely serve as cues used by squirrels to assess loss of acorn dormancy.

Similar to the seed coats of other higher plants, both oak and chestnut seeds contain waxes on the outer surface of the pericarp. The GCMS analyses indicated the presence of long chain fatty acids (hexadecanoic acid, linoleic acid, oleic acid and stearic acid), long chain fatty acid esters (docasonoic acid nonyl ester), alcohols (tetracosanol), and alkanes (heptacosane, tetracosane). All of these compounds are known or proposed components of plant cuticular waxes (Hwang et al. 2002; Coşkuner and Tekin 2003; Vogg et al. 2004; Pollard et al. 2008; Yeats and Rose 2013). I further confirmed the presence of waxes on the surface of the pericarp and plant sclereid cells by staining with Sudan-IV (Fig 4A-B).

Breaking of radicle dormancy is accompanied by an increase in permeability of the wax layer on the pericarp. Specifically, I quantified a reduction in proportion of alkanes for germinating versus dormant seed pericarps, which likely alters the composition of the wax and increases permeability. Recent reviews of plant cuticular wax studies have suggested that wax load is not correlated to water permeability; instead wax composition and specifically alkane content may be inversely related to cuticular permeability (Yeats and Rose 2013). Furthermore, I consistently isolated α- and β-amyrins from the shells of germinating chestnuts. β-amyrin is suggested to have a negative effect on cuticular waxes of *Arabidopsis* and other plants (Buschhaus & Jetter 2012) and may serve a similar function on the shells of chestnuts.

The mechanism resulting in loss of wax on pericarps of northern red oak is still unknown, although my results suggest a possible association with lipid peroxidation. I consistently isolated plant hormone Lup-20(29)-en-3-one and pyrogallol on the shells of germinating red oak. Lup-20(29)-en-3-one reduces activity of lipoxygenases as does α-amyrin, including plant soybean (*Glycine max*) lipoxygenase (Gutierrez-Lugo et al. 2004). Lipoxygenase catalyzes conversion of polyunsaturated fatty acids to fatty acid hydroperoxides (Brash 1999). Further, pyrogallol influences catalase-mediated hydrogen peroxide decomposition (Reed 1916; Jurd 1956). Finally, genetic elimination of lipoxygenase isozymes in soybean significantly reduces seed oil content (Narvel et al. 1998). Thus, the presence of both pyrogallol and Lup-20(29)-en-3-one on germinating shells suggests some relationship to lipid peroxidation.

Chemical changes that increase wax permeability likely lead to the corrosion or peeling of wax layers that I observed from the pericarp of seeds. Germinating seeds show an increased ‘peeling’ effect relative to control seeds. Furthermore, the treatment of dormant red oak pericarp with acetone increases peeling when compared to control seeds.

Therefore, the acetone washings of northern red oak shells likely remove key components from the wax of dormant seeds such as heptacosanol and heptacosane (Fig 2B), which further causes structural changes on the pericarp surface to increase wax porosity (Fig 4). Increased porosity is likely what occurred when acorns of northern red oak were washed with acetone and presented to eastern gray squirrels by Steele et al. (2001). They concluded that squirrels were more likely to consume seeds with acetone-treated pericarps. Coupled with findings from this study, I conclude that changes in porosity of pericarp wax may be detected by eastern gray squirrels and used as a cue to acorn dormancy status for northern red oak.

Escape of lower molecular weight aldehydes and ketones from germinating seeds are likely strong cues for lack of dormancy. Loss of kernel compounds to the pericarp following wax degradation may provide a means by which aldehydes and ketones are released to the pericarp. In chestnuts, the increased porosity of pericarp wax (caused by increased concentrations of compounds such as β-amyrin) also was accompanied by increased concentrations of kernel compounds on the shell. When I dipped dormant chestnut and oak seeds in an artificially created concoction of kernel compounds and presented them to squirrels, these treated seeds were more likely to be consumed in comparison to control seeds. However, detection of kernel compounds on the shell does not confirm the passage of kernel compounds through the pericarp; these compounds could have occurred in shell samples on the inner portion of the shell. Further, it is possible that only lower molecular weight kernel compounds can pass through the pericarp. To test if low molecular weight cell metabolism byproducts could influence seed fate, I presented seeds coated with acetaldehyde to squirrels. The results show that seeds treated with acetaldehyde were consumed more often than control seeds; thus escape of odorous chemical compounds are likely important cues of lack of dormancy. Future studies should use extraction protocols that are more suited to identifying lower molecular weight compounds from the outer surface of the pericarp, including solid phase micro extraction (SPME) methods to confirm which specific compounds signal lack of dormancy.

In addition to the compounds tested in this study, I isolated 6,7-dimethoxy coumarin (i.e, scoparone) from the shells of germinating chestnuts and found little evidence to suggest that this compound emerged from the kernel. Other studies have isolated scoparone from the inner shell of chestnuts (Noh et al. 2010), where it is considered to act as a fungal inhibitor in addition to potentially influencing lipid metabolism (Noh et al. 2010; Afek et al. 1986). Future research should consider presenting 6,7-dimethoxy coumarin in isolation as a potential cue of lack of seed dormancy. In contrast to chestnut seeds, germinating acorns of northern red oak showed little evidence of escaped kernel compounds on their shells. The only kernel compound consistently isolated on the shell of germinating red oak was pyrogallol (Fig 2B), and this compound had no significant effect on squirrel behavior.

My results suggest that seed manipulation in paws of squirrels provides a behavioral mechanism for detection of compounds on the pericarp. Squirrels spent longer time in seed manipulation behaviors with paws when handling seeds treated with acetaldehyde. From these results, I suggest that seed manipulation behaviors with paws have evolved to allow squirrels to detect release of chemicals from the pericarp. Specifically, manipulation that involves rotation of seeds in the squirrels’ mouth may allow gustatory receptors to detect chemicals occurring on the pericarp of the seed. Previous studies with fox squirrels have found that time spent in paw manipulation is longer for seeds that are consumed (Delgado et al. 2014). Further, these studies hypothesized that paw manipulation may allow squirrels to detect weakness in the pericarp. I was unable to support that observation since removal of wax did not influence time spent in paw manipulation.

Physical loss of wax from the pericarp only marginally improved chances of consumption (control seeds were 5 times more likely to be cached relative to scraped seeds), and squirrels spent marginally less time travelling with scraped seeds (logarithm of travel time reduced by 0.75 in scraped seeds relative to control seeds) prior to caching. Lower travel time suggests that squirrels cached scraped seeds at a shorter distance in comparison to control seeds so long as travel velocity remains relatively unchanged. Previous studies have found that early germinating seeds tend to be cached at shorter distances (Sundaram et al. 2015), probably because early germinating seeds are of lower caching value to squirrels (Steele et al. 2001b; Vander Wall 2010). The effects of scraping on travel time and seed fate suggest that loss of wax may be a cue signaling lack of dormancy. However, the relatively small effect sizes make it unlikely that loss of wax was the only cue squirrels used to evaluate condition of a red oak acorn.

I hypothesize that the combination of loss of wax and escape of chemical byproducts (such as acetaldehyde) in germinating red oak acorns serve as cues by which squirrels detect lack of dormancy. Although I did not evaluate these two cues in conjunction with one another, this hypothesis is based on the findings that scraping seeds and coating seeds with acetaldehyde influenced underlying squirrel foraging behaviors including travel time and seed manipulation with paws. In isolation these treatments did not appear to have a strong effect on a squirrel's decision to cache or consume a seed. However, they may exhibit additive or interactive effects on squirrel behavior, which could be tested in subsequent experiments.

5.5.1 Conclusions

Dormant oak and chestnut seeds contain waxes on the pericarp surface. These waxes become progressively more porous as seeds break dormancy and germinate. Eastern gray squirrels appear able to detect chemicals that escape from seeds, and potentially the physical loss of wax from the pericarp. I suggest that the escape of seed chemicals and loss of wax together provide squirrels with cues about lack of dormancy in seeds, thereby inducing behaviors related to and ultimately resulting in seed consumption. From an evolutionary perspective, the production of waxes on the pericarp by the parent tree allows seeds to escape predation and thus improves chances of dispersal, caching, and possible survival to germination. Therefore, understanding the mechanisms underlying detection of dormancy by animals furthers our knowledge of the seed dispersal process.

5.6 Figures

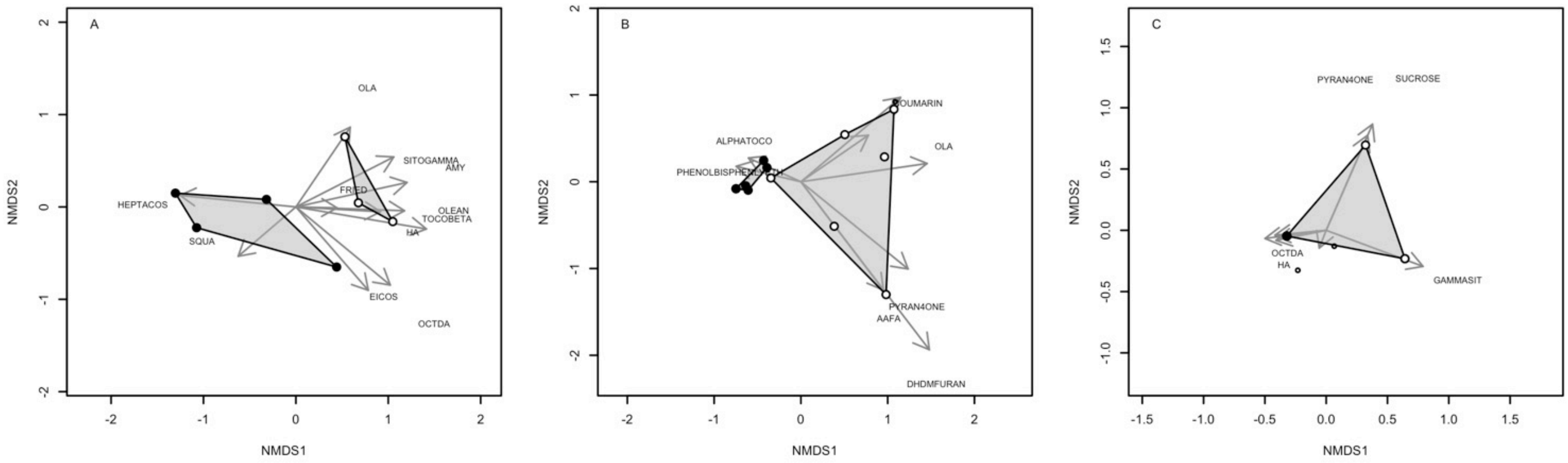

Figure 1. Results of non-metric multidimensional scaling (NMDS) performed on proportions of chemical compounds occurring on pericarps of chestnuts (pure *Castanea dentata*, *Castanea dentata* x *Castanea mollissima* hybrid) from (A) hexane washings, (B) acetone washings, and (C) methanol washings. Filled circles on plots represent dormant seeds. Open circles represent germinating seeds. Biplot arrows displayed for compounds weighted heavily by NMDS axes (>60% quantile cutoff of absolute loadings). Legend: ‘AAFA’ for Acetic acid, anhydride with formic acid, ‘ALPHATOCO’ for alphatocopherol, ‘AMY’ for α-amyrin and β-amyrin, ‘COUMARIN’ for 6,7-dimethoxy coumarin, ‘DHDMFURAN’ for 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, ‘EICOS’ for eicosanol, ‘FRIED’ for friedalone, ‘GAMMASIT’ for gammasitosterol, ‘HA’ for hexadecanoic acid, ‘HEPTACOS’

for heptacosane, ‘OCTDA’ for octadecanoic acid, ‘OLA’ for oleic acid, ‘OLEAN’ for Olean-12-en-3-one, ‘PHENOLBISPHENYLETH’ for 2,4-bis(1-methyl-1-phenylethyl)-phenol, ‘PYRAN4ONE’ for 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one, ‘SITOGAMMA’ for gammasitosterol, ‘SQUA’ for squalene, ‘SUCROSE’ for sucrose, and ‘TOCOBETA’ for betatocopherol.

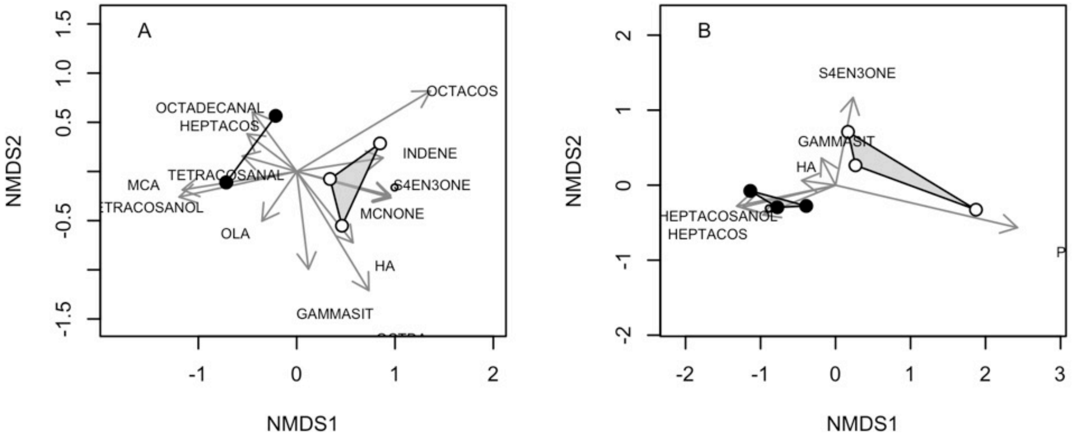

Figure 2. Results of non-metric multidimensional scaling (NMDS) performed on proportions of chemical compounds occurring on pericarps of northern red oak (*Quercus rubra*) from (A) hexane washings and (B) acetone washings. Filled circles on plots represent dormant seeds. Open circles represent germinating seeds. Biplot arrows displayed for compounds weighted heavily by NMDS axes (>60% quantile cutoff of absolute loadings). Legend: 'GAMMASIT' for gammasitosterol, 'HA' for hexadecanoic acid, 'HEPTACOSANOL' for hepacosanol, 'HEPTACOS' for heptacosane, 'INDENE' for 1-methylene-1H-Indene, 'MCA' for

24-Methylenecycloartanol, ‘MCNONE’ for 24-Methylenecycloartan-3-one, ‘OCTACOS’ for octacosane, ‘OCTADECANAL’ for octadecanal, ‘OLA’ for oleic acid, ‘TETRACOSANAL’ for tetracosanal, ‘TETRACOSANOL’ for tetracosanol, ‘P’ for pyrogallol, and ‘S4EN3ONE’ for Stigmast-4-en-3-one.

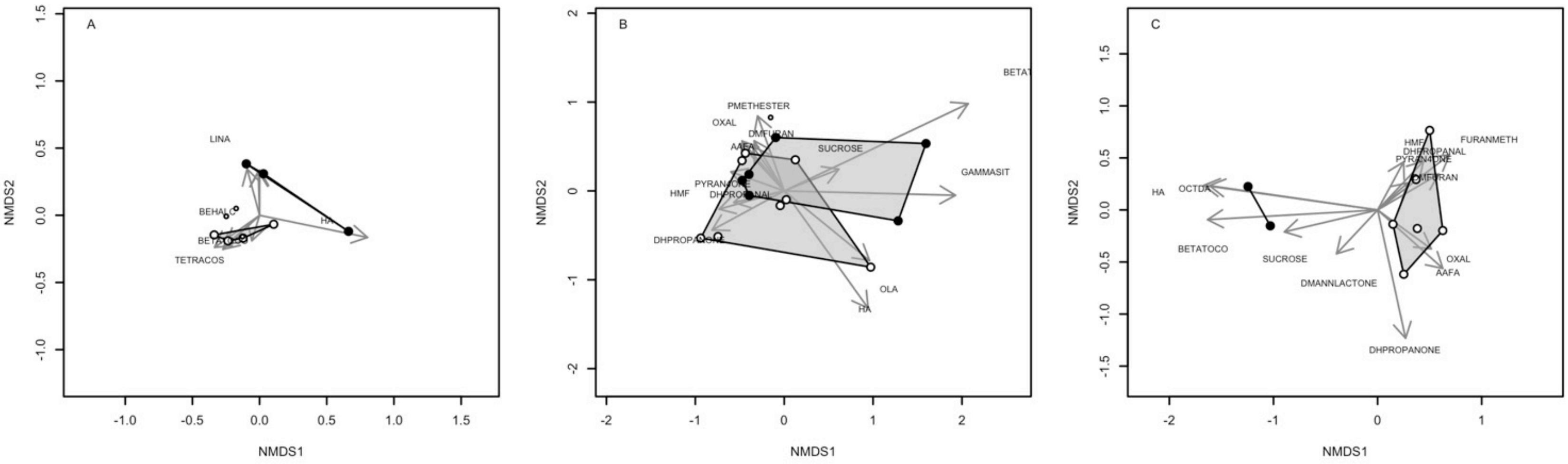

Figure 3. Results of non-metric multidimensional scaling (NMDS) performed on proportions of chemical compounds occurring in kernels of chestnuts (pure *Castanea dentata*, *Castanea dentata* x *Castanea mollissima* hybrid) from (A) hexane washings, (B) acetone washings, and (C) methanol washings. Filled circles on plots represent dormant seeds. Open circles represent germinating seeds. Biplot arrows displayed for compounds weighted heavily by NMDS axes (>60% quantile cutoff of absolute loadings). Legend: ‘AAFA’ for Acetic acid, anhydride with formic acid, ‘BEHALC’ for behenic alcohol, ‘BETATOCO’ for betatocopherol ,‘DHPROPANAL’ for 2,3-dihydroxy-propanal, ‘DHPROPANONE’ for 1,3-dihydroxy-2-propanone,

‘DMANNLACTONE’ for 3-Deoxy-d-mannoic lactone, ‘DMFURAN’ for 2,5-Dimethyl-4-hydroxy-3(2H)-furanone, ‘FURANMETH’ for 2-furanmethanol, ‘GAMMASIT’ for gammasitosterol, ‘HA’ is hexadecanoic acid, ‘HMF’ for 5-(hydroxymethyl)-2-Furancarboxaldehyde, ‘LINA’ for Linoleic acid, ‘OCTDA’ for octadecanoic acid, ‘OLA’ for oleic acid, ‘OXAL’ for oxalic acid, ‘PMETHESTER’ for 2-oxo-methyl ester propanoic acid, and ‘SUCROSE’ for sucrose.

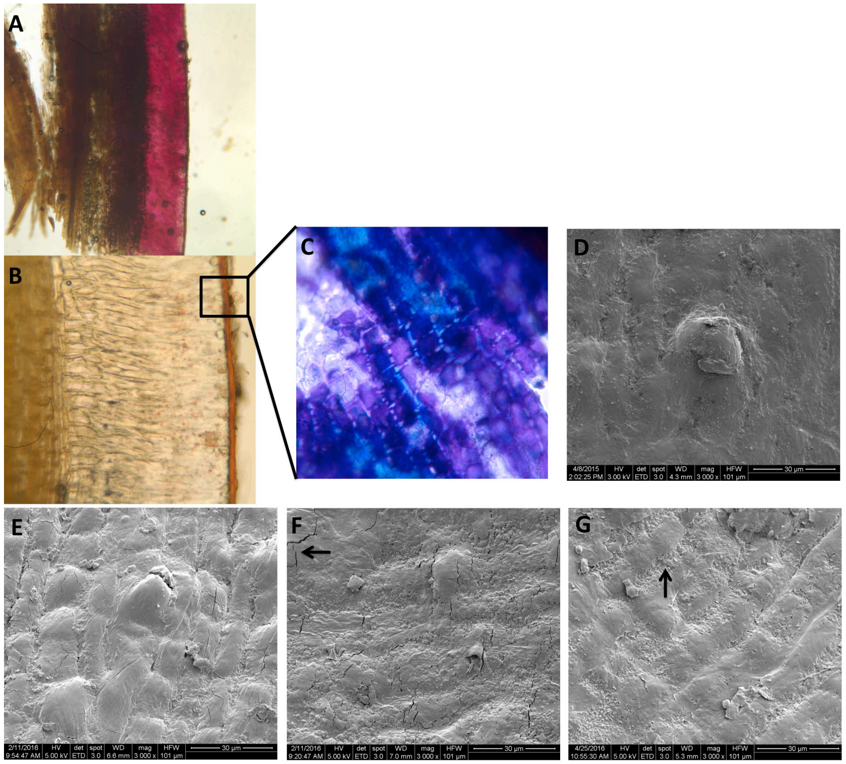

Figure 4. Microscopy images of northern red oak (*Quercus rubra*) shells. Cross-section of shell stained with phosphoglucinol-HCl to observe pink sclerid cells (A), cross-section stained with Sudan-IV to observe orange wax layer (B), surface of shell stained with Toluidine Blue (1%) to observe blue cells (C). Scanning electron microscopy images at 3000X of the surface of dormant red oak (D), stratified red oak at approximately 75 days (E), germinating red oak (F) and dormant red oak treated with acetone (G). Arrows added to point out breaks in the wax layer in (F) and corrosion in wax layer in (G).

5.7 Tables

Table 1. Results of permutation tests to compare numbers of squares of peeling across treatments in scanning electron microscopy (SEM) images. Separate tests were performed at magnifications of 1500 and 3000. Values above diagonal are the differences in average number of squares of peeling in the row treatment and the average numbers of squares of peeling in the column treatment. Values below diagonal are p-values computed by permuting treatments across SEM images 1000 times and recalculating differences across paired treatments. Italicized numbers are significant at an α of 0.1, and boldface numbers are significant at an α of 0.05.

Magnification	Treatment	Dormant & stratified red oak	Germinating red oak	White oak	Acetone-treated red oak
1500	Dormant & stratified red oak	-	*-6.333*	**-8.667**	**-10**
	Germinating red oak	*0.093*	-	-2.333	-3.667
	White oak	**0.014**	0.544	-	-1.333
	Acetone-treated red oak	**0.002**	0.347	0.697	-
3000	Dormant & stratified red oak	-	**-11.667**	**-12.333**	**-13.667**
	Germinating red oak	**0.013**	-	-0.667	-2
	White oak	**0.012**	0.893	-	-1.333

Acetone-treated red oak	**0.002**	0.684	0.82	-

Table 2. Results of 3 logistic regressions predicting seed fate (1 for seeds cached and 0 for seeds consumed) in field trials with eastern gray squirrels (*Sciurus carolinensis*). The first experiment tested whether treatment with kernel compounds altered fate of red oak (*Quercus rubra*) seeds relative to control seeds. The second experiment tested whether treatment with kernel compounds altered fate of American chestnut (*Castanea dentata*) seeds relative to control seeds. The third experiment tested whether treatment with pyrogallol, acetaldehyde and treatment by scraping off wax altered fate relative to control seeds. Italicized numbers are significant at an α of 0.1 and boldface numbers are significant at an α of 0.05.

Treatment	Variable	Estimate	SE	z-value	p
Red oak treated with kernel compounds	Intercept (Control)	0.223	0.474	0.47	0.638
	Treatment	-2.169	0.892	-2.431	**0.015**
American chestnut treated with kernel compounds	Intercept (Control)	1.54	0.636	2.421	**0.016**
	Treatment	-1.764	0.794	-2.222	**0.026**
Red oak treated with pyrogallol, acetaldehyde and by scraping	Intercept (Control)	2.89	0.726	3.979	**<0.001**
	Pyrogallol	-0.058	1.26	-0.045	0.964
	Scraped	-1.667	0.887	-1.879	*0.060*
	Acetaldehyde	-1.974	0.872	-2.263	**0.024**

Table 3. Results of negative binomial regression predicting number of head flicks, and linear regressions predicting time spent manipulating seeds in paws and travel time by eastern gray squirrels (*Sciurus carolinensis*) in seed presentation trials. Each behavior was predicted as a function of treatment groups, including 1. Control seeds (fit as the model intercept), i.e., dormant northern red oak (*Quercus rubra*) seeds dipped in distilled water, 2. Pyrogallol seeds (dormant red oak dipped in 0.02% pyrogallol solution), 3. Scraped seeds (dormant red oak seeds treated by scraping the wax off the pericarp with sandpaper) and 4. Acetaldehyde seeds treated by dipping seeds in 40ppb acetaldehyde solution. Italicized numbers are significant at an α of 0.1 and boldface numbers are significant at an α of 0.05.

Response variable	Predictor	Estimate	SE	z-value/t-value	p
Head flicks	Intercept (Control)	0.442	0.323	1.37	0.171
	Pyrogallol	-0.036	0.514	-0.071	0.944
	Scraped	-0.442	0.515	-0.857	0.391
	Acetaldehyde	-0.036	0.764	-0.048	0.962
Time spent in paw manipulation (mins; log transformation)	Intercept (Control)	-2.35	0.197	-11.93	**<0.001**
	Pyrogallol	0.237	0.322	0.736	0.47
	Scraped	-0.431	0.307	-1.404	0.175

	Acetaldehyde	0.907	0.483	1.88	*0.074*
Travel time (mins; log transformation)	Intercept (Control)	-0.866	0.245	-3.539	**0.001**
	Pyrogallol	0.205	0.383	0.535	0.596
	Scraped	-0.748	0.371	-2.015	*0.053*
	Acetaldehyde	1.275	0.67	1.902	*0.067*

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5.9 Appendix

Summary of chemical compound information for pericarps and kernels of all seed types studied.

Table A1. Artificial chemical concoction composition based on one sample of stratified Northern red oak (*Quercus rubra*) kernels. Compounds dissolved in distilled water and 1 liter solution below diluted to 1:5 before applying on dormant seeds.

Compounds	**Quantity in 1L**
Malic acid	4 mgs
Pencillamine	2 mgs
Benazoic acid	1 mg
Boric acid	20 mgs
Dodecanoic acid	2 mgs
Hexadecanoic acid	2 mgs
Octadecanoic acid	1 mg
Gamma tocopherol	2 mgs
Furfural	2 mgs
Itaconic anhydride	2 mgs
5-hydroxymethyl-2-furancarboxaldehyde	14 gms
1,2,3-benzenetriol	10 gms
L-lyxose	1 gm
1,1-diethoxyethane	2 mgs

Table A2. Chemical compounds occurring in pericarps of dormant and germinating seeds of Northern red oak (*Quercus rubra*), and early germinating white oak (*Quercus alba*).

Dormant *Q. rubra*	Germinating *Q. rubra*	*Q. alba*
1-Heptacosanol	1-methylene-1H-Indene	1-Eicosanol
1,30-Triacontanediol	1,30-Triacontanediol	1,30-Triacontanediol
24-Methylenecycloartanol	24-Methylenecycloartan-3-one	boric acid
Heptacosane	Heptacosane	Ethylene glycol
Heptacosane	Lup-20(29)-en-3-one	Glycolic acid
n-Hexadecanoic acid	n-Hexadecanoic acid	Heptacosane
n-Tetracosanol-1	Nonacosane	Hexacosane
Nonacosane	Octacosane	Malic acid
Octacosane	Octadecanal	n-Hexadecanoic acid
Octadecanal	Octadecanoic acid	n-Tetracosanol-1
Oleic Acid	Oleic Acid	Octacosane
Tetracosanal	Pyrogallol	Octadecanal
γ-Sitosterol	Stigmast-4-en-3-one	Octadecanoic acid
	Stigmast-4-en-3-one	Pentadecanal
	Tetracosanal	Phosphoric acid
	γ-Sitosterol	Stigmast-4-en-3-one
		Tetracosanal
		Vitamin E acetate
		α-Hydroxylsobutyric acid
		β-Sitosterol
		β-Tocopherol
		γ-Sitosterol
		γ-Tocopherol

Table A3. Chemical compounds occurring in kernels of dormant and germinating seeds of Northern red oak (*Quercus rubra*), and early germinating white oak (*Quercus alba*).

Dormant *Q. rubra*	Germinating *Q. rubra*	*Q. alba*
1-deoxy-Inositol	1-deoxy-Inositol	1-deoxy-Inositol
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	1-methylene-1H-Indene	2-Furanmethanol
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	1,30-Triacontanediol	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one
24-Methylenecycloartanol	2-Furanmethanol	2,3-dihydroxy-Propanal
5-(hydroxymethyl)-2-Furancarboxaldehyde	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
Anhydro-d-mannosan	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
Boric acid	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	5-(hydroxymethyl)-2-Furancarboxaldehyde
D-Glucose	3,5-dihydroxy-2-methyl-4H-Pyran-4-one	Acetic acid, anhydride with formic acid
Ethylene glycol	3,5-dihydroxy-2-methyl-4H-Pyran-4-one	Ethylene glycol
Gallic acid	5-(hydroxymethyl)-2-Furancarboxaldehyde	Furfural
Isocitric acid	5-(hydroxymethyl)-2-Furancarboxaldehyde	methyl 3,6-anhydro-α-D-Mannopyranoside
Malic acid	Acetic acid, anhydride with formic acid	Oxalic acid
n-Hexadecanoic acid	Acetic acid, anhydride with formic acid	Propanoic acid, 2-oxo-, methyl ester
Octadecanoic acid	Furfural	Sucrose
Oleic Acid	Levoglucosenone	α-Hydroxylsobutyric acid
Pentadecanal	Octadecanal	β-Tocopherol
Phosphoric acid	Oxalic acid	γ-Tocopherol
Pyrogallol	Pentadecanal	
Succinic acid	Pyrogallol	
Sucrose	Stigmast-4-en-3-one	
β-Tocopherol	β-Sitosterol	
γ-Sitosterol	β-Tocopherol	

Table A4. Chemical compounds occurring in pericarps of dormant and germinating seeds of American chestnuts (*Castanea dentata*), and hybrid of American and Chinese chestnut (*Castanea dentata* X *Castanea mollissima*).

Dormant	Germinating
1-Eicosanol	1-Eicosanol
2,4-bis(1-methyl-1-phenylethyl)-Phenol	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one
Friedelan-3-one	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
Heptacosane	5-(hydroxymethyl)-2-Furancarboxaldehyde
n-Hexadecanoic acid	6,7-Dimethoxycoumarin
Octadecanoic acid	Acetic acid, anhydride with formic acid
Oleic Acid	Friedelan-3-one
Squalene	Heptacosane
Sucrose	methyl 3,6-anhydro-α-D-Mannopyranoside
β-Tocopherol	n-Hexadecanoic acid
γ-Sitosterol	Octadecanoic acid
	Olean-12-en-3-one
	Oleic Acid
	Squalene
	Sucrose
	α-Amyrin
	β-Amyrin
	β-Tocopherol
	γ-Sitosterol

Table A5. Chemical compounds occurring in kernels of dormant and germinating seeds of American chestnuts (*Castanea dentata*), and hybrid of American and Chinese chestnut (*Castanea dentata* X *Castanea mollissima*).

Dormant	Germinating
1,3-dihydroxy-2-Propanone	1,3-dihydroxy-2-Propanone
2-Furanmethanol	1,30-Triacontanediol
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	2-Furanmethanol
2,3-dihydroxy-Propanal	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one
2,4-bis(1-methyl-1-phenylethyl)-Phenol	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	3-Deoxy-d-mannoic lactone
3-Deoxy-d-mannoic lactone	5-(hydroxymethyl)-2-Furancarboxaldehyde
5-(hydroxymethyl)-2-Furancarboxaldehyde	Acetic acid, anhydride with formic acid
Acetic acid, anhydride with formic acid	Behenic alcohol
Linoleic acid	Linoleic acid
n-Hexadecanoic acid	n-Hexadecanoic acid
Octadecanoic acid	n-Tetracosanol-1
Oleic Acid	Octadecanoic acid
Oxalic acid	Oleic Acid
Propanoic acid, 2-oxo-, methyl ester	Oxalic acid
Sucrose	Propanoic acid, 2-oxo-, methyl ester
y-tocopherol	Sucrose
β-Tocopherol	y-tocopherol
γ-Sitosterol	β-Tocopherol
	γ-Sitosterol

Table A6. Retention times (minutes) and NMDS axes loadings for compounds. Sample from which compound was isolated and solvent used during the extraction are also provided.

Retention Times	Compounds	NMDS 1	NMDS 2	Solvent	sample
14.781	Pyrogallol	3.726	-0.285	acetone	Red oak shells
20.935	n-Hexadecanoic acid	-0.683	-0.106	acetone	Red oak shells
31.467	γ-Sitosterol	-0.534	0.311	acetone	Red oak shells
32.466	24-Methylenecycloartanol	-1.113	-0.395	acetone	Red oak shells
32.566	Stigmast-4-en-3-one	-0.281	1.061	acetone	Red oak shells
27.109	1-Heptacosanol	-1.345	-0.504	acetone	Red oak shells
28.52	Heptacosane	-1.294	-0.502	acetone	Red oak shells
21.056	n-Hexadecanoic acid	0.510	-0.566	hexane	Red oak shells
22.741	Oleic Acid	-0.234	-0.432	hexane	Red oak shells
25.629	Octacosane	1.103	0.843	hexane	Red oak shells
26.665	Tetracosanal	-0.499	0.018	hexane	Red oak shells
27.113	n-Tetracosanol-1	-0.904	-0.221	hexane	Red oak shells
28.116	Octadecanal	-0.484	0.232	hexane	Red oak shells
28.527	Nonacosane	-0.408	0.031	hexane	Red oak shells
29.825	Heptacosane	-0.482	0.387	hexane	Red oak shells
30.899	1,30-Triacontanediol	-0.010	0.298	hexane	Red oak shells
31.65	γ-Sitosterol	0.193	-0.821	hexane	Red oak shells

32.499	24-Methylenecycloartanol	-0.917	-0.273	hexane	Red oak shells
22.942	Octadecanoic acid	0.705	-0.935	hexane	Red oak shells
32.138	Lup-20(29)-en-3-one	0.830	-0.097	hexane	Red oak shells
32.569	24-Methylenecycloartan-3-one	0.820	-0.086	hexane	Red oak shells
32.675	Stigmast-4-en-3-one	0.825	-0.075	hexane	Red oak shells
11.722	1-methylene-1H-Indene,	0.722	0.268	hexane	Red oak shells
15.741	Sucrose	0.315	0.721	methanol	Chestnut shells
20.951	n-Hexadecanoic acid	-0.348	-0.033	methanol	Chestnut shells
31.531	γ-Sitosterol	0.658	-0.245	methanol	Chestnut shells
20.951	α-D-Mannopyranoside, methyl 3,6-anhydro-	-0.047	-0.120	methanol	Chestnut shells
11.005	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	0.282	0.647	methanol	Chestnut shells
21.218	6,7-Dimethoxycoumarin	-0.341	-0.068	methanol	Chestnut shells
22.838	Octadecanoic acid	-0.416	-0.055	methanol	Chestnut shells
3.274	Acetic acid, anhydride with formic acid	0.792	-1.040	acetone	Chestnut shells
15.302	Sucrose	0.084	0.271	acetone	Chestnut shells
8.265	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	1.233	-1.613	acetone	Chestnut shells
11.118	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	1.029	-0.838	acetone	Chestnut shells
12.434	5-(hydroxymethyl)-2-Furancarboxaldehyde	1.233	-1.613	acetone	Chestnut shells
20.954	n-Hexadecanoic acid	0.648	0.447	acetone	Chestnut shells
22.638	Oleic Acid	1.212	0.176	acetone	Chestnut shells
25.906	2,4-bis(1-methyl-1-phenylethyl)-Phenol	-0.504	0.229	acetone	Chestnut shells
21.196	6,7-Dimethoxycoumarin	0.958	0.811	acetone	Chestnut shells
29.699	β-Tocopherol	-0.622	0.148	acetone	Chestnut

					shells
31.523	γ-Sitosterol	-0.575	0.003	acetone	Chestnut shells
21.808	n-Hexadecanoic acid	0.763	-0.044	hexane	Chestnut shells
22.689	Oleic Acid	0.490	0.719	hexane	Chestnut shells
21.798	1-Eicosanol	0.653	-0.752	hexane	Chestnut shells
22.876	Octadecanoic acid	0.851	-0.705	hexane	Chestnut shells
28.087	Squalene	-0.520	-0.445	hexane	Chestnut shells
28.523	Heptacosane	-1.045	0.104	hexane	Chestnut shells
29.738	β-Tocopherol	1.177	-0.199	hexane	Chestnut shells
31.609	γ-Sitosterol	0.883	0.451	hexane	Chestnut shells
31.728	Olean-12-en-3-one	0.980	-0.034	hexane	Chestnut shells
31.919	α-Amyrin	1.002	0.221	hexane	Chestnut shells
32.27	β-Amyrin	1.002	0.221	hexane	Chestnut shells
33.375	Friedelan-3-one	0.372	-0.009	hexane	Chestnut shells
5.55	2,3-dihydroxy-Propanal	0.208	0.385	methanol	Chestnut kernels
6.552	2-Propanone, 1,3-dihydroxy-	0.224	-1.027	methanol	Chestnut kernels
10.985	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	0.240	0.217	methanol	Chestnut kernels
12.317	5-(hydroxymethyl)-2-Furancarboxaldehyde	0.367	0.390	methanol	Chestnut kernels
16.096	Sucrose	-0.746	-0.175	methanol	Chestnut kernels
18.253	3-Deoxy-d-mannoic lactone	-0.329	-0.351	methanol	Chestnut kernels
20.953	n-Hexadecanoic acid	-1.346	0.195	methanol	Chestnut kernels
22.576	Octadecanoic acid	-1.391	0.197	methanol	Chestnut kernels
29.664	β-Tocopherol	-1.361	-0.078	methanol	Chestnut kernels

2.292	Oxalic acid	0.522	-0.468	methanol	Chestnut kernels
2.751	Acetic acid, anhydride with formic acid	0.430	-0.312	methanol	Chestnut kernels
5.962	2-Furanmethanol	0.575	0.442	methanol	Chestnut kernels
9.619	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	0.489	0.287	methanol	Chestnut kernels
2.187	Acetic acid, anhydride with formic acid	-0.282	0.471	acetone	Chestnut kernels
5.799	2-Furanmethanol	-0.356	0.413	acetone	Chestnut kernels
6.494	Propanoic acid, 2-oxo-, methyl ester	-0.395	0.468	acetone	Chestnut kernels
6.695	2-Propanone, 1,3-dihydroxy-	-0.676	-0.367	acetone	Chestnut kernels
10.977	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	-0.472	-0.108	acetone	Chestnut kernels
9.57	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	-0.293	0.415	acetone	Chestnut kernels
11.634	Propanal, 2,3-dihydroxy-	-0.500	0.180	acetone	Chestnut kernels
12.289	5-(hydroxymethyl)-2-Furancarboxaldehyde	-0.609	-0.171	acetone	Chestnut kernels
15.819	Sucrose	0.513	0.204	acetone	Chestnut kernels
20.967	n-Hexadecanoic acid	0.787	-1.099	acetone	Chestnut kernels
22.839	Oleic Acid	0.801	-0.653	acetone	Chestnut kernels
25.909	2,4-bis(1-methyl-1-phenylethyl)-Phenol,	-0.249	0.703	acetone	Chestnut kernels
29.688	β-Tocopherol	1.726	0.819	acetone	Chestnut kernels
31.473	γ-Sitosterol	1.606	-0.041	acetone	Chestnut kernels
2.424	Oxalic acid	-0.414	0.444	acetone	Chestnut kernels
8.176	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	-0.288	0.344	acetone	Chestnut kernels
20.985	n-Hexadecanoic acid	0.669	-0.137	hexane	Chestnut kernels
22.63	Linoleic acid	-0.008	0.273	hexane	Chestnut kernels
29.658	y-tocopherol	0.001	0.280	hexane	Chestnut

					kernels
31.595	γ-Sitosterol	-0.077	0.287	hexane	Chestnut kernels
25.666	Behenic alcohol	-0.279	-0.199	hexane	Chestnut kernels
27.194	n-Tetracosanol-1	-0.227	-0.211	hexane	Chestnut kernels
29.754	β-Tocopherol	-0.206	-0.182	hexane	Chestnut kernels
30.827	1,30-Triacontanediol	-0.049	-0.158	hexane	Chestnut kernels

VITA

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EDUCATION

Ph.D.	2011-Dec 2016, Quantitative Ecology, Purdue University
M.S.	2008-2011, Conservation Biology, Central Michigan University
B.S.	2004-2008, Life Sciences and Biochemistry, Mumbai University

PUBLICATIONS

Published papers or In press

Sundaram M, NI Lichti, MA Steele, HJ Dalgleish and RK Swihart. Frequency-dependent hoarding and its implications for seed survival. Journal of Mammalogy, In press.

Swihart RK, **Sundaram M**, Höök T, DeWoody JA. 2016. Factors affecting scholarly performance by Fisheries and Wildlife faculty. Journal of Wildlife Management 80:563-572.

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AWARDS AND FELLOWSHIPS

2014	American Society of Mammalogists Grants-in-Aid of Research ($1500)
2011-2016	Fred M. van Eck Scholarship, Hardwood Tree Improvement and Regeneration Center, Purdue University ($126,000)
2008-2011	Teaching & Research Assistantships, Department of Biology, CMU
2008-2010	Summer Research Grant, Department of Biology, CMU ($1000)
2009-2010	Presentation Travel Grant, Department of Biology, CMU ($700)
2005-2006	Science Honors Program Award for obtaining A+ grade in research module, Mumbai University

TEACHING EXPERIENCE

2011-Present Graduate Teaching Assistant at Purdue University

- Guided and encouraged student discussions for 1 semester of Global Environmental Issues
- Assisted in lab exercises for 2 semesters of Natural Resources Information Systems

2008-2011 Graduate Teaching Assistant at CMU

- Taught, wrote and graded quizzes and assignments for Introduction to Biology Laboratory courses for 5 semesters (20 sections, ~480 students)

EXTENSION AND SERVICE

2015 CS Academy, India

- Invited lecture ‘Being a researcher’ to grades 9, 10 & 11 of CS Academy, Coimbatore

2015 Outdoor Indiana magazine

- Interviewed on factors influencing selection of American chestnut seeds by squirrels

2014 CS Academy, India

- Invited lecture ‘Global biodiversity’ to grades 9 & 10 of CS Academy, Coimbatore
- Invited lecture ‘Global biodiversity’ to grades 9 & 10 of CS Academy, Erode

- Invited lecture ‘A student’s decision-making map to university’ to grades 11 & 12 of CS Academy, Erode

2014 Hardwood Tree Improvement Regeneration Center Advisory Committee, Purdue University

- Presentation on ‘American chestnut seed preference in squirrels’ to committee

2013 Purdue University

- Compass magazine article ‘Seed handling by eastern gray squirrels’ (Spring edition)

2009 Mumbai University

- Mentor undergraduate students interested in graduate research experiences outside of India