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Signaling from below: rodents select for deeper fruiting truffles with stronger volatile emissions

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Abstract. Many plant and fungal species use volatile organic compounds (VOCs) as chemical signals to convey information about the location or quality of their fruits or fruiting bodies to animal dispersers. Identifying the environmental factors and biotic interactions that shape fruit selection by animals is key to understanding the evolutionary processes that underpin chemical signaling. Using four *Elaphomyces* truffle species, we explored the role of fruiting depth, VOC emissions, and protein content in selection by five rodent species. We used stable isotope analysis of nitrogen ($\delta^{15}N$) in truffles to estimate fruiting depth, proton-transfer-reaction mass spectrometry to determine volatile emission composition, and nitrogen concentrations to calculate digestible protein of truffles. We coupled field surveys of truffle availability with truffle spore loads in rodent scat to determine selection by rodents. Despite presumably easier access to the shallow fruiting species, *E. americanus* (0.5-cm depth) and *E. verruculosus* (2.5-cm depth), most rodents selected for truffles fruiting deeper in the soil, *E. macrosporus* (4.1-cm depth) and *E. bartlettii* (5.0-cm depth). The deeper fruiting species had distinct VOC profiles and produced significantly higher quantities of odiferous compounds. *Myodes gapperi* (southern red-backed vole), a fungal specialist, also selected for truffles with high levels of digestible protein, *E. verruculosus* and *E. macrosporus*. Our results highlight the importance of chemical signals in truffle selection by rodents and suggest that VOCs are under strong selective pressures relative to protein rewards. Strong chemical signals likely allow detection of truffles deep within the soil and reduce foraging effort by rodents. For rodents that depend on fungi as a major food source, protein content may also be important in selecting truffles.

Key words: chemical ecology; communication; Elaphomyces; *fungal interactions; fungal volatiles; fungivory; mammal; truffle; volatile organic compounds.*

Introduction

Many plant and fungal species rely on animals to disperse their propagules. Dispersers are often attracted to the fruits or fruiting bodies that contain propagules by color or odor signals. The form or magnitude of these communication signals is impacted by both biotic interactions and environmental factors (Schaefer and Braun 2009, Beyaert and Hilker 2014). For example, in some dispersal mutualisms, signals are used by animals to assess the nutritional quality of food items provided as an incentive for propagule dispersal (Schaefer et al. 2008, Knauer and Schiestl 2015), whereas in other systems, signals may be used to increase contrast against the environmental background so dispersers can detect fruits or flowers within complex surroundings (Cazetta et al. 2009, Lomáscolo et al. 2010).

Chemicals, in the form of volatile organic compounds (VOCs), are common signals used to mediate interactions in various dispersal mutualisms including those between flowers and insect pollinators (Huber et al. 2005), fruits and mammal dispersers (Lomáscolo et al. 2010, Valenta et al. 2013), fungal spores and insect dispersers (Steinebrunner et al. 2008), and seeds and ant dispersers (Turner and Frederickson 2013). Animal selection on VOCs is a strong evolutionary force contributing to both divergence and convergence of odor signals among populations and species (Huber et al. 2005, Lomáscolo et al. 2010, Gross et al. 2016). Although less documented for chemical communication, the local environment may also exert strong evolutionary

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pressures on odor signals, similar to how differences in light levels and background foliage shape color signals in flowers and fruits (Beyaert and Hilker 2014, Valenta et al. 2017). Identifying how biotic interactions and environmental factors can shape chemical communication is key to understanding the evolutionary processes that underpin signaling in dispersal ecology (Rodriguez et al. 2013).

Unlike fruits and flowers that often use a combination of color and odor to attract dispersers or pollinators (Valenta et al. 2017), the belowground sporocarps, or fruiting bodies, of mycorrhizal fungi rely exclusively on chemical communication for animal dispersal. Mycorrhizal fungi are symbionts that colonize the roots of plants and aid in the transfer of soil nutrients and water (Smith and Read 1997). Many mycorrhizal taxa have evolved from having an aboveground sporocarp (mushroom) to having a belowground sporocarp (truffle) to protect against drought or freezing (Thiers 1984, Trappe 1988). Although mushroom spores may be wind-borne, hypogeous (below ground) fungi are sequestrate—the spores are entirely encased within the fruiting body—and require animals, especially mammals, to consume their fruiting bodies for spore dispersal (Johnson 1996). As such, truffles use chemical communication in the form of VOC emissions to alert mammals to their subterranean location (Maser et al. 1978, Donaldson and Stoddart 1994, Pyare and Longland 2001).

The evolution of truffles and subsequent dispersal by animals has been highly successful, independently arising many times across the world with some extant families consisting only of hypogeous forms (Johnson 1996, Peintner et al. 2001, Justo et al. 2010). This morphological divergence in fruiting habit has occurred rapidly, suggesting that strong selective pressures are driving changes in both fruiting habit and dispersal mechanisms (Thiers 1984, Bruns et al. 1989). Truffle odors have long been hypothesized to be a key form of communication that allowed this transition, yet the role of VOC signals and the belowground fruiting environment (e.g., fruiting depth) in shaping interactions between truffles and their mammalian dispersers are not well understood (Maser et al. 1978, Splivallo et al. 2011). Because truffles are nutrient-poor compared to mushrooms or seeds, truffles may rely more on VOC signals than nutritional rewards to secure dispersers (Cork and Kenagy 1989a, Wallis et al. 2012).

To understand the role of biotic and abiotic factors in shaping truffle-mammal interactions better, we studied selection of four *Elaphomyces* truffle species by five rodent species in eastern North America. The genus *Elaphomyces* is highly diverse, with over 50 described species, and occurs on all continents except Antarctica (Castellano et al. 2011, 2016, Paz et al. 2017). *Elaphomyces* forms mycorrhizal associations with both angiosperms and gymnosperms, and fruits as sequestrate sporocarps characterized by a spore-laden gleba encased in a thick peridium (Fig. 1a–d; Trappe 1979). *Elaphomyces* taxa often differ in their fruiting depth, with some species semiemergent in the leaf litter, whereas others are hypogeous deep within the organic horizon or mineral soil (Castellano et al. 2011, 2012, 2016). Additionally, odor emissions of *Elaphomyces* sporocarps can be quite variable, ranging from indistinct or mild in some species to musty, cabbage-like, or “disagreeable” in others (Castellano et al. 2011, 2018). Mammals, such as rodents (e.g., mice, voles, and chipmunks), excavate *Elaphomyces* truffles and consume the peridium along with spores which pass through the digestive system and are dispersed in scat (Fig. 1e–h; Cork and Kenagy 1989a, Castellano and Stephens 2017).

We used differences among *Elaphomyces* species to determine how truffles modify their chemical signal in relation to fruiting depth and how this relationship, along with truffle abundance and nutritional content, shapes selection by rodent species that differ in their reliance on fungi. We used stable isotope analysis of nitrogen ($\delta^{15}N$) in truffles to estimate soil fruiting depth, proton-transfer-reaction mass spectrometry to determine volatile emission composition (a measure of odor signals), and nitrogen concentration to calculate digestible protein of truffles (a measure of nutritional reward). To determine truffle selection by rodent species, we coupled field surveys of truffle abundance with spore loads in scat collected from live-trapped rodents. From these data, we ask the following questions: (1) Do VOC emissions differ among truffle species that fruit at different depths and does VOC composition influence selection by rodents? (2) Are chemical signals or nutritional rewards more important for truffle selection by rodents? We predicted that deeper fruiting truffle species would produce stronger chemical signals than shallower fruiting species to increase odor contrast against the soil. Because truffles rely exclusively on VOC emissions to facilitate detection, we predicted that strong VOC signals would be more important for rodent selection than nutritional rewards (Cork and Kenagy 1989a, Wallis et al. 2012).

Methods

Study system and sampling

Our study took place in mature forests at Bartlett Experimental Forest in the White Mountain National Forest of New Hampshire (44°3′7.2″ N, 71°17′25.1″ W). The climate is humid continental with warm summers (mean July temperature, 19°C), cold winters (mean January temperature, −9°C), and precipitation distributed evenly throughout the year (Richardson et al. 2007). Our work focused on four *Elaphomyces* species (*E. americanus*, *E. verruculosus*, *E. macrosporus*, and *E. bartlettii*), which are common in the forests of northeastern North America (Fig. 1a–d; Stephens et al. 2017*b*). Although *E. americanus* occurs across forest stands, the other *Elaphomyces* species are strongly associated with eastern

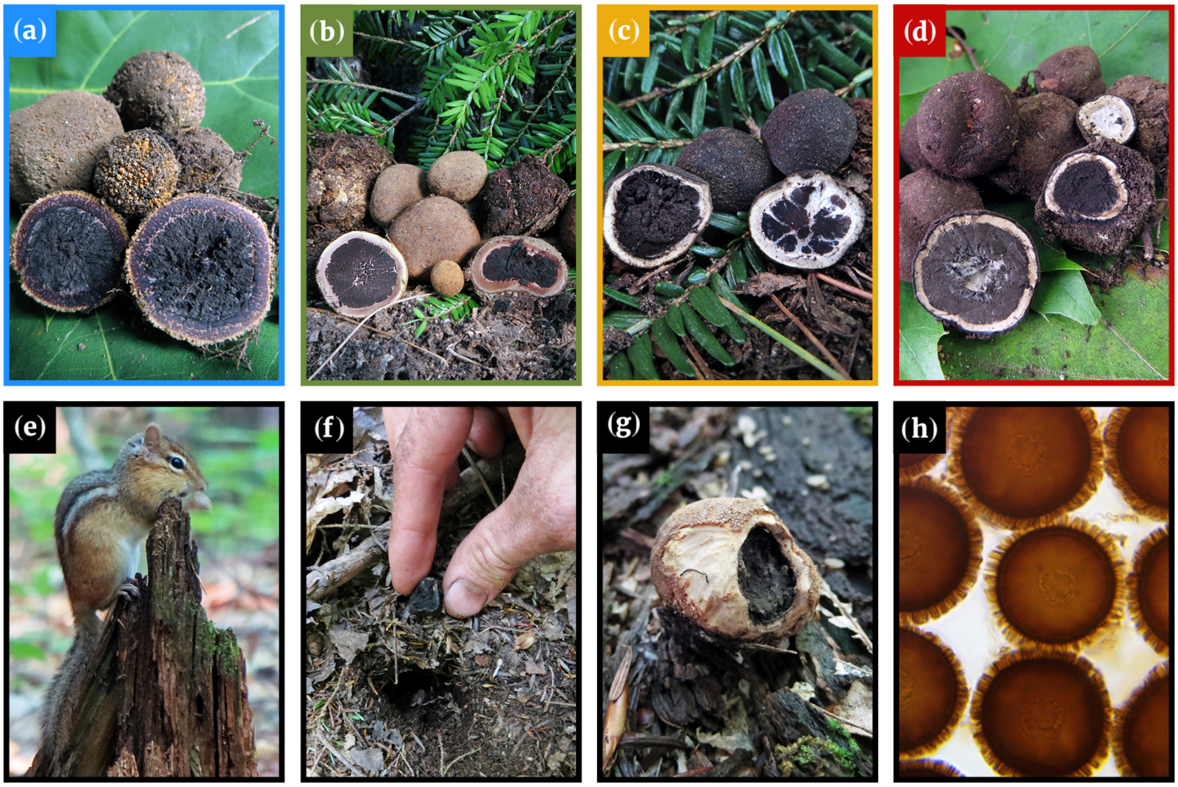

FIG. 1. (a) *Elaphomyces americanus*, (b) *E. verruculosus*, (c) *E. macrosporus*, and (d) *E. bartlettii* fruit as hypogeous sporocarps (truffles) characterized by a powdery gleba of spores encased in a thick peridium. Small mammals such as mice, voles, and chipmunks [(e) *Tamias striatus*] excavate sporocarps [(f) hole dug to reach an *E. bartlettii* sporocarp fruiting 7 cm below the soil surface (cataloged in Oregon State Mycological Collection 149818)], consume the peridium [(g); partially consumed sporocarp of *E. verruculosus*], and ingest the microscopic spores [(h); spores of *E. macrosporus*], which pass through the gut and are dispersed in scat.

hemlock (*Tsuga canadensis*; Stephens et al. 2017*b*). To ensure that rodents had access to all *Elaphomyces* species, we focused our sampling within eight forest stands where eastern hemlock made up 23–62% of the total basal area (mean 41%). Within each of these eight forest stands, we sampled rodents and truffles on a 1.1-ha grid that was divided into an 8 × 8 station arrangement with 15 m spacing between stations for a total of 64 sampling stations per stand. Soils were Spodosols with a thick organic layer extending to a depth of approximately 16 cm (Stephens et al. 2017*b*).

During June, July, and August of 2014, we indexed truffle abundance at 48 plots (4 m^2) stratified across sampling stations within each grid. Details of truffle sampling can be found in Stephens et al. (2017*b*). Briefly, during each month we sampled 16 plots on each grid using a short-tined garden cultivator and extracted truffles within 10 cm of the soil surface. Truffles were identified to species, counted, dried at 60°C for 24–48 h, and weighed to the nearest 0.01 g. Because the sporocarps of the *Elaphomyces* species included in our study are similar in size (Castellano and Stephens 2017) and mammals forage for individual sporocarps rather than biomass, we used sporocarp abundance for mammal selection analyses. Fruiting of *Elaphomyces* species varies little over the summer (Stephens et al. 2017*b*), and we calculated grid-level truffle abundance as the average number of sporocarps collected across all 48 plots, converted to truffles per hectare. Additionally, similar to other truffle taxa (e.g., Pyare and Longland 2001), sampling in subsequent summers indicated that fruiting location and relative abundance of *Elaphomyces* species were stable, and we used truffle sampling in 2014 as an index of truffle abundance for the entire study.

For truffle selection, we focused on five rodents that are among the most common species in the Northeast (Stephens et al. 2017*a*): woodland jumping mice (*Napaeozapus insignis*), white-footed mice (*Peromyscus leucopus*), deer mice (*P. maniculatus*), eastern chipmunks (*Tamias striatus*), and southern red-backed voles (*Myodes gapperi*). Although all five rodent species consume fungi during the summer, fungi only comprise about 15% of the diets of *N. insignis*, *P. leucopus*, *P. maniculatus*, and *T. striatus* whereas *M. gapperi* is a fungal specialist with over 60% of its diet from fungi (Stephens and Rowe, *in press*). To determine consumption of *Elaphomyces* species by rodents, we collected scat samples in June, July, and August of 2013–2015 on each grid.

Rodents were captured in Sherman live traps baited with bird seed and set within 1.5 m of each grid station. Polyester batting was added for insulation and traps were checked twice daily for 4 d. Each individual was marked with uniquely numbered ear tags (model 1005-1; National Band and Tag Co.), passive integrated transponder tags (model HPT9; Biomark, Boise, Idaho, USA), or both. Scat samples were collected from the trap upon first capture of an individual during each month and frozen at −18°C. Traps with captures were washed and replaced to avoid contamination from other individuals. For spore microscopy, in each month and year of trapping we selected up to 10 scat samples for a rodent species on a grid. Our trapping protocol was approved by the University of New Hampshire Animal Care and Use Committee (protocols 120708 and 140304) and followed guidelines recommended by the American Society of Mammalogists (Sikes et al. 2016).

Truffle fruiting depth

Determining truffle fruiting depth is difficult because hypogeous sporocarps have no aboveground visual cues and truffle collection via raking disturbs soil layers. To overcome this issue, we estimated fruiting depth using an isotopic approach (Hobbie et al. 2014) that related the δ^{15}N of truffle peridia to soil depth from a subset of carefully excavated soil profiles. Soils dominated by ectomycorrhizal fungi and their plant associates show strong and predictable patterns of ^{15}N enrichment with depth due to accumulation of ^{15}N-depleted leaf litter at the surface coupled with preferential retention of ^{15}N-enriched compounds during decomposition (Hobbie and Ouimette 2009). Sporocarp δ^{15}N signatures of mycorrhizal fungi reflect the depth at which their hyphae occur, with some ^{15}N enrichment due to preferential transfer of ^{15}N-depleted nitrogen to plants (Hobbie et al. 2014). Thus, because hypogeous sporocarps fruit underground near their hyphae, their sporocarp ^{15}N should reflect the depth at which they fruit. To test this, we sampled truffles and soils in August of 2015 from soil profiles at a subset of truffle plots (n = 13) where truffles were abundant in 2014. Soil profiles, approximately 15 cm in diameter and 30 cm deep, were extracted using a narrow spade and the organic horizon divided into 1–2-cm sample increments. The identity of truffles present within each depth increment was recorded, and co-occurring soils (collected from the same depth) were sieved to 2 mm to remove roots and debris. From the 13 soil profiles, this yielded 1 *E. americanus*, 17 *E. verruculosus*, 14 *E. macrosporus*, and 5 *E. bartlettii*. Soils and truffle peridia were then dried at 60°C, ground to a fine powder using a puck mill, and analyzed for δ^{15}N and %N.

We confirmed that soil δ^{15}N was highly and positively correlated with depth (Appendix S1: Fig. S1a; adjusted r^2 = 0.82, P < 0.0001), indicating that it could be used as a natural tracer of truffle fruiting depth. Subsequently, with our known-depth truffles we used a regression analysis to establish a relationship between truffle depth and truffle δ^{15}N across all four *Elaphomyces* species. Truffle δ^{15}N was included as a quadratic term because its relationship with depth was nonlinear (Appendix S1: Fig. S1b). We also included truffle %N in the regression because protein content affects fungal δ^{15}N (Hobbie et al. 2012). We then used this regression relationship to estimate fruiting depth of a larger set of sporocarps collected during the 2014 surveys, which included 21 *E. americanus*, 55 *E. verruculosus*, 42 *E. macrosporus*, and 16 *E. bartlettii*. Each species was sampled from an average of 12 plots (range 6–18) with a maximum of six sporocarps selected from a given plot.

All truffle peridia and soils were analyzed for nitrogen stable isotope ratios (δ^{15}N) and elemental composition (%N) at the University of New Hampshire Stable Isotope Lab (www.isotope.unh.edu) on an Elementar Americas (Ronkonkoma, New York, USA) Pyrocube elemental analyzer coupled to a GeovisION isotope ratio mass spectrometer. The ratio of sample analyses to in-house standards analyzed was less than 4:1. The measurement uncertainty of the instrument as determined by repeated analyses of in-house QA/QC standards was ± 0.20 ‰ (± 1σ) for δ^{15}N (see Appendix S1 for more details).

Truffle protein and size

To estimate total protein of truffles, we multiplied the %N of truffle peridia (from samples used to calculate fruiting depth) by 5.6 following Mariotti et al. (2008). We determined digestible protein of *Elaphomyces* peridia as 55% of total protein (Cork and Kenagy 1989a), which is similar to other truffle taxa (Wallis et al. 2012). For truffle size, we used the average dry mass of single sporocarps (immature truffles excluded) collected during surveys in 2014. This included 68 *E. americanus*, 117 *E. verruculosus*, 69 *E. macrosporus*, and 10 *E. bartlettii*.

Truffle selection by rodents

We determined truffle selection of rodent species by comparing truffle consumption relative to availability. When consuming the peridium of *Elaphomyces* sporocarps, rodents ingest the spore-laden gleba (Fig. 1f–h), subsequently passing spores in their scat. The abundance of spores in scat is a product of how many, and how often, truffles are eaten, and can be used as an index of truffle consumption (Stephens and Rowe, *in press*). Following methods in Stephens et al. (2017*b*), we calculated the number of spores per gram of rodent scat for each *Elaphomyces* species. Spores were distinguished based on distinctive morphology, including size, color, and ornamentation (Castellano and Stephens 2017).

Although *Elaphomyces* spp. are among the most commonly consumed fungal taxa by rodents at Bartlett Experimental Forest, there are over 30 other fungal taxa consumed (Stephens and Rowe, *in press*). Additionally, fungi only comprise about 15% of the diet for most of the rodent species during the summer (Stephens and Rowe, *in press*). Thus, because all four *Elaphomyces* species were rarely consumed on a daily basis by an individual rodent, and spores are expelled within 3 d (Cork and Kenagy 1989b, Danks 2012), it is difficult to determine selection for *Elaphomyces* spp. from a single scat sample. Consequently, we measured population-level consumption on each grid as the average spore load of each *Elaphomyces* species in the scat of each rodent species. We used square-root-transformed spore abundance because of overdispersion of the raw data.

Spore abundance (square-root transformed) and sporocarp abundance were used to calculate Jacob's index of selection on each grid (Jacobs 1974). Compared to other selection indexes, Jacob's index is relatively unbiased toward rare food items (Chesson 1978). It is symmetrical around 0 and is bounded between −1 and 1, with −1 indicating avoidance, 0 indicating consumption relative to availability, and 1 indicating strong selection (Jacobs 1974). For a given rodent species and *Elaphomyces* species *i*, Jacob's index is defined as

$$D_i = \frac{r - p}{r + P - 2rp}$$

where r is the proportion of spores from *Elaphomyces* species i in the scat of the rodent species and p is the proportion of sporocarps from *Elaphomyces* species i available to the rodent species. A requirement of Jacob's index is that food items must be available in order to be consumed. For three grids, spores of *E. bartlettii* were detected in rodent scat, but no sporocarps were found during truffle surveys. To be able to account for the consumption of *E. bartlettii* on these grids, we assigned an availability of 25 truffles per hectare, half of the lowest availability found for a truffle species on a grid. For each rodent and truffle species, we calculated mean and 95% confidence intervals of Jacob's index from the eight sampling grids. Although preferential feedings on truffle species may have influenced estimates of sporocarp abundance used in selection calculations, it is unlikely to have shaped the species abundance distributions. Given that consumption of *Elaphomyces* sporocarps is generally low compared to other truffle taxa (North et al. 1997) and that abundance of sporocarps differed by as much as several orders of magnitude between species (see Results), it is unlikely that rodent feeding greatly influenced our estimates of truffle abundance.

Truffle VOC emissions

For truffle VOC analysis, we collected fresh truffles at Bartlett Experimental Forest on July 17–18, 2016 by searching near recent rodent digs or where truffles were abundant in the 2014 surveys. Truffles were carefully excavated using a short-tined garden cultivator, sorted by ripeness based on peridium color, packed in soil, and placed on ice in the field. Truffles were refrigerated in the lab and shipped overnight on ice to Montana State University on July 20, 2016.

We used proton-transfer-reaction mass spectrometry (PTR-MS) to analyze VOC profiles on 10–15 sporocarps from each species on July 22 and 25 (see Appendix S1: Fig. S3). Individual truffles were carefully removed from the soil and placed on paper towels, then loose and compacted soil was removed from the truffle surface using dry and damp kimwipes. Care was taken to remove as much soil as possible to avoid any confounding volatile emissions or adsorption that would change the signature while also avoiding damaging the tissues, which would induce a volatile wound response. After cleaning, truffles were refrigerated prior to measurement. For analysis, individual truffles were removed from the refrigerator and kept at room temperature for 30 min before being placed in separate 0.4-L glass jars (Ball Corp., Broomfield, Colorado, USA) and capped under a fume hood. Each truffle was sealed in the chamber with a cap modified to accommodate a septum through which two 1/16-inch outside diameter Teflon lines were inserted (inlet and outlet), each ~ 75 cm long. Because only one sample could be analyzed at a time, samples were capped ~15 min apart to account for the time to obtain a steady-state mass spectra for each sample and to ensure a equal build-up of volatiles in the headspace among samples. Each truffle was left in the sealed chamber for approximately 1 h to allow volatiles to accumulate in the headspace prior to analysis using PTR-MS.

After a 1-h equilibration period, headspace volatiles were measured using a high-sensitivity PTR-MS (IONICON Analytik GmbH, Innsbruck, Austria), the details of which have been described in several reviews (Lindinger and Jordan 1998, de Gouw and Warneke 2007, Blake et al. 2009). For the sampling process, 10-sccm (standard cubic centimeters per minute) of medical-grade zero air was introduced into one of the lines. The second line served as the outlet and was connected to a stainless steel tee where an additional flow of 190 sccm of zero air was added, which provided a diluted flow that was then introduced to the PTR-MS. All flows were delivered using MKS mass flow controllers. Each jar was then sampled for approximately 6 min, which allowed five mass spectral scans (20–160 amu) to be recorded. The drift tube of the PTR-MS was operated at 2 mbar and heated to 40°C with a reduced electric field to number density (E/N) of 103 Td. The measured ion intensities were converted to concentrations using a sensitivity factor of 10 normalized counts per second per ppb (ncps/ppb), which is a representative conversion factor for this instrument derived from calibrated gas standards. Conversion from measured intensities to concentration was performed only to cast all of the trials to a common base so that they could be compared directly. No effort was made to quantify any of the individual components that are reported. Identification of the volatile compounds was based primarily on elemental compositions derived from ion mass-to-charge ratio (m/z). These elemental compositions were then compared to see if they corresponded to compounds reported in the existing truffle volatile literature (see Appendix S1: Compound identification).

Most studies of truffle VOCs use dogs to select sporocarps at their peak odor emission stage (e.g., March et al. 2006). We did not have access to a truffle dog, and even truffles with mature gleba may not be at a peak odor emission stage. Preliminary analyses suggested that a number of sporocarps may have been at a different maturational stage relative to other sporocarps we collected, as evidenced by emission of VOCs not found in other samples such as m/z 29 (Appendix S1: Fig. S3). These sporocarps would confound species comparisons and thus were identified and removed using the function "pcout" in the R package mvoutlier, which is an algorithm developed for identifying outliers in high-dimensionality data sets (Filzmoser and Gschwandtner 2017). We removed three *E. americanus*, six *E. verruculosus*, and one *E. bartlettii* (Appendix S1: Fig. S3), and discuss outlier removal further in the discussion section. This left a total of 12 *E. americanus* (seven mature, five immature), nine *E. verruculosus* (eight mature, one immature), 15 *E. macrosporus* (13 mature, two immature), and nine *E. bartlettii* (seven mature, two immature). Mature sporocarps had a gleba composed of fully developed spores and immature sporocarps had a gleba composed of white hypha with no developed spores.

We tested for differences in VOC profiles among truffle species and ripening stages with a Bray-Curtis dissimilarity matrix of VOC compounds and PERMANOVA (multivariate repeated-measures ANOVA—function "adonis" in the R package vegan; Oksanen et al. 2014). We assessed significance through comparisons with 999 randomized data sets and followed up with pairwise comparisons to determine differences between truffle species. Significance levels for pairwise comparisons were adjusted with Holm correction (Holm 1979). To visualize VOC profiles among truffle species, we used nonmetric multidimensional scaling, a robust unconstrained ordination method (NMDS—function "metaMDS" in the R package vegan; Oksanen et al. 2014). Additionally, to visualize point density and overlap among VOC profiles of truffle species better, we generated contours using two-dimensional kernel density estimation (function "stat_density_2d" in the R package ggplot2; Wickham 2009).

We used indicator species analysis to determine VOC compounds associated with a particular truffle species or group of truffle species (Dufrêne and Legendre 1997). Because sample sizes varied among truffle species, we used the group-equalized indicator value function (IndVal.g) with "multipatt" in the R package "indicspecies" and assessed significance ($\alpha = 0.05$) with Monte Carlo tests based on 999 randomizations (Cáceres and Legendre 2009). We only included compounds that made up an average of at least 1% of total ions for one or more truffle species.

All statistical analyses were performed in R version 3.5.1 (R Development Core Team 2016).

Results

Sporocarp fruiting depth and availability

Across the eight sampling grids, sporocarps of *Elaphomyces* species differed in their availability, with *E. verruculosus* an order of magnitude more abundant (mean ± SE sporocarps per ha; 26,699 ± 4,960) than the other *Elaphomyces* species: *E. macrosporus* (2,702 ± 1,303), *E. americanus* (762 ± 209), and *E. bartlettii* (182 ± 104) (Fig. 2a).

Using the regression equation of truffle $\delta^{15}N$ and %N against truffle depth (Appendix S1: Fig. S1b; adjusted $r^2 = 0.69$, $P < 0.0001$) we calculated fruiting depths of truffles based on their peridium $\delta^{15}N$ and %N values (Appendix S1: Fig. S2). With $\delta^{15}N$ as a tracer for fruiting depth (Appendix S1: Figs. S1 and S2), the *Elaphomyces* species were stratified across the first 8 cm of the organic horizon (Fig. 2b). Overall, fruiting depths of *E. americanus* (mean ± SE; 0.5 cm ± 0.1) and *E. verruculosus* (2.5 cm ± 0.2) were relatively shallow, whereas *E. macrosporus* (4.1 cm ± 0.2) and *E. bartlettii* (5.0 cm ± 0.5) fruited deeper below the soil surface.

Sporocarp protein content and size

The *Elaphomyces* differed in their nutritional content with *E. verruculosus* and *E. macrosporus* averaging about 50% more digestible protein (10.5% and 11.1%, respectively) than either *E. americanus* or *E. bartlettii* (7.3% and 7.0%, respectively; Fig. 2c). Although there was variation in the dry mass among individual truffles, *Elaphomyces* species did not differ in size with all species having a mass of ~1 g (Fig. 2d).

VOC emissions

After grouping masses that belonged to the same compound or that had a ^{13}C isotopologue, the PTR-MS identified 60 volatile chemical species (represented as mass/charge [m/z]) released by truffle sporocarps. Based on PERMANOVA, truffle species explained 33% of variation in volatile composition ($F_{3,40} = 6.60$, $P < 0.001$) whereas stage of ripeness only contributed 0.01% and was not significant ($F_{1,40} = 0.44$, $P = 0.837$). Pairwise PERMANOVA comparisons between truffle species indicated that volatile composition was similar between *E. americanus* and *E. verruculosus* ($F_{1,19} = 0.65$, $P = 0.591$), but was significantly different between *E. americanus* and both *E. macrosporus* and *E. bartlettii* ($F_{1,25} = 9.32$, $P = 0.006$; $F_{1,19} = 3.73$, $P = 0.026$, respectively), between *E. verruculosus* and both *E. macrosporus* and *E. bartlettii* ($F_{1,22} = 11.35$, $P = 0.006$; $F_{1,16} = 6.54$,

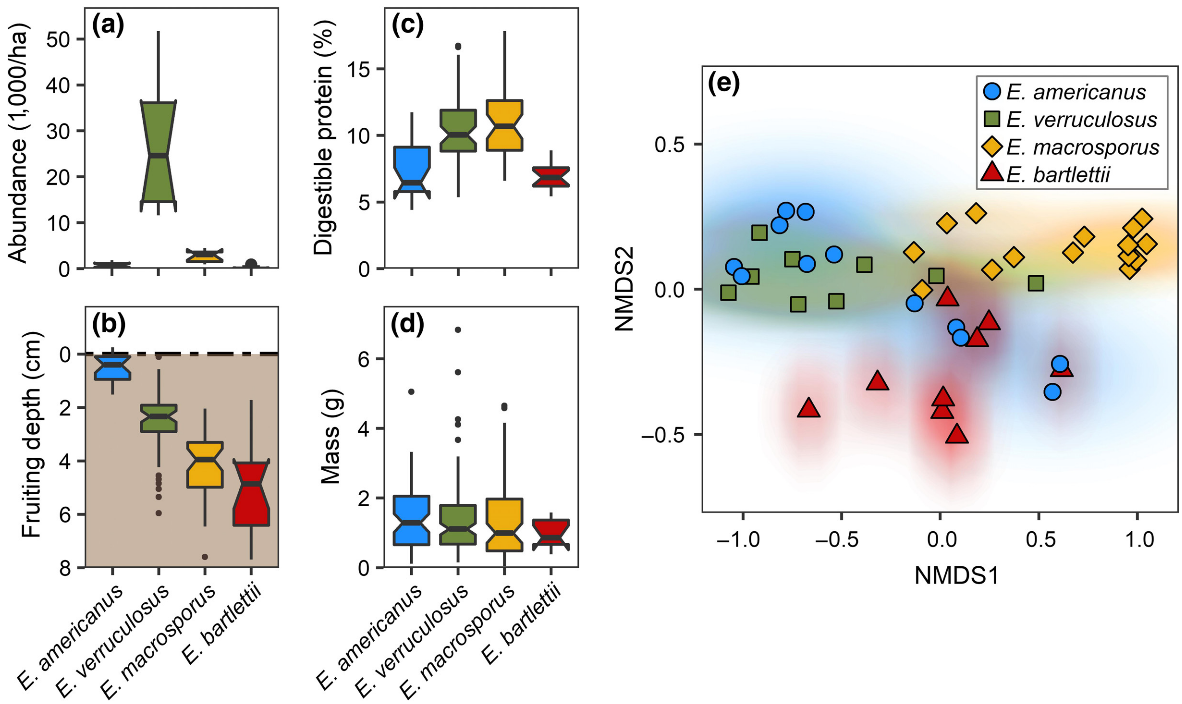

Fig. 2. Truffles of *Elaphomyces* species differed in abundance, fruiting depth, digestible protein, and VOC emission composition, but not mass. (a) Truffle abundance across sampling grids, (b) truffle fruiting depth as estimated from $\delta^{15}N$ and %N values of peridia, (c) truffle digestible protein, (d) dry mass of mature truffles, (e) and two-dimensional nonmetric multidimensional scaling (NMDS) of truffle volatile composition. Thick horizontal lines of boxplots represent medians, with box limits denoting the first and third distributional quartiles and whiskers extending to the most extreme data point within 1.5 times the interquartile range. Dots represent outliers and notches denote 95% confidence intervals around medians ($1.58 \times$ interquartile range/$\sqrt{n}$) with nonoverlapping notches indicating significant differences between medians (McGill et al. 1977). In the NMDS, colored contours around truffle points are based on 2D kernel density estimation and are shown to visualize relative volatile community space occupied by each truffle species.

$P = 0.012$, respectively), and between *E. macrosporus* and *E. bartlettii* ($F_{1,22} = 8.12$, $P = 0.006$). These patterns were apparent in the NMDS ordination, with high overlap between *E. americanus* and *E. verruculosus* and clear separation between *E. macrosporus* and other species on NMDS axis 1 with additional separation of *E. bartlettii* and other species on NMDS axis 2 (two-dimensional NMDS, stress = 0.061; Fig. 2e).

In total, 15 compounds made up at least 1% of total ions for one or more truffle species (Table 1). Of these, 11 were identified as indicator species and were associated with either *E. macrosporus* or *E. bartlettii*. *Elaphomyces macrosporus* was significantly associated with methanol, acetone, and phenylethanol, whereas *E. bartlettii* was associated with ethanol, acetaldehyde, hexanal, and bis (methylthio)methane, among others.

Rodent selection

Of the 967 total rodent scat samples analyzed, spores from one or more *Elaphomyces* species occurred in 799, (82.6%) from 97 *N. insignis* (77.0%, $n = 126$), 145 *P. leucopus* (82.3%; $n = 176$), 253 *P. maniculatus* (80.1%; $n = 316$), 134 *T. striatus* (87.6%; $n = 153$), and 170 *M. gapperi* (86.7%; $n = 196$). There was an average of 20.0 ± 12.1 (range 3–49) scat samples from each rodent species on a grid that contained *Elaphomyces* spores; these were used to measure population-level consumption of truffle species. Patterns in truffle selection were similar among rodent species (Fig. 3). Despite the close proximity of *E. americanus* to the soil surface and the high abundance of *E. verruculosus*, most rodents selected *E. americanus* relative to its availability and avoided *E. verruculosus*. Rodents strongly selected for both *E. macrosporus* and *E. bartlettii*, truffle species with higher VOC emissions and deeper fruiting depths. Selection was not associated with protein content, with the exception of the fungal specialist, *M. gapperi*, which consistently consumed both *E. verruculosus* and *E. macrosporus*.

Discussion

The evolutionary success of truffles has been attributed to their hypogeous fruiting habit that relies almost entirely on chemical communication with mammals for spore dispersal. Studying hypogeous fungi and their interactions with mammals is inherently difficult, and

TABLE 1. Mean (SE) ppb of volatile compounds that comprise at least 1%, on average, of total emissions for one or more *Elaphomyces* species. Bold values indicate compounds that are significant indicator species for a truffle species. Bold compounds reflect the putative identification for the observed ion(s), whereas nonbolded compounds are tentative assignments. See Appendix S1 for details on identifications.

Measured ions† (m/z)	Identity	Species			
		E. americanus	*E. verruculosus*	*E. macrosporus*	*E. bartlettii*
33, 51	**Methanol**	271.9 (85.9)	202.5 (93.2)	**1,216.3 (180.8)**	396.5 (81.6)
91	Fragment ion of benzene methanol	71.2 (21.5)	50.4 (9.9)	106.1 (21.3)	53.4 (7.5)
59, 77	**Acetone/propanal**	37.9 (7.7)	44.3 (8.8)	**99.8 (10.8)**	61.2 (18.8)
111	Fragment ion of C8-volatile	63.7 (29.6)	9.6 (1.3)	21.6 (3.8)	**125.9 (28.1)**
93	Unknown	22.4 (5.8)	16.7 (2.9)	31.2 (5.9)	20 (2.4)
47, 65	**Ethanol**	11.2 (0.9)	9.7 (0.8)	11.9 (0.8)	**68.5 (19.1)**
45, 63	**Acetaldehyde**	13.6 (2.7)	10.5 (1.1)	11 (0.8)	**30.9 (4.3)**
109	Benzene methanol	14.7 (3.8)	10.7 (1.5)	20.7 (3.5)	12.2 (1.3)
69	Fragment ion of C8-volatile, second hydrate methanol	15.2 (6.5)	3.2 (0.5)	8.1 (1.2)	**35.1 (5.8)**
101	Possible sulfur compound	11.7 (1.4)	10.7 (1.5)	9.9 (1.2)	**14.9 (1.0)**
43	Fragment ion	5.8 (0.7)	5.8 (0.8)	9.1 (0.6)	**10.2 (1.4)**
61	bis (methylthio)methane	7.1 (0.8)	6 (0.6)	7.7 (0.6)	**10.8 (1.4)**
105	Fragment ion of phenylethanol	6.3 (1.9)	5.5 (1.3)	**12.3 (2.9)**	4.1 (0.5)
83	Hexanal	4.2 (0.4)	3.3 (0.3)	3.4 (0.4)	**7.9 (2.0)**
125	2-acetyl-5-methylfuran, 2-butylfuran, (methylthio)dimethyl sulfoxide	5.7 (0.2)	5.6 (0.1)	5.3 (0.2)	

† Major ion(s). Listings showing multiple ions include the first hydrate $MH^+(H_2O)$. All abundances include the contribution from their measured ^{13}C isotopologue. Where appropriate contributions from ^{18}O isotopologue are also included.

our study is among the first to investigate factors (i.e., fruiting depth, VOC emissions, and protein content of truffles) that shape selection by mammals. We found that deeper fruiting truffles had more distinct VOC profiles and stronger odor signals and that these truffles were selected for by rodents. These findings suggest that fruiting depth and detection by rodents are likely both strong evolutionary forces that help shape truffle VOC composition. Moreover, truffle VOC signals are more important than protein rewards for most rodent species.

Truffle fruiting depth and VOC emissions

We used nitrogen isotopes to estimate the fruiting depth of *Elaphomyces* truffles, which ranged from just below the soil surface to 8 cm belowground. These differences in fruiting depth may relate to interspecific interactions among mycorrhizal fungal species or tradeoffs in edaphic characteristics. For example, Mujic et al. (2015) found that interspecific competition between truffle-producing species in the genus *Rhizopogon* determined the depth at which they colonized tree roots, with *R. vesiculosus* excluding *R. vinicolor* from the upper soil depths. Changes in edaphic characteristics by soil depth may also represent tradeoffs in acquiring nutrients versus more protected conditions for fruiting. Soil nutrients vary markedly by depth, with easily accessible inorganic nitrogen more available closer to the soil surface compared to the less accessible organic nitrogen available deeper in the soil (Hobbie et al. 2014). Plant root density also declines with soil depth (Jackson et al. 1996), reducing the opportunity for deeper fruiting mycorrhizal species to colonize roots of their obligatory hosts. Despite the advantages of being closer to the soil surface, shallow truffles are likely less buffered from ambient conditions such as drought or freezing (Trappe 1988), and may be more exposed to airborne pathogens. For example, we often observed *Tolypocladium* spp. parasitizing sporocarps of the shallow fruiting species, *E. americanus* and *E. verruculosus*, but rarely found parasitized sporocarps of the deeper fruiting species, *E. macrosporus* or *E. bartlettii*.

Independent of competitive interactions or nutrient tradeoffs in fruiting depth, truffles that are deeper below the soil surface would presumably be more difficult for rodents to detect and extract, ultimately reducing spore dispersal. Yet, we found that all rodent species preferentially selected deeper-fruiting over shallower-fruiting truffle species (Fig. 3). Although all four *Elaphomyces* species produced similar VOC compounds, the deeper-fruiting species released overall greater emissions for many of the compounds, giving them distinct signals (Table 1). In fruit–mammal dispersal mutualisms, high fruit VOC emissions are important mediators of interactions with mammals such as mouse lemurs (*Microcebus* spp.) and fruit bats (*Pteropus* spp.) (Lomáscolo et al. 2010, Valenta et al. 2013). Additionally, Donaldson and Stoddart (1994) found that Tasmanian bettongs (*Bettongia gaimardi*) preferred the odor produced by the entire community of truffle VOC compounds relative to individual VOC compounds of truffles from *Mesophellia* spp. This suggests that multiple compounds are

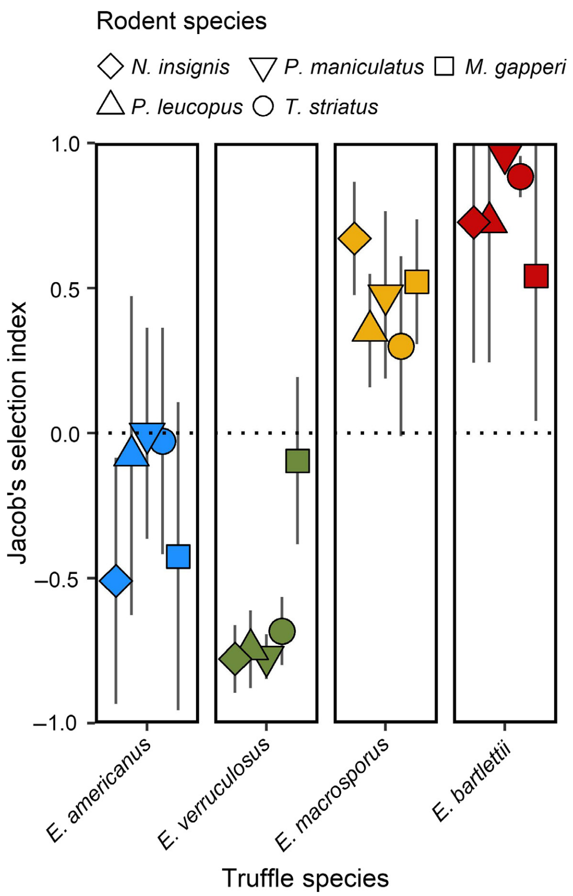

Fig. 3. Selection of truffle species by rodent species. Jacob's selection index ranges from −1 to 1, with 1 indicating strong preference, 0 indicating consumption relative to availability (dotted line), and −1 indicating strong avoidance. Shapes denote mean population-level selection by rodent species from eight sampling grids. Bars denote 95% confidence intervals.

important for providing signals that mediate interactions between truffles and mammals. For example, at our study site, both of the deeper-fruiting species (*E. macrosporus* and *E. bartlettii*) emitted higher levels of compounds containing short carbon chains such as methanol, acetone, and ethanol (Table 1). These compounds volatilize readily and can likely move quickly through the soil profile and into the atmosphere where they can disperse long distances to alert rodents to their general presence and make it easier to home in on their subterranean location. Additionally, compared to the other *Elaphomyces* truffle species, *E. bartlettii* emitted higher levels of C8 volatiles and potential sulfur-containing compounds (Table 1). Sulfur compounds in particular have an extremely low olfactory threshold and produce a strong odor (Guadagni et al. 1963) that may be used by rodents to home in on the exact location and depth of truffles, even for deep-fruiting species. Indeed, the sulfur-containing compound dimethyl sulphide is a key compound for pigs and trained dogs to detect the commercially valuable black truffle, *Tuber melanosporum* (Talou et al. 1990) and another sulfur-containing compound, 3-methyl-4,5-dihydrothiophene, was the most important contributor to the human-perceived aroma of the white truffle, *T. borchii* (Splivallo and Ebeler 2015).

If a stronger odor signal increases the chances of being detected and dispersed, why then did we not observe these high VOC emissions in the shallow-fruiting truffle species? One explanation is that the shallow-fruiting species do not need to invest in creating strong VOC signals for dispersal. This may be because they require less effort to locate by rodents or because they rely, in part, on passive dispersal from soil disturbance (erosion or mammal activity) with subsequent movement of the powdery spores by the wind (Reynolds 2011). VOC synthesis requires energy and resources as they are derived from precursor molecules such as amino acids and fatty acids (Splivallo et al. 2011). Thus, releasing lower levels of VOCs would perhaps benefit fungi by allowing species to allocate resources to other metabolic activities. Another explanation is methodological: outlier removal may have changed the observed VOC signal. The outliers, including one *E. bartlettii*, had extremely different VOC compositions compared to the other truffles, irrespective of species, and were likely at a different maturation stage. Thus, it is possible that at a certain stage of maturation, these species do emit high levels of some VOCs. However, we believe that overall *E. americanus* and *E. verruculosus* do produce lower VOC emissions. For example, during truffle surveys we handled sporocarps from 248 *E. americanus* and 5,346 *E. verruculosus* and never encountered one with an odor that was more than slightly musky or skunky (Castellano and Stephens 2017, Stephens et al. 2017*b*). In contrast, many of the 574 *E. macrosporus* sporocarps collected during field surveys had a sweet to citrus odor and most of the 58 *E. bartlettii* were extremely pungent, even at stages that were not fully mature. This field observation is consistent with PTR-MS results suggesting that the odor produced by the shallower fruiting species is less than that of the deeper fruiting species.

In addition to allocating fewer resources to VOC production, the lower emissions of the shallow fruiting species may also reduce the chance of detection by rodents before the sporocarps are fully mature. We found that stage was not a significant predictor of VOC composition, suggesting that odor begins to develop before the truffle is fully mature. Thus, having a strong signal close to the soil surface may be detrimental to dispersal, as it would allow rodents to detect and consume sporocarps before spores are fully developed. We found evidence of this during field sampling with relatively shallow fruiting sporocarps of *E. macrosporus* (approximately 3 cm below the soil surface) that were partially consumed before they had developed a gleba of mature spores (Oregon State University Herbarium 149825 and 149826, respectively; Castellano and Stephens 2017).

Additionally, about 5% of *M. gapperi* and 7% of *T. striatus* scats used in this study contained large amounts of immature *Elaphomyces* spores (likely from *E. macrosporus*), indicating consumption of immature truffles.

Nutritional rewards

Truffles are low-quality food source compared to other natural food sources such as seeds. For example, golden-mantled ground squirrels (*Callospermophilus lateralis*) get about three times as much digestible nitrogen and twice the energy from Douglas-fir seeds (*Pseudotsuga menziesii*) than from *E. granulatus* truffles (Cork and Kenagy 1989a). It has been hypothesized that despite the low nutritional quality of truffles, rodents consume truffles because VOC signals make them an easy food source to locate (Cork and Kenagy 1989a). Although it is not possible to totally decouple VOC signals and nutritional reward in our study, our results do suggest that even among truffle species, simply finding sporocarps is more important than the amount of digestible protein or digging needed to reach them. For rodent species, slight increases in the amount of digestible protein (Fig. 2c) gained from selecting *E. verruculosus* or *E. macrosporus* relative to the other two *Elaphomyces* species may be negligible. Instead, the strong VOC signals emitted by *E. macrosporus* and *E. bartlettii* likely make them easier to locate, which would reduce foraging time. Even with more digging required to reach these deep fruiting species, targeting stronger chemical signals may be a more effective foraging strategy for rodents than traveling longer distances in search of less odiferous truffles. This may be similar to myrmecochory mutualisms, where ants select seeds with elaiosomes of stronger VOC emissions rather than those of higher nutritional quality (Turner and Frederickson 2013).

The factors that shape truffle selection may vary among rodent species, depending on their reliance on fungi as a food source. Four of the five rodent species we studied consume fungi as a relatively small part of their diet (i.e., *N. insignis*, *Peromyscus* spp., and *T. striatus*), whereas fungi comprise the majority of the diet of *M. gapperi*. Although we cannot disentangle the effects of higher digestible protein content and sporocarp availability of *E. verruculosus*, it is clear that the fungal specialist, *M. gapperi*, selected this truffle species more than the other rodent species. In addition to being higher in digestible protein than other *Elaphomyces* species, *E. verruculosus* fruits in large clusters, making it a relatively good food source that occurs in high abundance (Stephens et al. 2017*b*). This clustering may affect behavior; another fungal specialist, the northern flying squirrel (*Glaucomys sabrinus*), is thought to return to forage at patches where truffles were previously found (Pyare and Longland 2001). Returning to soil patches where *E. verruculosus* truffles were already detected may make it easier for *M. gapperi* to home in on weaker VOC signals. Such spatial foraging fidelity is also common in flower pollinators such as bees (e.g., Ogilvie and Thomson 2016).

The *Elaphomyces* species in this study had sporocarps that were of similar mass, making it unclear if sporocarp size affects truffle selection by rodents. Among genera of hypogeous fungi, sporocarp size can range from just a few millimeters to several centimeters in diameter (Castellano et al. 1989). Similar to plant fruits, larger truffles would likely have higher VOC emissions and offer more rewards than smaller truffles (Beyaert and Hilker 2014) and such differences may be important in disperser selection, particularly among mammals that vary in size.

Conclusions

We found that deeper-fruiting truffle species produce stronger VOC signals and were preferentially selected by rodents, suggesting that both fruiting depth and rodent selection likely play an important role in shaping VOC composition among *Elaphomyces* species. This interplay between environmental and biotic factors may help explain the interspecific variation observed in VOC composition among the commercially valuable *Tuber* spp. (Splivallo et al. 2011). Considerable effort has focused on the factors that influence the VOC emissions of *Tuber* spp. and has revealed that geographic location, genetics, season, host species, and bacterial communities contribute to aroma (Molinier et al. 2015, Splivallo et al. 2015, Vita et al. 2018), although the role of fruiting depth and mammal dispersers has yet to be explored (Splivallo et al. 2011). In addition to odor, truffle taxa are diverse in sporocarp traits such as abundance, size, color, and odor as well as in spore characteristics such as size, ornamentation, degree of melanization, and wall thickness (Castellano et al. 1989, Maser et al. 2008), suggesting different selective pressures are acting on the evolution of hypogeous fungi. More fully integrating mammal dispersers into the study of hypogeous fungi will shed light on the factors that helped shape the evolution and diversity of truffles.

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