An electrophysiological "fingerprint" response of dung beetles (Coleoptera: Aphodiidae, Geotrupidae, Scarabaeidae) to chemical volatiles of common dung types in the Iberian Peninsula *

Una respuesta electrofisiológica tipo "huella dactilar" en coleópteros coprófagos (Coleoptera: Aphodiidae, Geotrupidae, Scarabaeidae) hacia los compuestos químicos volátiles de excrementos comunes en la Península Ibérica

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ABSTRACT

A group of species of dung beetles from the Aphodiidae, Geotrupidae, and Scarabaeidae families were selected to explore their electrophysiological response to a set of volatile organic compounds from three common dung types (cow, horse, and rabbit). It was postulated that those species with shared or similar trophic preferences would elicit similar physiological results when presented with these volatiles. The results showed that each species responded in a unique manner to the compounds, creating what is described as an electrophysiological "fingerprint". These findings point to the value of the plume of volatiles emitted from a food source in mediating food-searching behavior.

Key words: Electroantennography, dung beetles, food searching behavior, Scarabaeoidea, volatile compounds.

RESUMEN

Un grupo de especies de escarabajos estercoleros de las familias Aphodiidae, Geotrupidae y Scarabaeidae fueron seleccionadas para analizar sus respuestas electrofisiológicas a un conjunto de compuestos volátiles orgánicos característicos de tres tipos de excrementos comunes (vaca, caballo y conejo). La hipótesis de partida fue que aquellas especies con preferencias tróficas similares o compartidas presentarían resultados fisiológicos similares al ser expuestos a estos compuestos. Los resultados muestran que cada especie responde de manera singular a estos volátiles, creando lo que se describe en este estudio como una "huella dactilar" electrofisiológica. Los resultados apuntan a la importancia que el conjunto de volátiles emitidos de una fuente de alimento en forma de mezcla de volátiles (aroma) pueden tener la fuente de alimento para determinar la atracción y preferencia de los coleópteros coprófagos.

Palabras claves: electroantenografía, coleópteros coprófagos, compuestos volátiles, comportamiento de búsqueda de alimento, Scarabaeoidea.

INTRODUCTION

A characteristic trait in coprophagous beetles is the use of their antennal olfactory system to detect and fly towards a food source (HALFFTER & MATTHEWS, 1966). Their acute olfactory senses have been described as the key to exploit randomly distributed resources, particularly mammalian herbivore dung (SIMMONS *et al.*, 2011). Yet up until recently, few studies had attempted to analyze the chemical interaction between the olfactory system of dung beetles and their food. In their work with *Geotrupes auratus* (Dallatorre, 1879) INOUCHI *et al.* (1988) discovered five specific volatile compounds from cow dung, namely 2-butanone, phenol, ρ-cresol, indole, and skatole, that released food searching behavior in this species. Other studies, while not specifically addressing the chemical reasons behind the observed attractions to various types of dung in the field, have pointed to the importance these volatile plumes could have in determining the observed preferences (MARTIN-PIERA & LOBO, 1996; GITTINGS & GILLER, 1998; WHIPPLE & HOBACK, 2012).

It is pretty well established that dung beetles are considered polyphagous dung feeders (HALFFTER & MATTHEWS, 1966; SCHOLTZ *et al.*, 2009; SIMMONS *et al.*, 2011), yet it is hard not to point out the numerous studies in the recent decades that have begun to explore the feeding preferences that have been observed under various field and laboratory settings (FINN & GILLER, 2002; DORMONT *et al.*, 2004, 2007; JONES *et al.*, 2012; FRANK *et al.*, 2017; FRANK *et al.*, 2018a; CORREA *et al.*, 2020; TONELLI *et al.*, 2021). Originally applied to the feeding pattern found in oribatid mites (SCHNEIDER & MARAUN, 2005), the term "choosy generalism" has recently gained traction to describe the feeding preferences observed in dung beetles as well (WURMITZER *et al.*, 2017; WEITHMANN *et al.*, 2020). However, to what degree the volatile organic compounds (VOCs) from a dung source influence the trophic preference of certain species, or whether species with a similar preference respond in the same olfactory manner to the same suite of chemicals remain unknown.

Addressing the latter question, this study focused on exploring how dung beetles with shared trophic preferences responded to a selected set of VOCs through electrophysiological bioassays. To amplify the reach of the study, three dung types (cow, horse, and rabbit) were chosen for the chemical analyses due to their high distribution and abundance along the Iberian Peninsula (GALANTE & CARTAGENA, 1999; DELIBES-MATEOS *et al.*, 2009), allowing an increased number of species of dung beetles to be considered for the bioassays. By first identifying the chemical composition of each dung type through gas chromatography-mass spectrometry, a set of VOCs representing each type can then be selected and used as synthetic standards in the electroantennogram assays to measure and contrast the physiological responses of each species of dung beetle. Assuming that the chemical makeup of each dung type is significantly different from one another, the hypothesis of this study proposes that for species with similar or shared trophic preferences, their antennal physiological reaction to the presented volatiles should be more or less similar, allowing for the differentiation of the groups of species with different trophic preferences based on this criterion. Should the tested hypothesis be proven accurate, the findings would further aid in explaining the observed patterns of resource preferences in previous field studies with similar species.

MATERIALS AND METHODS

Collection and analyses of dung samples

Cow and horse dung were collected at the Picos de Europa National Park (Asturias, Spain), from three different individuals for each dung type. To avoid dung colonization by invertebrates and to minimize physical-chemical transformation, the dung samples were collected in the early hours of the morning. During the moment of collection, a headspace solvent extraction (HSSE) static sampling technique was applied to each sample. Each stir bar, under the commercial name Twister® (Gerstel GmbH & Co. KG), was coated in polydimethylsiloxane (PDMS) as the sorptive

extraction phase. The Twisters® were exposed to the samples for one hour at ambient temperature (22°C). Fresh rabbit excrement was collected at Sierra de la Carrasqueta (Valencia, Spain) and in the vicinity of the University of Alicante (San Vicente del Raspeig, Alicante, Spain), where wild rabbit activity is common year-round. Volatile extraction of rabbit dung was performed in an incubator at the laboratory at 37°C for 24 hours. The extraction times and temperatures set here were based on previous assays.

Thermal desorption and gas chromatography

The stir bars were thermally desorbed on a thermal desorption system (Gerstel TDS-2, Gerstel GmbH & Co. KG), for 10 minutes at 300°C with a helium flow rate of 55 ml/min connected to a gas chromatographer-mass spectrometer system (Agilent 6890GC and Agilent 5973MS respectively; Agilent Technologies, CA, USA). The GC-MS data was processed using the MSD ChemStation software (Agilent Technologies). Tentative compound identification of secretion components was done by comparison of mass spectra in the WILEY and NIST mass spectral libraries. Retention indices were calculated using a series of linear alkanes C7 – C30 (Sigma-Aldrich 49451-U), obtained under the same chromatographic conditions, and compared against literature values (ADAMS, 2017). Identifications were confirmed by comparison of spectra and retention times with those of authentic standards when available. Commercial standards were obtained from chemical suppliers (Fluka, Sigma-Aldrich, Avocado, Acros), with ≥ 98% purity and were run under the same conditions as the samples. The compounds clearly identified (≥ 90% quality and confirmed by fragmentation pattern analysis) were expressed as the percentage of the total content of compounds (relative abundance) and classified under functional groups.

Collection of dung beetle fauna

Fifteen species of dung beetles, collected from different geographical and altitudinal gra-

dients of the lower half of the Iberian Peninsula, were selected for the bioassays (See Table 1). Based on the literature and *a priori* knowledge, many of the species have known attractions to at least one of the three tested dung types and were searched and found in cow pats, horse dung and rabbit latrines (KLEMPERER, 1984; MARTIN-PIERA & LOBO, 1996; GALANTE & CARTAGENA, 1999; VERDÚ & GALANTE, 2002, 2004; VERDÚ *et al.*, 2007; ZAMORA *et al.*, 2007). Effort was made to include species from all three families (Aphodiidae, Geotrupidae, and Scarabaeidae). Each species was placed in its own terrarium inside a climate chamber at 16 ± 1:10 ± 1 °C (L:D), 80 ± 5 % RH with a photoperiod of 14:10 (L:D). The specified conditions were set to mimic the habitat condition from the site of collection and these were adjusted according to the season of collection (See Table 1 for details). This work conforms to the Spanish legal requirements including those relating to conservation and welfare.

Electroantennography bioassays

For the physiological assays a group of statistically significant and characteristic VOCs from each dung type were selected (See Data Analysis for details). Electroantennogram signals were recorded with an EAG system (Syntech, Kirchzarten, Germany) consisting of a universal single-ended probe (Type PRG-2), a data acquisition interfaces board (Type IDAC-02), and a stimulus air-controller (CS-55). A Syntech PC-based signal processing system was used to amplify and process the EAG signals. The signals were further analyzed using the EAG 2010 software (Syntech, Kirchzarten, Germany). Each compound was prepared at 1% in hexane (HPLC-grade, Sigma-Aldrich Co.) and stored at -20°C until needed. The number of antennae tested per species ranged from 8 to 12 using different individuals. The EAG responses were initially measured as the maximum amplitude of depolarization in millivolts (mV) caused directly by a stimulus. For each value generated by a test compound, the closest control response (hexane) was subtracted from it to eliminate the effect of

Table I. Dung beetles used in the study
Tabla I. Escarabajos coprófagos utilizados en este estudio

Species	Collection Site	Trophic Preference[1]
Aphodiidae		
Ammoecius elevatus (Olivier, 1879)	Cañada de los Potros, Sierra Nevada National Park, Andalusia, Spain.	Cow
Anomius baeticus (Mulsant and Rey, 1869)	Cañada de los Potros, Sierra Nevada National Park, Andalusia, Spain.	Rabbit
Aphodius fimetarius (Linnaeus, 1758)	La Sauceda, Los Alcornocales Natural Park, Andalusia, Spain.	Cow-Horse
Geotrupidae		
Ceratophyus hoffmannseggi (Fairmaire, 1856)	Doñana Biological Reserve, Doñana National Park, Andalusia, Spain.	Cow-Horse
Jekelius hernandezi (Lopez-Colon, 1988)	Corral Rubio, Albacete, Castilla la Mancha, Spain.	Common
Sericotrupes niger (Marsham, 1802)	Cañada de los Potros, Sierra Nevada National Park, Andalusia, Spain.	Cow-Horse
Typhaeus typhoeus (Linnaeus, 1758)	La Sauceda, Los Alcornocales Natural Park, Andalusia, Spain.	Horse
Thorectes valencianus (Baraud, 1966)	Font Roja Natural Park, Alicante, Valencia, Spain.	Rabbit
Scarabaeidae		
Ateuchetus cicatricosus (Lucas, 1846)	Doñana Biological Reserve, Doñana National Park, Andalusia, Spain.	Cow
Bubas bison (Linnaeus, 1767)	Charco Redondo, Cádiz, Andalusia, Spain.	Cow-Horse
Copris hispanus (Linnaeus, 1764)	Charco Redondo, Cádiz, Andalusia, Spain.	Horse
Onthophagus emarginatus (Mulsant & Godart, 1842)	Sierra de la Carrasqueta, Jijona, Valencia, Spain.	Horse
O. fracticornis (Preyssler, 1790)	Les Angles, Pyrénées-Orientales, France.	Cow-Horse
O. maki (Illiger, 1803)	Doñana Biological Reserve, Doñana National Park, Andalusia, Spain.	Cow
O. melitaeus (Fabricius, 1798)	La Sauceda, Los Alcornocales Natural Park, Andalusia, Spain.	Cow-Horse

[1] Dung type most preferred according to the literature (See Materials and Methods).
[1] El tipo de excremento más preferido según la literatura (Ver Materiales y Métodos).

the solvent (hexane) on the response. If an antenna had undergone more than one complete round of stimulus testing, the responses for said antenna were averaged after compensating for the solvent. This process was repeated for each species.

Data analysis

To analyze the potential differences in the chemical composition among the three dung types a non-parametric multiple analysis of variance (NPMANOVA or PERMANOVA) on a Bray-Curtis dissimilarity (BCD) matrix was applied. This was followed by a *post hoc* multilevel pairwise comparison from package "vegan" (OKSANEN *et al.*, 2020) in R Studio®. For the selection of the compounds that were used in the electrophysiological assays the Indicator Species Analysis (IndVal) of DUFRÊNE & LEGENDRE (1997) was applied. This test identified the VOCs that had significant specificity (and fidelity) values ($\alpha = 0.05$, IndVal ≥ 0.45, range: $0.0 - 1.0$) to a particular dung type. Given that some species had a trophic preference for more than one dung type, volatiles shared among two or three types of dung were also considered in the selection process.

Table II. Chemical composition of the different dung types analyzed by HSSE/GC-MS
Tabla II. Composición química de los diferentes tipos de excrementos analizados por HSSE/GC-MS

Compound	Family group	Identified[a]	Composition (%)[b]		
			Dung Type		
			Cow	Horse	Rabbit
2-Heptanone	Ketone	MS, RI, STD	ND	1.2*	ND
Nonane	Hydrocarbon	MS, RI, STD	ND	0.3*	ND
Heptanal	Ketone	MS, RI, STD	0.5*	ND	ND
α-Pinene	Monoterpene	MS, RI, STD	8.6*	ND	8.2*
Camphene	Monoterpene	MS, RI, STD	ND	ND	4.1*
(E)-2-Heptenal	Aldehyde	MS, RI	1.4	1.0	ND
Sabinene	Monoterpene	MS, RI, STD	3.1*	4.6*	1.7*
6-Methyl-5-hepten-2-one	Ketone	MS, RI, STD	2.2*	4.7*	ND
2-Octanone	Ketone	MS, RI	ND	2.0*	ND
3-Octanol	Alcohol	MS, RI	ND	0.9*	ND
p-Cymene	Monoterpene	MS, RI, STD	7.3*	1.2*	5.8*
Limonene	Monoterpene	MS, RI, STD	2.2*	1.6*	2.2*
Eucalyptol (1,8-Cineole)	Monoterpene	MS, RI, STD	ND	ND	9.4*
Y-Terpinene	Monoterpene	MS, RI, STD	2.0	2.5	1.8
Acetophenone	Ketone	MS, RI, STD	ND	6.9*	ND
p-Cresol	Phenol	MS, RI, STD	28.0*	25.0*	ND
2-Nonanone	Ketone	MS, RI, STD	ND	0.6*	ND
Undecane	Hydrocarbon	MS, RI, STD	ND	1.4*	ND
Nonanal	Aldehyde	MS, RI, STD	2.7*	1.1*	2.2*
Camphor	Monoterpene	MS, RI, STD	ND	ND	5.3*
Isopinocamphone	Monoterpene	MS, RI	ND	ND	3.1*
2-Decanone	Ketone	MS, RI	ND	0.3*	ND
Dodecane	Hydrocarbon	MS, RI, STD	ND	0.6*	ND
Decanal	Monoterpene	MS, RI, STD	0.9*	0.4*	ND
Verbenone	Monoterpene	MS, RI	ND	ND	14.3*
β-Cyclocitral	Monoterpene	MS, RI	1.6*	0.5*	0.7*
Benzothiazole	Miscellaneous	MS, RI, STD	ND	ND	4.5*
1H-Indole	Miscellaneous	MS, RI, STD	13.3*	ND	ND
2-Undecanone	Ketone	MS, RI	ND	0.7*	ND

Table II. *(Continuation)*
Tabla II. *(Continuación)*

α-Cubebene	Sesquiterpene	MS, RI, STD	0.1*	0.3*	3.5*
α-Ylangene	Sesquiterpene	MS, RI, STD	ND	1.2*	ND
α-Copaene	Sesquiterpene	MS, RI, STD	1.3*	1.8*	ND
Skatole	Miscellaneous	MS, RI, STD	ND	5.2*	ND
β-Bourbonene	Sesquiterpene	MS, RI, STD	0.9*	ND	ND
Longifolene	Sesquiterpene	MS, RI, STD	ND	ND	8.0*
(E)-β-Caryophyllene	Sesquiterpene	MS, RI, STD	10.9*	13.4*	8.9*
β-Copaene	Sesquiterpene	MS, RI, STD	ND	0.7*	ND
α-trans-Bergamotene	Sesquiterpene	MS, RI	ND	0.6*	ND
Dihydro-β-ionone	Ketone	MS, RI, STD	ND	0.3*	ND
α-Humulene	Sesquiterpene	MS, RI, STD	1.5*	1.9*	6.2*
9-epi-(E)-Caryophyllene	Miscellaneous	MS, RI	ND	0.4*	ND
cis-Muurola-4(14),5-diene	Sesquiterpene	MS, RI	4.7*	1.6*	ND
γ-Himachalene	Sesquiterpene	MS, RI, STD	ND	1.2*	ND
Germacrene D	Sesquiterpene	MS, RI, STD	ND	1.3*	ND
(E)-β-ionone	Sesquiterpene	MS, RI, STD	1.3*	1.1*	ND
Valencene	Sesquiterpene	MS, RI	1.4*	3.6*	ND
Pentadecane	Hydrocarbon	MS, RI, STD	1.2*	3.8*	ND
β-Bisabolene	Sesquiterpene	MS, RI	0.6*	1.4*	ND
Tridecanal	Ketone	MS, RI	0.8*	ND	ND
γ-Cadinene	Sesquiterpene	MS, RI, STD	0.6*	0.9*	ND
δ-Cadinene	Sesquiterpene	MS, RI, STD	0.3*	1.3*	10.2*
Tetradecanal	Ketone	MS, RI	0.6*	0.3*	ND
Total compounds			**27**	**40**	**18**

[a] Method of identification: MS, identified by comparison with mass spectra databases; RI, identified by retention indices; STD, comparison with the retention times and mass spectra of available standards.
[b] Relative abundance calculated from GC-MS peak areas. ND, not detected.
* Statistically significant IndVal scores (IndVal of at least 0.45 and $P < 0.05$).
Compounds in bold were those selected for the EAG bioassays.
[a] Métodos de identificación: MS, identificado al compararlo con la base de datos del espectrómetro de masas; RI, identificado por los índices de retención; STD, comparación con los tiempos de retención y la masa espectral de estándares disponibles.
[b] Abundancias relativas calculadas de las áreas bajo los picos en el GC-MS. ND, no detectado.
* Valores de IndVal estadísticamente significativas (IndVal de al menos 0.45 y $P < 0.05$).
Compuestos en negrita fueron aquellos seleccionados para los ensayos de EAG.

Grouping the electrophysiological data by species, a Shapiro-Wilk test (α = 0.05) was applied to check for Gaussian distribution. Differences in median value responses were analyzed using Kruskal-Wallis rank sum tests (α = 0.05) given the heteroscedastic nature of the standard deviations. *Post hoc* Dunn tests (α = 0.05) with a Bonferroni *P*-value adjustment for multiple pairwise comparisons were performed following significant differences in EAG responses to the set of volatiles. All statistical analyses were performed in R Studio®.

RESULTS

Chemical composition of cow, horse, and rabbit dung

A total of 51 VOCs were identified from cow, horse, and rabbit dung samples. Of these, 27 were found in cow dung, 40 in horse dung and 18 in rabbit dung (See Table 2). Of the total number of compounds identified, 22 were found in more than one dung type, with cow and horse sharing the most number of volatiles, though as expected, the overall chemical profile of each type of dung significantly differed from one another (PERMANOVA on BCD, permutations = 9999, *d.f.* = 2, *SS* = 1.206, r^2 = 0.83, *pseudo-F* = 15.00, *P* = 0.004). These differences were further confirmed when analyzed at a pairwise level (*post hoc*; Cow *vs.* Horse: *t* = 2.87, P(perm) = 0.113, *P*(MC) = 0.011; Cow *vs.* Rabbit: *t* = 4.26, P(perm) = 0.105, *P*(MC) = 0.005; Horse *vs.* Rabbit: *t* = 4.13, P(perm) = 0.095, *P*(MC) = 0.005). Thirty chemicals were significantly associated with a particular dung type (IndVal > 0.45, *P* < 0.05; see Table 2). Various families of compounds were identified including the monoterpenes, ketones, and sesquiterpenes with a few hydrocarbons, aldehydes, indoles, alcohols, and phenols.
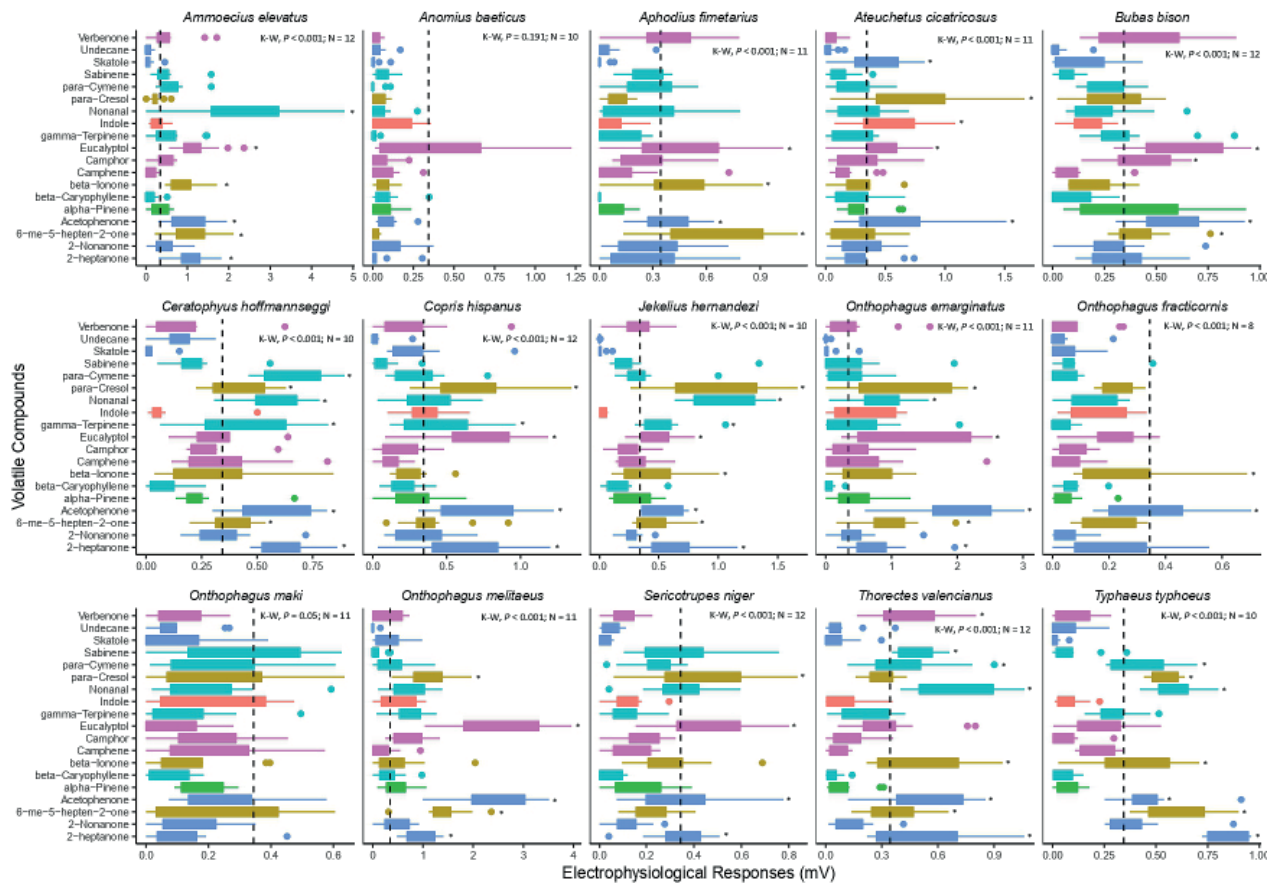
Electrophysiological bioassays

Nineteen VOCs were selected for the EAG bioassays. All of the species presented a non-Gaussian distribution in their EAG responses (Shapiro-Wilk test, *P* < 0.05), with values skewing strongly to the right of the zero value. Between 8 and 12 antennae were tested for each species. All 19 compounds generated a response, and despite the shared trophic preferences in some of them, every species presented a unique assemblage of electroantennographical responses to the compounds (See Figure 1). Two species, *Anomius baeticus* and *Onthophagus maki*, did not show significant differences in their antennal responses to the volatiles (*A. baeticus*: Kruskal-Wallis, *P* = 0.191, Statistic: 22.985, N = 10; *O. maki*: Kruskal-Wallis, *P* = 0.05, Statistic: 29.319, N = 11). Of the remaining 13 species, the number of significant pairwise comparisons varied greatly from as little as 3 in *O. fracticornis* to as many as 53 in *T. valencianus*. The range of electrophysiological responses varied from species to species with some like *Sericotrupes niger* never producing values higher than 0.8 mV to species like *Ammoecius elevatus* and *Onthophagus emarginatus* producing values as high as 5.0 mV and 6.0 mV respectively (See Figure 1). In at least 8 out of the 15 species, several compounds such as acetophenone, 6-methyl-5-hepten-2-one, eucalyptol, 2-heptanone, ρ-cresol, and nonanal produced higher than average electroantennographical results (See Fig. 1).

DISCUSSION

As expected, both qualitatively and quantitatively, the differences among the three dung types were significant. Many of the identified volatiles in cow and horse dung such as ρ-cresol, 1H-indole, and skatole have been previously cited in other studies where both of these dung types were analyzed (KIMURA, 2001; DORMONT *et al.*, 2010; STAVERT *et al.*, 2014; WURMITZER *et al.*, 2017; FRANK *et al.*, 2018b). Among the volatiles, ρ-cresol showed the highest relative abundance both in cow and horse dung (28% and 25% respectively), mirroring the findings in similar studies (FRANK *et al.*, 2018b). For volatiles that are the product of amino acid metabolism by intestinal anaerobes (MACKIE *et al.*, 1998) such as ρ-cresol, being the product of tyrosine fermentation (FRANK *et al.*, 2017),

Fig. 1. Boxplot results for the electroantennogram bioassays of each of the 15 species of coprophagous beetles analyzed in this study. The x-axis measures the electrophysiological response in millivolts (mV) to each of the 19 tested VOCs. The y-axis gives the names the tested volatile compounds. The dashed (- -) vertical line denotes the overall average antennal response for each species. The Kruskal-Wallis (K.W.) *P*-values are given for each species. The *N* is the number of replicas for each species. The asterisks mark the compounds that had statistically significant differences in the *post hoc* pairwise comparisons (Dunn test with Bonferroni adjustment of the *P*-value) and produced higher than average electrophysiological responses. The colors in the boxplots represent under which type of dung(s) the compound was identified in: Cow, Horse, Rabbit, Cow-Horse, Cow-Rabbit and Generalist. (To see this figure in color, access the online version).

Fig. 1. Diagramas de cajas de los ensayos de electroantenografía para cada una de las 15 especies de escarabajos coprófagos. El eje X mide la respuesta electrofisiológica en milivoltios (mV) a cada uno de los 19 COVs utilizados. La eje Y da los nombres de los compuestos volátiles. La línea vertical discontinua (- -) denomina la respuesta fisiológica promedio para cada especie. Los valores de *P* de los Kruskal-Wallis (K.W.) son dados para cada especie. La *N* es el número de réplicas para cada especie. Los asteriscos marcan los compuestos que tienen diferencias estadísticamente significativas en los análisis *post hoc* en pares (prueba de Dunn con ajuste de Bonferroni a los valores de *P*) y que dieron respuestas electrofisiológicas por encima de la media para una especie. Los colores en los diagramas de cajas representan en qué tipo de excremento(s) se identificaron los compuestos: Vaca, Caballo, Conejo, Vaca-Caballo, Vaca-Conejo y Generalista. (Para ver esta figura en color acceder a la versión online).

or like 1H-indole and skatole, the end products of tryptophan metabolism (SAITO *et al.*, 2018), they can be assumed to be common to various dung types. Though horse dung contained the highest number of VOCs, it also had the highest number of shared compounds with cow dung, possibly explaining the slightly higher *P*-value obtained in the *post hoc* tests (See Results). In his thesis, SLADECEK (2017) analyzed the che-

mical composition of cow dung and found that 2-heptanone was among the first compounds to be emitted from this dung type but as it aged the more volatile chemical families such as the aldehydes, alcohols, and ketones would decrease in abundance, while phenols and terpenoids would become more abundant, possibly explaining why 2-heptanone was only found in cow dung and in such a low abundance in the current study.

From a diet perspective, both animals feed and graze on similar types of pastures in the Iberian Peninsula, which could explain the high number of shared volatiles among them, despite the ruminant method of digestion by cows and the monogastric digestive system of horses.

Similar to ρ-cresol, various compounds had surprisingly large relative abundances across the dung samples such as α-pinene which was only found in cow and rabbit dung (8.6% and 8.2% respectively) or acetophenone, which was only found in horse dung, at a relative abundance of 6.9%. This last compound, being an aromatic ketone, is naturally found in different plants such as *Trifolium pratense* (Linnaeus, 1753), *Cistus ladanifer* (L., 1753), and *Philadelphus coronarius* (L., 1753) (ADAMS, 2017), all of which can make up the diet of grazing, non-ruminant animals like horses. Rabbit dung, though it had the lowest number of volatiles identified, had various unique and highly abundant compounds such as eucalyptol (9.4%), verbenone (14.3%), camphor (5.3%), and longifolene (8.0%). α-Humulene and δ-Cadinene, though they are not unique to rabbit dung, had much higher relative abundance values than that of cow or horse dung at 6.2% and 10.2% respectively. It can again be pointed to the diet of this animal to understand the highly distinct chemical makeup of its dung. For example verbenone can be found naturally in *Rosmarinus officinalis* (L., 1753) while 1,8-cineole is normally derived from the leaves of the Eucalyptus genus (*Myrtaceae*, *Eucalypteae*) (ADAMS, 2017), both of which can be consumed by the Iberian-native *Oryctolagus cuniculus* (L., 1758) (GALANTE & CARTAGENA, 1999; BHOWAL & GOPAL, 2015). Additionally, it can probably be assumed that because rabbits use caecotrophy to maximize their nutrient intake, the chemical composition of their dung could be highly influenced by this feeding method (GOODRICH *et al.*, 1981).

Contrary to what the main hypothesis stated, no uniformity in electrophysiological responses among species with shared trophic preferences was recorded (See Fig. 1). In fact, no uniformity in physiological responses was observed with any of the 15 species of dung beetles. This was surprising considering that some species, such as *Ammoecius elevatus*, *Ateuchetus cicatricosus*, or

Onthophagus maki, which have strong preference towards cow dung (See Table 1), did not share a common pattern of antennal responses to the same set of 19 volatiles that were tested here. Another curious result was seen with *Thorectes lusitanicus* and *Anomius baeticus*, two species with strong preferences for rabbit dung, but where the former responded stronger to non-rabbit-based compounds like nonanal and aceto-phenone, while the latter showed no difference in physiological responses to any of the tested volatiles. While *Copris hispanus*, *T. typhoeus*, and *O. emarginatus* exhibit preferences to horse dung, the only horse dung-specific volatile that generated a strong antennal response was aceto-phenone, while the rest were either shared with cow dung (ρ-cresol and nonanal) or found only in rabbit dung (1,8-cineole). Likewise, the same results were found for the other trophic preference groups, were each species exhibited a unique assemblage of electrophysiological responses, with varying degrees of antennal depolarization values anywhere from 0.1 mV to over 5.0 mV for different compounds (See Fig. 1). However, from an evolutionary perspective, the results would show two important aspects of dung beetle biodiversity: (1) the ability (and the benefits this brings) of a species being able to respond to any or most of the chemical constituents of a dung plume, regardless of type, in order to exploit this temporally and spatially limited resource and (2) the lack of a uniform olfactory response among species with similar trophic preferences would allow each species to navigate towards the same food source by detecting different sets or blends of key volatiles from the plume that would generate the strongest behavioral response for each species.

The ability of dung beetles to respond to all of the tested compounds in this study could have a deep link with the theory of trophic genera-lism proposed by previous studies (HANSKI & CAMBEFORT, 1991; SIMMONS *et al.*, 2011; WHIPPLE & HOBACK, 2012; FRANK *et al.*, 2018a) since it would be implied that having the ability to detect and respond to various dung-derived compounds is what allows them to reach and exploit various types of dung, even those not normally preferred by a species. Plenty of ideas

have been proposed to explain the origin of this generalist behavior, such as the pressure imposed on dung beetles after the mass extinction event of the Cretaceous-Paleogene (K-Pg) period to exploit any available resource (GALETTI *et al.*, 2018) or the r-Theory reproductive strategy of various species as a result of the unpredictable conditions of their environment, with particular reference to the ephemeral nature of dung (SCHOLTZ *et al.*, 2009). Regardless of the origin, the wide range of dung types that have attracted dung beetles, and the lack of specialization or dependence on any one type of dung, has been well documented by now (DORMONT *et al.*, 2004; JONES *et al.*, 2012; CORREA *et al.*, 2020). On the other hand, RAINE & SLADE (2019) observe that so far, the field and laboratory experimental methods that have been conducted do not provide conclusive evidence to support the argument of trophic generalism given that many of the methods have involved too few dung types to allow a decisive result. Nevertheless, the fact that all 15 species, even those with particular trophic preferences, responded with non-zero values to all 19 compounds in this study could reinforce the ecological advantage of trophic generalism, the avoidance of over-dependence on any one dung type, and the plasticity of their diet in the face of a stochastic food source like herbivorous mammalian dung.

When looking closer at the *post hoc* analyses of the electrophysiological assays, a trend began to appear with several compounds across various species. Acetophenone, 6-methyl-5-hepten-2-one, eucalyptol, 2-heptanone, ρ-cresol, and nonanal were among the volatiles that frequently elicited higher than average electrophysiological response values in at least 8 out of the 15 species (see Figure 1). When comparing the relative abundances of the previously cited compounds with the rest of the volatiles utilized in the EAG assays, it is clear that apart from ρ-cresol and eucalyptol, they do not stand out with larger or smaller relative abundance values. On the other hand, compounds such as 1H-indole (13.3%), α-pinene (8.2% – 8.6%), β-caryophyllene (8.9% - 13.4%), or verbenone (14.3%) though they had notably higher relative abundances, did not generate higher-than-average antennal responses, evidencing that the abundance

of a compound does not necessarily correlate with a strong physiological response. A perfect example of this would be with 2-heptanone, a compound found only horse dung with a relative abundance of 1.2%, but which elicited higher than average EAG responses in 10 out of the 15 tested species of beetles in this study. In his work with Afrotropical Coleoptera, DAVIS (1994) found that while in pig feces, compounds such as 1H-indole, skatole, and trimethylamine hydrochloride were highly abundant, they did not attract dung beetles nearly as much as 2-butanone, a compound with a small relative abundance in this type of dung. Even the blends of synthetic compounds that were employed for the bait traps in the work by FRANK *et al.,* (2018b) consisted of compounds with small relative abundances, with the exception of ρ-cresol (see Supplementary Material 3 of FRANK *et al.*, 2018b). And despite this, the chemical blend was just as attractive as the baits that contained samples of different dung types. It could be that the volatiles identified in the current study, though likely not the only ones, act as chemical signals to the presence of a food source for a large number of dung beetles. Needless to say, further bioassays would be necessary for a more in-depth understanding of this phenomenon.

While it is undoubtedly interesting to identify a set of compounds that induce strong electrophysiological responses in dung beetles, it is perhaps even more interesting to see that each species, even those with similar trophic preferences, display a unique assemblage of antennal responses to the same set of volatiles. These assemblages have been termed electrophysiological "fingerprints" in order to describe the species-specific interactions of these volatiles with their olfactory systems under electroantennogram bioassays. Though in their environment the foraging decisions made by dung beetles could be mediated by complex profiles of dung-emitted compounds (STAVERT *et al.*, 2014), this study shows which VOCs could potentially be more important within the detected dung plume to initiate food-searching behavior. It should be noted however, that no compound alone would be important enough to drive this behavior, as the EAG results have shown with the tested volatiles. Rather, as CORTEZ *et al.*

(2016) point in their description, and distinction, of generalist and specialist olfactory response neurons (ORNs) in insects, the fact that the latter group of ORNs can detect an individual odor component in a plume does not mean that said odor is a single substance, but a mix of compounds since in nature single volatile chemicals are virtually nonexistent. Taking this into account, it could be plausible that in a group of species with a shared preference for the same type of dung, each one has tuned their olfactory system to respond to different blends of compounds emanating from the same food source in order to search, arrive, and exploit it in different times depending on the order of emissions of the compounds, the relative abundance of each one, and the mixing of the plume in the air with the rest of the natural chemical-emission sources, along with other factors.

Though the ecophysiological assays did not support the initial hypothesis, the results were even more compelling given that any assumption of olfactory uniformity in the complex chemical environment of dung beetles is implausible at best. In their description of the Scarabaeinae, HALFFTER & MATTHEWS (1966) stated that given the similar size of the antennal clubs in both sexes of most species of this subfamily, it is reasonable to suggest that their primary function is the detection of dung odors, highlighting the significance of this ecophysiological trait. Since then, a plethora of studies have discovered just how complex the interaction is between the olfactory system of the dung beetle and the chemical signals emanating from their food source (INOUCHI *et al.*, 1988; DAVIS, 1994; DORMONT *et al.*, 2004, 2007, 2010; VERDU & GALANTE, 2004; VERDU *et al.*, 2007; TSHIKAE *et al.*, 2008; WHIPPLE & HOBACK, 2012; STAVERT *et al.*, 2014; FRANK *et al.*, 2017, 2018b). Studies like the one by DORMONT *et al.,* (2010) have suggested that resource selection in coprophagous insects could be based on innate olfactory preferences. This study has attempted to analyze how this innate olfactory preference could be explained through the interaction with a sample of chemical constituents from three common dung sources. While each dung type presented significantly different chemical compositions, the electroantennographical results showed that each species assembled a unique array of antennal responses to the tested compounds, termed in this study as an electrophysiological "fingerprint". Further studies are certainly encouraged in order to better elucidate the physiological significance of certain compounds such as acetophenone, 6-methyl-5-hepten-2-one, eucalyptol, 2-heptanone, ρ-cresol, and nonanal which seem to produce strong antennal responses across various species exhibiting different trophic preferences.

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