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SUMMARY

The effects of insect larval diet on adult olfactory responses to host-plant or food volatiles are still debated. The induction of adult host preferences has been studied in insects with diverse ecologies, including parasitoids, flower-visitors and phytophagous species. We investigated this question for the first time in a coprophagous insect species. Larvae of the French scarab dung beetle *Agrilinus constans* were reared on four different artificial substrates containing dung from cattle, horse, sheep or wild boar, and responses of imagos to dung volatiles were then behaviourally tested in an olfactometer. We also reported the first analysis of the composition of different mammal dung volatiles. We showed that adult beetles were more attracted to cattle and sheep dung odours, and that larval feeding experience had no effect on the adult olfactory responses to dung volatiles. A second experiment showed that the presence of other insects inside the dung resource affects the process of dung selection by adults. We identified 64 chemical compounds from dung emissions, and showed that dung volatiles clearly differed among different mammal species, allowing olfactory discrimination by dung beetles. Our results suggest that resource selection in coprophagous insects may be based on innate olfactory preferences. Further experiments should examine whether *Agrilinus* adults can learn new dung odours, and whether larval diet may influence the behaviour of adults in other coprophagous species.

Key words: dung, coprophagous, *Agrilinus constans*, larval diet, olfaction.

INTRODUCTION

The influence of experience during immature stages on adult physiology and behaviour in holometabolous insects has long intrigued ecologists and still remains under debate. In particular, host preference and oviposition behaviour by females may be determined either by larval diet ['Hopkins' host selection principle' or 'pre-imaginal conditioning' (Barron, 2001; Gandolfi et al., 2003; Chow et al., 2005)], by environment experienced during the pupal stage or even by imago stage shortly after emergence ['early-adult experience' or 'neo-Hopkins' principle' (Jaenike, 1988; Turlings et al., 1993; Barron and Corbet, 1999)]. One possible mechanism that may explain the induction of adult host preference is 'the chemical legacy hypothesis' (Corbet, 1985): the presence of chemicals at particular 'sensitive periods' of insect development may dramatically affect adult chemosensory responsiveness.

Regarding specifically the olfactory responses of adults to volatiles derived from host plants or other food sources, to what extent larval experience may influence adult behaviour remains poorly understood. In parasitoid species, learning of olfactory cues has been shown to result mainly from early adult experience (Turlings et al., 1993; Du et al., 1997) but learning during pre-imaginal stages has also been suspected (Smith and Cornell, 1979). In many flower-visiting species, foraging behaviour may proceed from a combination of innate preferences for certain flowers and learning abilities that guide insects towards new rewarding flower species (Kelber, 2002; Riffell et al., 2008). For example, floral odour learning by adults has been reported in hawkmoth species (Raguso and Willis, 2002; Cunningham et al., 2006) and bees (Giurfa, 2007; Wright et al., 2007; Riveros, 2009). In phytophagous insects, the olfactory preferences of adults have been reported to be induced by both larval and early-adult experience (Rietdorf and Steidle, 2002) but olfactory learning by adults has been shown to strongly influence insect responses to plant volatiles (Fan et al., 1997; Cunningham et al., 2004; Jorgensen et al., 2007).

While many studies have examined the induction of adult host preferences in parasitoid, phytophagous or flower-visiting insects, coprophagous species, e.g. dung beetles, have received very little attention. Most dung beetles exploiting the droppings of mammals are considered opportunistic, using a wide variety of excrement types without much discrimination (Hanski and Cambefort, 1991). Consequently, the process of resource selection by coprophagous insects has been poorly investigated. However, recent studies have provided evidence for clear trophic preferences in many dung beetle species. For example, field experiments conducted with dung of various herbivorous animals showed significant differences in the abundance of beetles among dung types (Lumaret and Iborra, 1996; Martin-Piera and Lobo, 1996; Galante and Cartagena, 1999; Finn and Giller, 2002). Such patterns of resource partitioning depend on the insects' abilities to detect and select different resource types but very little information exists on the influence of volatile compounds emitted by dung in the long- and short-range attraction of insects. Adult dung beetles are commonly supposed to be attracted by volatile components from various kinds of faeces without any preference (Hanski and Cambefort, 1991; Martin-Piera and Lobo, 1996). However, laboratory olfactometer bioassays showed that many dung beetles are capable of making a choice between volatiles

emitted by different types of faeces (Dormont et al., 2004; Dormont et al., 2007). Given that the resource-selection process in dung beetles is probably mediated by olfactory cues emitted from dung, three particular questions remain unanswered: does the behaviour of resource selection in dung beetles result from innate odour preferences or from larval feeding experience? Does the presence of other insects within the dung influence the olfactory responses of adult beetles to the dung resource? What are the differences in the chemical composition of dung volatile emissions that can allow adult beetles to distinguish, in flight, faeces from different herbivore species?

We investigated these questions in a very common French scarab dung beetle, *Agrilinus constans* Duft. (Coleoptera: Aphodiidae), in which egg, larval and pupal development takes place entirely within the dung (Lumaret, 1975). Recent studies have incorporated laboratory rearing of this species (Lumaret et al., 2007; Römbke et al., 2007), and a protocol is now available that enables successful rearing of larvae of *A. constans* under different artificial conditions, e.g. including extracts from different dung types. This species is known to colonise faeces of various mammalian herbivores (Lumaret, 1990). Field experiments on trophic preferences using a series of pitfall traps baited with different kinds of dung showed significant feeding preferences of *A. constans* for cattle or sheep dung over horse or Roe deer dung (Dormont et al., 2004; Dormont et al., 2007).

The purpose of this study was thus to evaluate the ability of the dung beetle *A. constans* to discriminate among dung odours from faeces of different mammals (three herbivores and one omnivore), and to examine the possible effects on adult responses to dung volatiles when larvae previously developed within faeces of each of the four different mammal species. In the French Mediterranean region, faeces of cattle, horse, sheep and wild boar are among the most important resources for dung beetle communities. Our objectives were thus: (i) to rear larvae of *A. constans* in four different artificial substrates containing dung from cattle, horse, sheep or wild boar, (ii) to test, using laboratory olfactometer bioassays, the behavioural responses of adult beetles emerging from the four different larval environments, (iii) to test whether the presence of other insects, congeners of *A. constans* or not, within the dung influences the behaviour of adults in the choice of the dung resource, and (iv) to analyse and compare the chemical composition of dung volatiles emitted by faeces of the four different animals.

MATERIALS AND METHODS

Laboratory rearing of *A. constans*

Agrilinus constans is a small (4.5–6 mm) dung beetle widely distributed in Europe. The female of *A. constans* oviposits directly in dung pats, and the entire development from egg to pupa takes place in dung. Eggs were collected in south-central France, in a site located about 30 km north of Montpellier, near Saint-Martin de Londres (43°48′N, 3°43′E, 235 m elevation). This area has a Mediterranean climate. Eggs of *A. constans* were obtained from fresh pats dropped by cattle, enabling easy and rapid collection of eggs in large quantities. Egg sampling from other dung types (horse, wild boar, sheep) would not have provided a sufficient number of eggs (with a similar physiological age on the same day) necessary for experiments (400 eggs needed). Eggs collected were immediately transported to the laboratory, then deposited and reared in the laboratory on various artificial substrates, including different kinds of dung. The substrate was made of one-third Vermiculite (K3), one-third commercial garden soil and one-third fresh dung from animals, which had not been treated with anthelminthics. Four types of substrate were prepared, using four different sources of dung, from cattle, horse, sheep and wild boar. The moisture content of this mixture was maintained constant. The weight of each rearing box was measured daily so that water could be sprayed to adjust the moisture of the substrate to close to 70%. Transparent plastic boxes (27 cm×20 cm×12 cm) were used for larval rearing, each box containing one of the four types of substrates. The boxes were covered with gauze (200 μm mesh) to allow permanent air exchange. The boxes were incubated at 20±2°C until emergence of the imagos. This rearing method respects a standard procedure for the breeding of *A. constans* that has been elaborated for testing the effects of veterinary pharmaceuticals on this species (Hempel et al., 2006; Lumaret et al., 2007). Ten eggs were deposited in each box, and 10 boxes were used for each dung type: a total of 400 eggs were deposited on the different substrates, i.e. 100 eggs per dung type. The boxes were surveyed daily, and emerging imagos were placed in separate individual boxes for subsequent behavioural tests.

Olfactory responses of *A. constans* to dung volatiles

Behavioural bioassays were carried out to test the responses of adult beetles to volatile compounds from different kinds of dung. Tests were performed using adults freshly emerged from the rearing boxes described above. Insects emerging from the four different kinds of dung were kept in separate boxes until the behavioural tests.

Behavioural tests were done using an olfactometer design derived from those described by Dormont and Roques (Dormont and Roques, 2001). The design consisted of a plastic rectangular arena (30 cm×12 cm×8 cm) with two holes cut in the arena floor. The holes were 2.5 cm in diameter, spaced 20 cm apart, and covered by a small circular wire mesh. A circular Plexiglas® container (6 cm in diameter, 12 cm high) was placed under each hole and pierced at the bottom in order to allow air entry. Air flow was generated by a pump connected to the olfactometer at the centre of the floor, which provided a continuous movement of air from outside through each container's grid holes, as well as within the arena. The air flow rate was measured using an air flow meter placed between the pump and the olfactometer, and was maintained at 500 ml min^{-1}. Air flow movements within the arena were assessed using chemical smoke (a mixture of ammonia and hydrochloric acid, 1:1), and air flow rate was adjusted so that insects at the centre of the arena could perceive both odour sources without any air turbulence within the arena. The tests were done in a darkened room equipped with two red lights (40 lx) placed 50 cm above the arena. The source of volatile compounds consisted of two different fresh dung samples (50 g each) placed in the different containers. In order to record insect movements, the arena floor was covered with a white paper sheet that was divided into four equal sections designated A, B, C and D. Sections A and D included the holes connected to the containers with dung samples. Following each test, the paper sheet was replaced and the entire assembly was washed using a solvent mixture of ethanol–acetone (1:1). Room temperature was held at 21±2°C during the experiments.

One hour prior to each experiment, the individual plastic boxes containing the beetles were transferred to the testing room. Fresh samples of two different types of dung were placed in the separate containers. Dung samples were collected each morning from pastures close to the site of egg collection (see above). Each experiment then consisted of releasing an individual in the centre of the arena (between sections B and C). Each beetle's position was continuously observed, and the total length of time (in seconds) spent by an individual in each section of the olfactometer was summed over a 10-min period. All beetles were submitted in a random order

to the two following series of tests, which were applied successively: (i) no dung sample in either of the two containers, in order to record beetle activity in the absence of an odour source; (ii) a dung sample from the dung type in which the beetle was reared *vs* a dung sample from another mammal species, in order to test for olfactory preferences among volatiles from these two different dung types. Four dung types (from cattle, sheep, horse and wild boar) were used for the tests. Each insect was successively submitted (in random order) to three tests, each test consisting of sampling the dung from which the beetle was reared against one of the three other kinds of dung. A fourth test (control) consisted of placing in each container a similar dung sample (dung type in which the beetle was reared).

A second set of similar experiments was conducted in order to test the influence of the presence of other insects in dung, using the 'sheep population' (25 *A. constans* adults that had emerged from the boxes holding sheep dung). Olfactometer tests were performed as described above but using only fresh sheep dung in which had been placed individuals of *A. constans* or other coprophagous insects. For each test, one container of the olfactometer was 'odorised' using a fresh 100 g sheep dung sample free of insects, while the opposite container included a similar dung sample in which 10 individuals of one of the four species of coprophagous beetles had been introduced just before the test. The following four species were used for these tests: *A. constans*, *Otophorus haemorroidalis*, *Colobopterus erraticus* and *Onthophagus lemur*. Beetles were collected using dung-baited pitfall traps in the same site where eggs of *A. constans* were collected (see above). For each species, and before each test, a 100 g dung sample was placed in the plastic box containing the beetles, and the dung sample was then used for olfactometer tests once 10 insects had been observed to enter into the dung sample. For each of the four kinds of tests (four species used), 25 successive individual tests were performed, using the 25 available *A. constans* adults.

Headspace and solid-phase microextraction sampling of dung volatiles

Dung samples were collected from animals grazing in pastures close to the site of egg collection (see above). Fresh dung samples were brought to the laboratory in separate cool boxes, and were immediately processed one hour after they were collected *in situ*. For each of the domesticated vertebrate species, fresh dung from 10 different animals was sampled. For wild boar, we collected many different fresh faeces samples in different clearings but without assurance that these samples came from 10 distinct individuals.

Volatile emissions from dung were sampled using two different techniques: dynamic headspace method, consisting of trapping airborne volatiles onto an absorbent (porous polymer Supelpak2®, Supelco Sigma-Aldrich®, Saint-Quentin Fallavier, France), and solid-phase microextraction (SPME). The two methods were applied simultaneously on each dung sample.

Dynamic headspace extraction was performed using a manifold effluvial headspace sampler that enabled the processing of eight samples simultaneously. Dung samples were individually sealed inside bags made of Nalophan® (Kalle®, Essex, UK), a non-reactive plastic. The bags were perforated with air inlet holes at both extremities. Teflon® tubing with air-tight brass couplings was used to connect the sample bags to the glass cartridges filled with Supelpak2®. The glass cartridges were then connected by Teflon® tubes to a vacuum pump. Seven different dung samples, and a control consisting of an empty Nalophan® bag, were simultaneously connected to the pump during each experiment. The effluvial sampling ran for three hours. Once the effluvial sampling was completed, each glass cartridge was eluted with 3 ml of dichloromethane (CH_2Cl_2) for a 2-hour period. The resulting mixture was analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

Sampling by SPME was performed using 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibres (Supelco®). The PDMS fibre was introduced with a manual holder into the Nalophan® bag containing the dung sample, as described above. The fibre was exposed for 30 min in close proximity to the dung (1 cm). The fibre was then immediately inserted into the GC injector (temperature 260°C) for desorption.

Dung samples into which insect individuals had been introduced for particular olfactometer tests (see above) were also used to sample dung volatiles, both with dynamic headspace and SPME methods, in order to compare odours from dung that was free of insects with that of dung containing insect species. Unfortunately, no specific volatile compound could be detected from extracts of dung samples that included insects.

Gas chromatography and gas chromatography–mass spectrometry of dung volatiles

GC–MS analyses of the crude mixture were performed using the electronic impact ionisation mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian CP-3800 apparatus (Palo Alto, CA, USA). The Varian CP-3800 was equipped with a 1079 split-splitless injector (250°C) and a 30 m×0.25 mm×0.25 µm film thickness ID WCOT CPSil-8CB fused silica capillary column (Chrompack®, Bergen op Zoom, The Netherlands), with helium as the carrier gas (1 ml min^{-1}), and programmed 2 min isothermal at 50°C, 50°C to 220°C at 4°C min^{-1}. Mass spectra were recorded in electronic impact (EI) at 70 eV, and identified by comparison with data of the NIST 98 software library (Scientific Instrument Services®, Ringoes, NJ, USA). Comparison of the chromatographs of the dung samples with that of the control from the same experiment allowed for the removal of the peaks corresponding either to adsorbent or to degradation of Nalophan®, or to volatiles present in the air surrounding the bags. Quantitative analyses were carried out in a Varian Star 3380 chromatograph equipped with a 1177 split-splitless injector, a flame-ionization detector (FID) and a fused silica capillary column WCOT CPSil-5CB 0.32 mm×25 m (Chrompack®, Middelburg, The Netherlands). The carrier gas was helium with a flow rate of 1 ml min^{-1}. The temperature of both the injector and detector was 250°C. The oven temperature program was 2 min isothermal at 50°C, then programming at 4°C min^{-1} to 220°C. Dung volatiles were identified based on retention time of external standards or with GC–MS analyses. Peaks were quantified using Star Chromatography Software® (Varian®, Palo Alto, CA, USA). The relative importance of each compound was expressed with respect to total volatiles in order to compare the volatile profile of the samples.

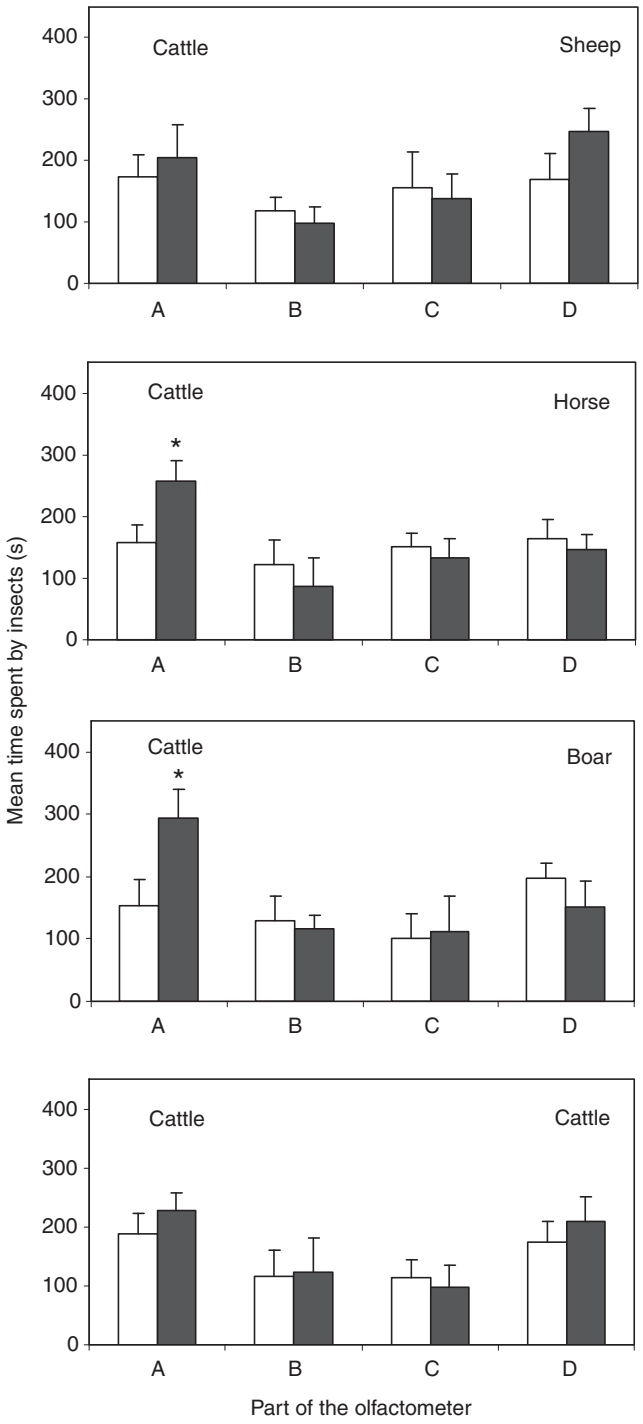
Data analyses

During laboratory rearing, the mean number of adults that emerged per box was compared between two different dung types using the non-parametric test of Mann–Whitney ($P<0.05$). For the beetle behavioural responses to dung volatiles, the mean cumulative time spent by insects in each part of the olfactometer was compared between the two olfactory situations (test with no odour source *vs* test with two different dung odours) using the Mann–Whitney *U*-test ($P<0.05$). A correspondence analysis was used to compare dung types according to their volatiles. All analyses were performed with Statistica 6.0 (Stat Soft Inc., Tulsa, OK, USA).

RESULTS

Rearing of *A. constans*

A total of 263 imagos emerged from the artificial substrates. Of the 100 eggs initially deposited per type of substrate, a total of 63, 72, 80 and 48 adults were observed to emerge from the substrate containing cattle, sheep, horse and wild boar dung, respectively. The mean number of emergences per box was found to be not significantly different among the different dung types, except for the two following cases: sheep dung (mean ± s.e.: 7.2±0.9) *vs* wild boar dung (4.8±0.7) (Mann–Whitney *U*-test, P=0.0065), and horse dung (8.0±1.0) *vs* wild boar dung (4.8±0.7) (P=0.0024).



Behavioural responses of beetles to dung volatiles

115 insects were used in olfactory tests: 28 individuals that were reared on cattle dung, 30 on horse dung, 34 on sheep dung and 23 on wild boar dung.

Considering the 'cattle' population (adults that emerged from the substrate containing cattle dung), insects were attracted significantly more often to cattle dung odours than to odours from horse or wild boar dung (Fig. 1) but showed no preference for cattle dung odours *vs* sheep dung odours. Adults from the 'sheep' population were significantly more attracted to sheep faeces than to volatiles from horse or wild boar dung (Fig. 2) but showed no preference for sheep dung odours *vs* cattle dung odours. Regarding insects that were reared on horse dung, adults significantly preferred cattle or sheep dung odours over horse dung odours (Fig. 3) but were more attracted to horse dung odours when insects had the choice between wild boar and horse dung. The 'wild boar' population was not strongly attracted to volatiles from wild boar dung, and showed a significant preference for cattle, sheep or horse dung volatiles when insects were submitted to two dung odour stimuli, including odour from wild boar dung (Fig. 4). Adults of *A. constans* were thus similarly attracted to volatiles from sheep and cattle dung, less attracted to horse dung odours and little attracted to wild boar dung odours. Preferences were not influenced by the origin of insects, i.e. adults did not change their olfactory behaviour whatever the kind of substrate from which they emerged (containing cattle, sheep, horse or wild boar dung extracts).

The presence of other insects within the sampled dung greatly influenced the olfactory responses of *A. constans* to dung volatiles (Fig. 5). Adults of *A. constans* were significantly more attracted by volatiles from dung containing other *A. constans* individuals than by volatiles from dung containing no insects. By contrast, adults of *A. constans* oriented preferentially to odours from dung free of insects than to dung containing individuals of another insect species (*O. haemorroidalis*, *C. erraticus*, *O. lemur*).

Chemical composition of dung volatiles

The number of volatile compounds detected in emissions from dung of the four herbivore species varied little. A total of 26 compounds were found in volatiles emitted from the dung of sheep whereas 36, 32 and 25 compounds were detected in the dung volatiles from cattle, horse and wild boar, respectively (Table 1 and Fig. 6). Nine compounds were found to be present in the volatiles of all four dung

Fig. 1. Olfactory responses of *Agrilinus constans* to dung volatiles: behavioural tests conducted with beetles that were reared on cattle dung (N=28 insects tested). The graphs present the mean time spent by insects in each part of the olfactometer when submitted to different dung odour stimuli: no odour source (control, white bars) and odours from two different faeces (black bars). Each column of a graph presents the mean (±s.e.) number of seconds spent by beetles in the corresponding part of the arena over a 10 min period. The arena floor was divided into four equal sections designated A, B, C and D. For the experiment with dung odour sources (black bars), section A included the hole corresponding to the container with the dung type that was used for rearing, section D, diagonally opposite, included the hole corresponding to the container with another dung type (or with the same dung type for the control test). Four dung types were used during these tests: dung of cattle, sheep, horse and wild boar. For a species, the mean number of seconds spent by beetles was compared between the two situations (no odour source *vs* dung odours) for each part of the olfactometer, using a Mann–Whitney *U*-test (*: P<0.05). Before the behavioural tests, *A. constans* larvae were reared from egg to adult emergence on artificial substrates including extracts from one of the four dung types.

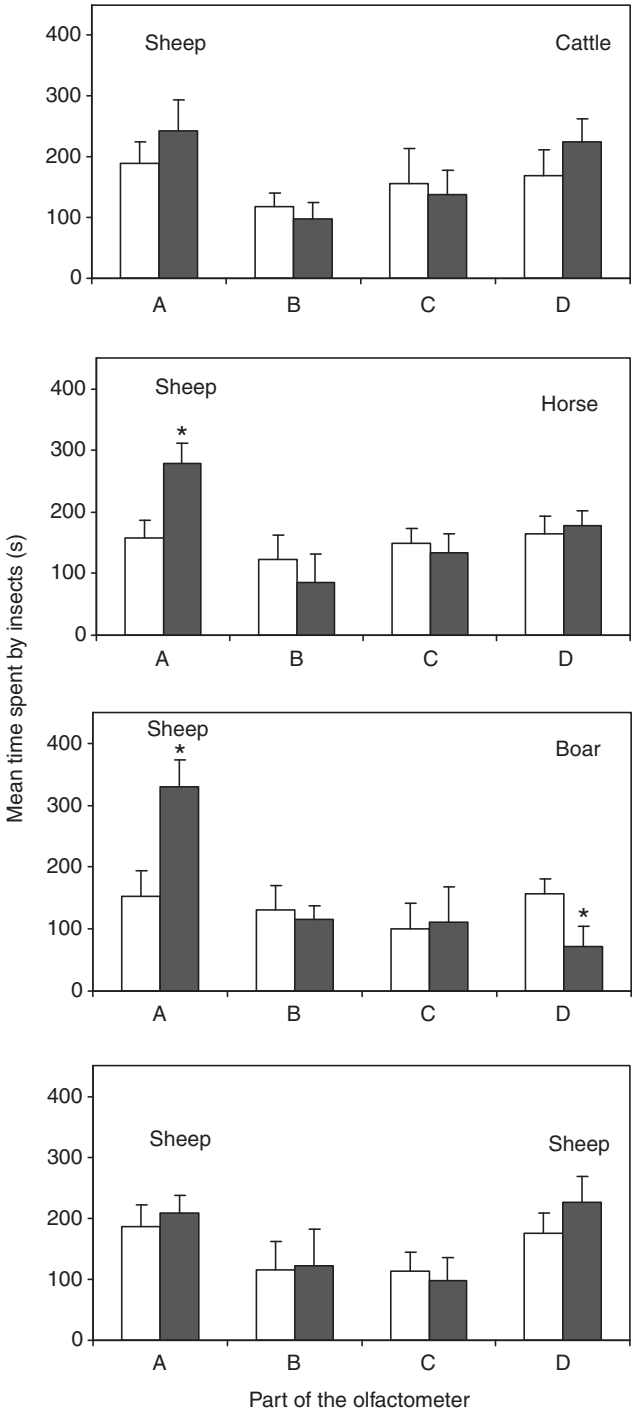


Fig. 2. Olfactory responses of *Agrilinus constans* to dung volatiles: behavioural tests conducted with beetles that were reared on sheep dung (*N*=34 insects tested). Experimental design and procedure are described in Fig. 1.

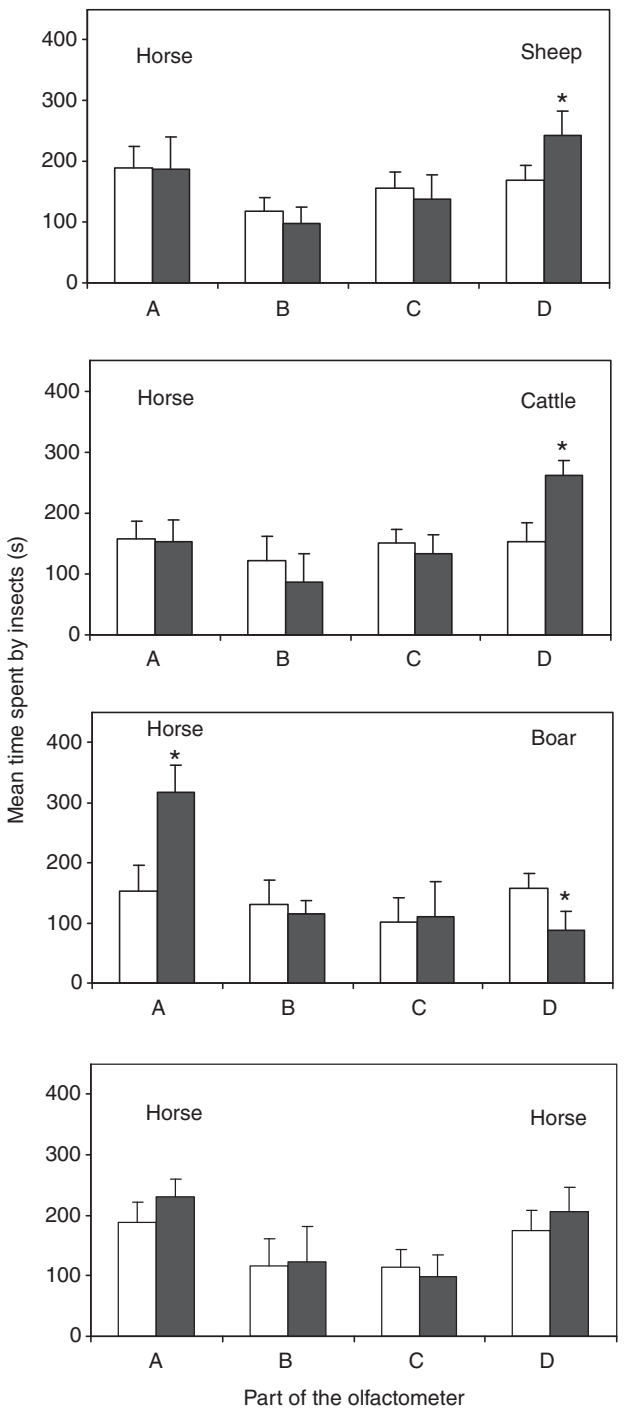


Fig. 3. Olfactory responses of *Agrilinus constans* to dung volatiles: behavioural tests conducted with beetles that were reared on horse dung (*N*=30 insects tested). Experimental design and procedure are described in Fig. 1.

types. The correspondence analysis allowed the separation of the volatile profiles of the four different dung types (Fig. 7). Sheep dung odours could be distinguished from other dung odours when considering the first axis, while the second axis separated the volatile profile of dung of the omnivorous wild boar from dung of herbivorous mammals. The compounds 2-phenylethanol, β-copaene and α-(E,E)-farnesene (on axis 1) and methylthioanisole (on axis 2) largely contributed to this pattern. The most common component, which was found to be abundant in all dung samples, was a shikimic product, *p*-cresol. In emissions of dung from sheep, the major components were *p*-cresol, 5-methyl-3-heptanone, dihydrolimonene, 2-phenylethanol and β-caryophyllene. The chemical composition of cattle dung odours consisted mainly of terpene products. In all of the samples of cattle dung volatiles, α-pinene and *p*-cresol

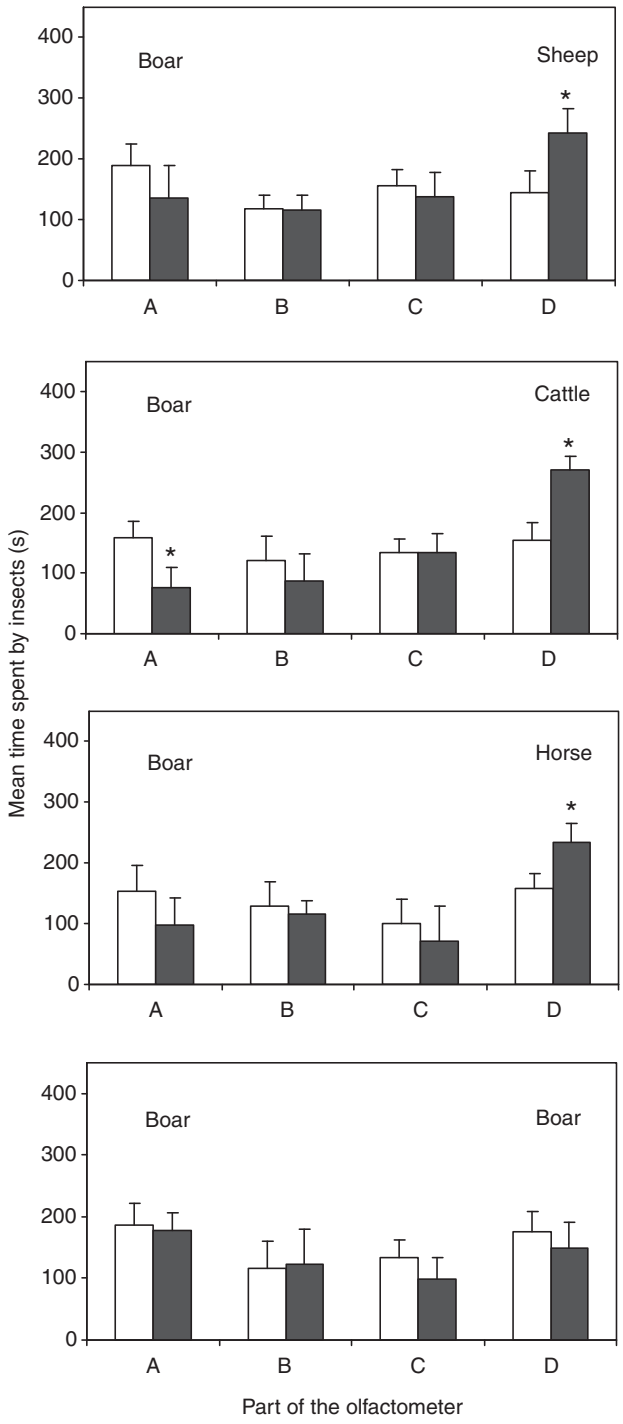


Fig. 4. Olfactory responses of *Agrilinus constans* to dung volatiles: behavioural tests conducted with beetles that were reared on wild boar dung (*N*=23 insects tested). Experimental design and procedure are described in Fig. 1.

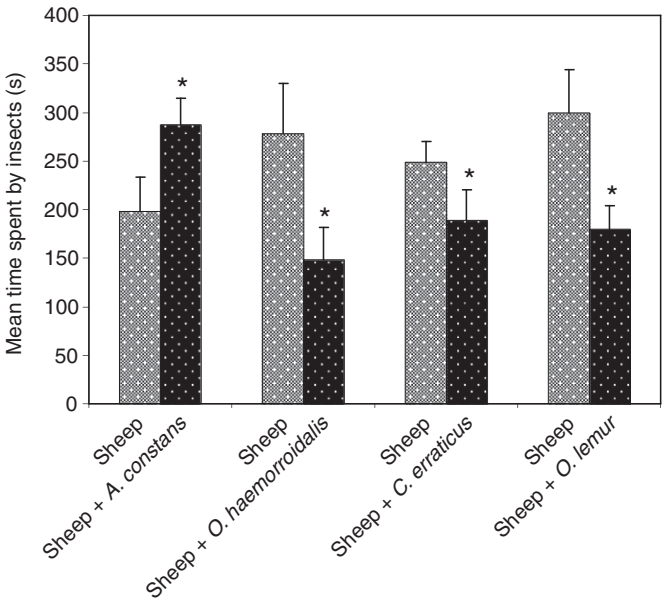


Fig. 5. Olfactory responses of *Agrilinus constans* to dung volatiles: behavioural tests conducted with beetles that were reared on sheep dung (*N*=25 insects tested). The graphs present the mean time spent by insects in two different parts of the olfactometer when submitted to two different dung odour stimuli, fresh sheep dung (grey bar) *vs* fresh sheep dung inhabited by 10 adult beetles of a single species (black bar). Four series of tests were performed (*n*=25 repetitions for each series), using four insect species: *Agrilinus constans*, *Otophorus haemorroidalis*, *Colobopterus erraticus* and *Onthophagus lemur*. Each pair of columns summarises one test, for which insects were submitted to odours from two different dung samples placed beneath opposite sections A (sheep dung, left column) and D (sheep dung with insects, right column). The mean time spent by beetles in the two middle parts (B and C) of the olfactometer is not shown on the graph, and tests with no odour source are not shown on the graph. Experimental design and procedure are described in Fig. 1.

dominated. In the volatiles of horse dung samples, *p*-cresol and β-citronellene were strongly prevalent in all of the samples, while many compounds originating from the lipidic pathway were also found (dodecane, tridecane, tetradecane, nonane, etc.). In wild boar dung, the volatile profile was dominated by four components, dihydrolimonene, *p*-cresol, indole and skatole – the last three originating from the shikimic pathway.

In sheep, cattle and wild boar, the dung volatile profiles showed limited variation among individuals, although a few components present as traces were not detected in all samples. By contrast, the volatile emissions from the different samples of horse faeces varied both quantitatively and qualitatively, except for the two major components.

The SPME method allowed the trapping of many more dung volatile compounds than did adsorption on a porous polymer: 92% (i.e. 47 compounds of the total 51 compounds) of the total number of volatile compounds were detected with SPME whereas only 76% (39 of the total 51 compounds) were detected using the headspace technique with the porous polymer.

DISCUSSION

Three important results emerge from this study: (1) larval nutrition did not affect the adult olfactory responses to dung volatiles, suggesting that the process of resource selection in dung beetles may proceed from innate olfactory preferences; (2) the presence of other insects inside the dung resource affected the process of dung selection by *A. constans*; (3) and volatiles emitted by dung clearly differed among different mammal species, allowing olfactory discrimination by dung beetles.

The results obtained with olfactometer bioassays showed the key importance of dung volatiles in the process of resource selection by scarab dung beetles. The species *A. constans* responded positively

Table 1. Volatile compounds emitted by fresh faeces of sheep, cattle, horse and wild boar

RT*	RI†	N‡	Compound	Sheep	Cattle	Horse	Boar
6.62	900	1	Nonane			**	
6.90	909	2	Butyl propanoate		**		
7.20	918	3	Methyl hexanoate		**		
7.50	923	4	γ-Valerolactone		*	**	
7.51	927	5	4-Methyl-3-heptanone			***	
7.60	930	6	5-Methyl-3-heptanone	***		*	
7.70	936	7	α-Pinene	**	****	**	*
7.80	939	8	β-Citronellene		***	****	
8.28	953	9	Camphene		*		
8.65	965	10	Pinane trans		**		*
8.80	969	11	Trimethyldisulphide	*	**		**
9.09	977	12	β-Pinene		*		
9.25	982	13	Pinane cis		**	**	**
8.90	990	14	3-*p*-menthene	**			*
9.41	992	15	2-Octanone			*	
9.79	998	16	Dihydrolimonene	***	**	**	***
10.09	1000	17	Decane			*	
10.09	1008	18	α-Terpinene	*	*		
10.61	1023	19	*p*-cymene	**	***		
10.79	1026	20	Limonene	**	**	*	*
11.35	1045	21	(E)-ocimene		*	*	*
11.80	1056	22	γ-Terpinene		*		**
12.10	1067	23	Acetophenone			*	
12.30	1073	24	*p*-cresol	***	****	****	****
12.73	1087	25	Terpinolene	**	**	*	*
12.91	1092	26	2-Nonanone			**	
13.18	1100	27	Undecane			**	*
13.40	1103	28	Nonanal	**		*	
14.00	1123	29	2-Phenylethanol	***			
16.69	1200	30	Dodecane		**	**	**
19.15	1291	31	Methylthioanisole				*
19.98	1297	32	Indole	**	*	**	***
20.11	1300	33	Tridecane	*	*	**	*
21.73	1349	34	α-Cubebene	*	**		
22.69	1377	35	α-Copaene	**	**	*	*
22.70	1378	36	Skatole	*	*	**	***
22.96	1387	37	β-Bourbonene	*	**	*	
23.29	1397	38	Tetradecene		*	**	
23.30	1396	39	Cyclodecene		*	**	
23.40	1400	40	Tetradecane		**	**	
24.13	1423	41	β-Caryophyllene	***	**	**	**
25.14	1427	42	β-Copaene	*			
25.25	1459	43	α-Humulene	**	**	*	
25.26	1461	44	2-Methyltetradecane	*		**	*
25.82	1478	45	Germacrene D	*	*		*
26.48	1499	46	Nor-ionone		*		*
26.49	1500	47	Pentadecane		*	**	*
27.00	1501	48	γ-Cadinene	*	*		
28.18	1502	49	α-(E,E)-farnesene	**			*
27.13	1506	50	δ-Cadinene	*	*		
29.43	1600	51	Hexadecane		*	*	*

*RT=retention time. †RI=retention index. ‡N=peak number.

For each of these mammal species, dung from 10 different individuals was sampled. Dung volatiles were collected using both solid-phase microextraction (SPME) and dynamic headspace with a porous polymer, and analysed by GC–MS. The relative importance of each compound was expressed with respect to the total content of volatile compounds: *<1% ; **1–5% ; ***10–30% ; ****>30%.

and selectively to dung volatiles in laboratory experiments, and oriented preferentially towards the volatiles from cattle and sheep dung. Horse dung volatiles were attractive only when adult beetles made a choice between horse *vs* wild boar dung. Wild boar faeces were little attractive and rarely chosen by *A. constans* when a choice was proposed. It is interesting to note that wild boar is the only omnivorous mammal among those included in this study, and that horse is the only non-ruminant herbivore of those we studied. It could be hypothesised that horse dung may contain more undigested chemical or mechanical defences of plants. The foregut fermentation of ruminants may allow them to destroy numerous secondary metabolites during digestion (Aguiar and Wink, 2005). These olfactory tests confirmed the trophic preferences of *A. constans* observed during field trapping experiments, in which adults of this species were collected in much greater abundance in pitfall traps baited with cattle or sheep dung than in traps baited with horse or deer dung (Dormont et al., 2004; Dormont et al., 2007). Our study also showed that *A. constans* can detect the presence of other insects

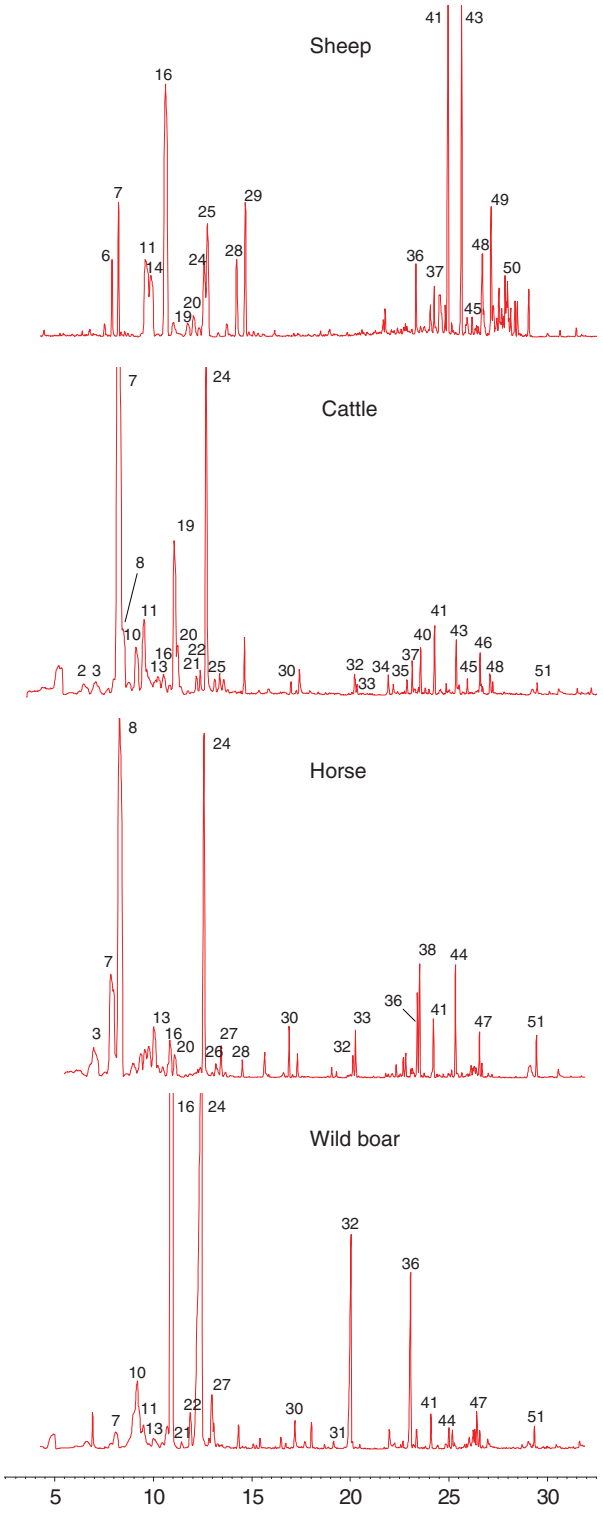


Fig. 6. Gas chromatograms of dung volatiles from sheep, cattle, horse and wild boar. Peak designations are given in Table 1.

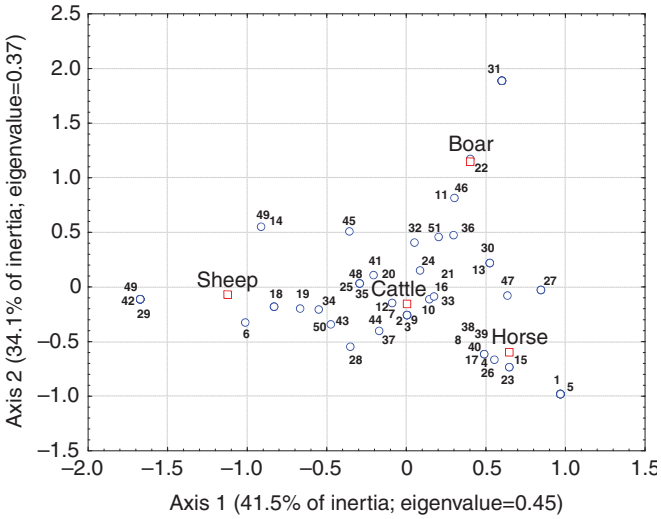


Fig. 7. Plot of dung types (red squares) and volatile compounds (blue circles) on axes 1–2 of the correspondence analysis. Numbers correspond to the compounds listed in Table 1.

within the dung, being either attracted (when other *A. constans* were present in the dung sample) or repelled (when the dung was inhabited by other insect species). Similarly, other *Aphodiinae* species have been reported to prefer to lay eggs in sheep dung uninhabited by fly larvae (Hirschberger and Degro, 1996). What kinds of volatiles could induce such behaviour in *A. constans* is not known, because no specific volatile compound was isolated or identified from extracts of dung samples that included insects.

A surprising result is that larval feeding experience had no effect on adult behavioural response to olfactory signals. During olfactometer bioassays, all of the adult beetles showed similar olfactory responses to dung volatiles, independently of which substrate had been used for larval rearing. These results suggest that resource selection behaviour of adults is probably not influenced by larval diet in *A. constans*. Moreover, because imagos were not immediately placed in separate boxes after emergence within rearing boxes (freshly emerged adults could rest a few hours in the boxes because the rearings were surveyed only twice a day), we can hypothesise that adult olfactory behaviour is probably not influenced by chemicals perceived during early-adult experience. Although the possible influence of chemicals shortly after emergence should be examined through more rigorous experiments to discriminate between larval and early-adult experience (Gandolfi et al., 2003), learning during the young-adult phase just following emergence seems unlikely to occur in *A. constans*. The apparent absence of any influence of larval or early adult experience on the olfactory response of *Aphodiinae* adults contrasts with the situation observed in phytophagous insects. Similar experiments with other phytophagous insects from several families showed that adult insects often chose the plant species on which they had developed as larvae (Schoonhoven et al., 1998; Barron, 2001; Rietdorf and Steidle, 2002).

These behavioural experiments confirmed that odours from faeces are clearly involved in the process of resource location and selection by dung beetles. In this study, we report the first analysis of the volatile compounds emitted by dung from different mammals, and this constitutes an important step in understanding the role of chemical cues in dung beetles. Comparison of the chemical composition of dung odours revealed notable differences among dung from sheep, cattle, horse and wild boar. Each dung type was characterised by a distinct profile of volatiles consisting of about 30 components, including compounds common to all dung types as well as a few compounds specific to each dung type. Only one compound was found to be abundant in all four dung types: *p*-cresol (4-methyl phenol). *p*-cresol is known as a malodorous compound

with a typical faecal odour, and has already been isolated in volatile emissions from cattle faeces (Aii et al., 1980; Kite, 1995), pig manure (Hobbs et al., 1999; Bicudo et al., 2002) and human faeces (Moore et al., 1987; King et al., 2009). Bazemore et al. reported *p*-cresol as a 'cattle-faeces aroma' in emissions from spent mushroom compost (Bazemore et al., 2000). This compound has also been found in the floral odours of diverse plant families (Knudsen et al., 2006), such as Araceae (Kite, 1995; Gibernau et al., 2004). Interestingly, *p*-cresol was found to be present in great quantity in the volatile emissions of *Arum maculatum* (Kite, 1995), a species exhibiting a typical 'faecal' odour, and this compound is suspected to attract the main pollinator of *A. maculatum*, *Psychoda phalaenoides*, which breeds exclusively in cattle dung. *p*-cresol has also been shown to elicit a strong olfactory response in the Japanese dung beetle *Geotrupes auratus* (Inouchi et al., 1988). Regarding other important dung volatiles, β-citronellene, which dominated the volatile profile of horse dung, has only been reported in volatiles from *A. maculatum* (Kite, 1995). 'Dihydrolimonene', one of the main volatiles found in wild boar dung, probably includes several close compounds with quite similar chemical structures. These two last compounds, together with pinane-cis and -trans, are partially hydrogenated compounds that correspond to the first steps of reduction reactions but whether they contribute to the typically faecal odour is unknown.

Surprisingly, some other compounds that are commonly believed to be responsible for faecal odour, such as methyl sulphides, volatile fatty acids, skatole or indole (Monroe, 1985; Suarez et al., 1998; Sato et al., 2001), were either not found in our study or only present in low levels in some dung extracts (indole, skatole, dimethyltrisulphide and methylthioanisole). However, these compounds are known to produce a very strong odor, even at very low concentration. O'Neill and Phillips reported, for example, that skatole and indole had an odour detection threshold by the human nose of less than $1\,\mu g\,m^{-3}$ (approximately 1 p.p.m.) (O'Neill and Phillips, 1992). Such compounds have often been reported to be dominant in volatiles from faeces of mammals, e.g. humans (Moore et al., 1987; Garner et al., 2007), dogs (Arnould et al., 1998) or pigs (Smith et al., 2000).

However, comparing our results with data reported in the literature, which have mostly addressed faeces volatiles from carnivorous or omnivorous mammals, requires a number of precautions because of the large differences both in diets of the animals and in bacterial transformations in the gut (Prins, 1977). Moreover, the chemical composition of herbivore faeces may also be greatly influenced by the processes of fermentation and microbial synthesis in the rumen. It is not surprising that many terpene products and alkanes (alkanes are long-chain hydrocarbons commonly occurring in plant cuticles) were detected in our study, as herbivore faeces contain many residues of plant material (Murphey et al., 1981). Although further investigations will have to address possible variation in dung volatiles as a function of animal diet, we observed little variation among individuals of the same mammal species, except for horse faeces volatiles.

We thus showed in this study that clear qualitative differences occurred between dung volatiles from different mammal species, and that *Aphodius* beetles can distinguish between faeces from different mammal species on the basis of olfactory cues. Further electroantennographic (EAG) studies will have to examine what compounds or what groups of compounds are implicated in dung selection by these insects. What is interesting is that olfactory choices by adult *Agrilinus* were shown to result from apparent innate preferences. Our results deviate from Hopkins' host selection principle and the chemical legacy hypothesis. Following these principles, adults of *A. constans* would have exhibited a preference for the odours of the dung in which they had developed. Our experiments showed that olfactory behaviour of *A. constans* adults did not vary when the larvae were previously reared in distinct artificial diets.

However, the ability of adult beetles to orient towards and to learn novel dung volatiles in the field has not been evaluated in this study. Olfactory learning by adults has been demonstrated in many insect species (Fan et al., 1997; Cunningham et al., 2004; Cunningham et al., 2006; Jorgensen et al., 2007; Giurfa, 2007; Wright et al., 2007; Riveros, 2009). To what extent associative learning (association of new odours with an adequate substrate for feeding and reproduction) influences the apparent prevalence of innate olfactory preferences in dung beetles remains open to question. In many flower-visiting insects, adults commonly exhibit innate preferences for floral scents but can also learn to associate new floral cues with nectar rewards (Kelber, 2002; Cunningham et al., 2004; Riffell et al., 2008). The ability to learn is considered advantageous for insects facing scarce or changing resources. However, in what cases learning is better than innate behaviour in enabling insects to face variable ecological factors remains difficult to demonstrate (Dukas, 2008). Dung from mammals represents a relatively scarce and patchy food resource in most ecosystems. In the French Mediterranean region where this study was conducted, livestock herds traditionally consist of domesticated cattle and sheep. Ranches (horse) have also recently developed, and the population density of wild boar has increased considerably during the past two decades. Although precise information on the densities of different mammal species is not available from literature, abundance of dung resource can be considered roughly equivalent among the four dung types considered in this study. Although vertebrate faeces may consistently vary in chemical composition, they do not 'defend' themselves against insects, contrary to plants. In consequence, polyphagy, which appears clearly advantageous for locating and exploiting such rare dung resources, is the predominant feature of dung beetle feeding patterns (Hanski and Cambefort, 1991). Feeding specialisation, i.e. restriction of beetle species to dung of a particular mammal species, has been observed in a very few cases (Lumaret and Iborra, 1996; Galante and Cartagena, 1999).

The carcasses of vertebrates used by necrophagous insects represent a similar case of a scarce food resource, lacking active chemical defences (although possible competition with bacteria should be expected to produce some particular compounds that may influence beetle behaviour). There are very few specialised species in burying beetle communities, and most species are polyphagous (Scott, 1998). Carrion beetles have been reported to respond positively to a group of common volatile compounds emitted by different types of fresh carcasses, i.e. sulphur-containing gases, such as methanethiol, dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide (Kalinova et al., 2009). It would be interesting to know whether necrophagous beetles, like coprophagous beetles, orientate towards carcasses through innate olfactory preferences or if larval diet may influence the choices made by adults.

We can thus imagine that polyphagous dung beetles are primarily attracted to a blend of characteristic compounds emitted by most dung volatiles, such as p-cresol, indole and skatole. Again, EAG experiments are needed to examine this hypothesis. In particular, it would be interesting to test also the EAG activity of compounds emitted by carcasses, because volatile compounds from carcasses have been observed to attract many species of dung beetles in the field (J.-P.L., personal observation). Learning abilities would confer a very

limited advantage, given that very few types of dung (i.e. mammal species) are locally available for insects, when compared with the plant diversity with which phytophagous insects are confronted. Further experiments should examine whether *Aphodiinae* adults can learn new dung odours, and whether spontaneous olfactory preferences also occur in other scarab dung beetles.

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