

**Research papers**

**Geographic variations in *Helleborus foetidus* elaiosome lipid composition: implications for dispersal by ants**

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**Summary.** Geographic variations in the correspondence between diaspore phenotypes and disperser behavior are thought to determine the evolution of plant-animal dispersal mutualisms. *Helleborus foetidus* is a widely distributed plant in Western Europe, which seeds bear a lipid rich elaiosome attracting ant dispersers. Laboratory cross-tests were conducted to check the correspondence between diaspore phenotypes and ant preference in two localities of the Iberian Peninsula, Caurel and Cazorla, separated by 750 km. Diaspores from Caurel were systematically preferred to those from Cazorla by *Formica lugubris* (the major disperser at Caurel), and *Aphaenogaster iberica* and *Camponotus cruentatus* (both major dispersers at Cazorla). Further bioassays conducted on *A. iberica* only showed that differences in elaiosome traits were sufficient to explain ant preference. Separation of the lipid fractions composing the elaiosome revealed that triglycerides, diglycerides and free fatty acids were all dominated by oleic acid. The elaiosomes from Caurel contained relatively more free oleic acid but were less concentrated in linoleyl-containing triglycerides, free palmitic acid and free linoleic acid than those from Cazorla. The three lipid fractions were attractive to ants but dummies soaked with the free fatty acids extracted from Caurel were preferred to those from Cazorla. Taken together, these results reinforce the idea that oleic acid is a major releaser of seed collection by ants and suggest that geographic variations in free fatty acid composition affect the probability of diaspore removal by ants which in turn potentially determine plant demography.

**Key words.** Myrmecochory – oleic acid – seed-dispersal – coevolution – Formicidae – Ranunculaceae

**Introduction**

Dispersal constitutes a determinant step in the life cycle of most organisms. For plants, it is also an ecological challenge that they frequently overcome using animal movement in exchange of food rewards. In the last decades, the processes governing the evolution of plant-animal dispersal mutualisms have been the topic of important investigations. The

observation that plants were generally dispersed by more than one animal species led authors to question the initial vision of pairwise tightly coevolved associations (Howe 1984; Herrera 1995; Levey & Benkman 1999; Beattie & Hughes 2002). However, theoretical developments considering spatial variations of interactions suggest that even such diffuse mutualisms may be driven by coevolutionary processes (Thompson 1994). Hence, plant-disperser coadaptations likely vary with space and time depending on the species involved in the mutualism in conjunction with abiotic (e.g. climate) and biotic (e.g. presence of predators or parasites) constraints. The expected result is a geographic mosaic in which well-matched adaptations evolve locally and further extend to other populations while unfavorable associations tend to be eliminated.

Among invertebrates, ants are the major group of seed-dispersers. Over the world, more than 3000 species of herbs, shrubs and trees are known to be principally ant-dispersed (Beattie & Hughes 2002). Ant-dispersed plants (also called myrmecochores) produce seeds bearing a lipid-rich appendage, the elaiosome, which serves as food reward for ants. In general, ants collect the entire diaspores (seed + elaiosome), carry them to their nest and feed the larvae with the elaiosomes (Fischer *et al.* 2005) while the intact seeds are rejected either outside the nest or in an abandoned chamber. Myrmecochory potentially provides various advantages for plants (Handel 1978; Heithaus 1981; Beattie & Culver 1982; Bond & Breytenbach 1985; Hughes & Westoby 1992b; Valverde & Silvertown 1995; Christian & Stanton 2004) which suggests that traits enhancing diaspore collection may be under selection by ants. In a recent study on *Helleborus foetidus* L. (Ranunculaceae) in the Iberian Peninsula, Garrido *et al.* (2002) found important between-locality variations in the correlation between seed mass and ant size. They interpreted this pattern as a mosaic of good and bad diaspore adaptations resulting from geographic variations in the strength of selection by ants. This explanation attempt was then reinforced by the finding that variations in seed losses due to field mouse predation could locally hamper ant selective pressure on plants (Fedriani *et al.* 2004).

The previous statements rest on the idea that seed size affects diaspore dispersal. To date however, clues that within-species variation in seed or diaspore size limit dispersal are scarce. By contrast, at least one study explicitly

identified variations in elaiosome size as a major determinant of diaspore removal (Mark & Olesen 1996). Moreover, several lines of evidence indicate that, besides morphological characters, elaiosome chemical composition affects the probability of removal (Mayer *et al.* 2005). Hence, the abundant quantities of oleic acid contained in the elaiosome, either as neutral lipid or as free fatty acid, were repeatedly shown to release ant removal behavior (Marshall *et al.* 1979; Skidmore & Heithaus 1988; Brew *et al.* 1989; Hughes *et al.* 1994; Sheridan *et al.* 1996). Linoleic acid, which most insects are unable to synthesize *de novo* and need to incorporate in their diet, was also suggested to stimulate diaspore-carrying behavior (Lanza *et al.* 1992).

In the present study, we explore geographic variations in diaspore adaptation to removal by ants. To that end, we compared the probability of removal of *H. foetidus* diaspores from two distant populations by local and non-local ant dispersers. Then, we conducted several bioassays and chemical analyses in order to know if ant preferences could be explained by differences of elaiosome traits. Specifically, we asked whether diaspore, elaiosome and elaiosome lipid fractions from two populations had different rewarding effects on ant dispersers.

## Materials and methods

### Plants and Ants

The stinking hellebore *H. foetidus* is a perennial herb widely distributed in European mountains. Plants used in the present study were collected in two populations separated by 750 km. Caurel and Cazorla. Both localities differ completely by their climate and ant communities. Cazorla is located SE Spain and has a Mediterranean climate with short winters and long dry hot summers. In this population, *H. foetidus* is mostly dispersed by *Camponotus cruentatus*, *Aphaenogaster iberica* and *Pheidole pallidula* (Authors, unpublished results). By contrast, Caurel is located NW Spain and is characterized by temperate climate with relatively long winters and precipitations all over the year. The former ant species are absent from this population and diaspores are dispersed by wood ants, mainly *Formica lugubris* (Authors, unpublished results). Details on the biology and distribution of the plant system are described extensively elsewhere (Garrido *et al.* 2002).

At the end of the 2002 and 2003 ripening seasons 10 to 20 plants per population were cut off at the base and kept in paper bags at 9 °C to slow fruit maturation. Fruit opening was then checked daily. Freshly liberated diaspores were either frozen at -18 °C for subsequent chemical analyses or kept at 9 °C for bioassays (no more than 2 months later) or maintained at room temperature during 2 months for elaiosome semi-dry mass measurements.

Colonies of *C. cruentatus* and *A. iberica* were collected in Sierra de Cazorla and colonies of *F. lugubris* were collected in Sierra de Caurel. The colonies of *C. cruentatus* and *F. lugubris* contained between 1000 to 2000 workers approximately while those of *A. iberica* contained between 250 and 400 workers. All of them had at least one queen and a brood. Nevertheless, uncontrolled between-colony differences were suspected for brood and worker age and brood size. Colonies were installed in formicariums each composed of an artificial nest (18 × 18 × 6 cm) connected to a foraging area (18 × 18 × 6 cm) through a 5 cm tygon tube. Ants were kept in the laboratory at 25 °C and fed twice a week with sugar (20 % v/v) and abundant dead mealworms.

### Bioassays

Bioassays were designed to evaluate the probability of removal by ants as a function of diaspore origin and elaiosome traits. In a first

series, 15 diaspores (seed + elaiosome) from Caurel and 15 diaspores from Cazorla were placed simultaneously in the foraging areas. The diaspores were previously marked with a dot of color paint (Unipaint, Mitsubishi Pencil Co. Ltd Japan) to identify their population of origin. A preliminary analysis confirmed that ants were not influenced by the paint. After 75 min the number of diaspores removed to the nest was counted. The tests were conducted in 2002 on *A. iberica*, *C. cruentatus* and *F. lugubris* (5 colonies per species) and were repeated in 2003 on *A. iberica* only using 5 different colonies.

The second series of tests followed a similar protocol but the ants were presented simultaneously with 15 elaiosomes (seeds removed) from Cazorla and 15 elaiosomes from Caurel. The experiment used 7 colonies of *A. iberica*. The other species were not tested. The choice of *A. iberica* was determined by the result of the first series (see results) in which this species appeared more prompt in removing diaspores than *F. lugubris* and *C. cruentatus*.

The last series aimed at testing the probability of removal of the triglyceride, diglyceride and free fatty acid fractions extracted from elaiosomes from Cazorla and Caurel (see next section). Dummies were created by absorbing 3 elaiosome-equivalents of triglycerides, diglycerides or free fatty acids on small pieces of filter paper (4 mm<sup>2</sup> approximately) previously pencil marked to identify the population. Ten dummies from Caurel, 10 dummies from Cazorla and 10 controls (paper soaked with dichloro-methane: methanol (2:1)) were placed in the foraging area of 9 colonies of *A. iberica*. Each colony was used 3 times in random order to test triglyceride, diglyceride and free fatty acid dummies.

### Elaiosome mass and lipid content analyses

Measures of elaiosome mass were made on samples of 100 and 200 elaiosomes per population in 2002 and 2003 respectively. These samples were randomly selected from larger sets containing diaspores from 10 plants per population in 2002 and 20 plants per population in 2003. Elaiosomes were cautiously removed from their seed and weighed on a 0.1 mg precision balance.

To analyze elaiosome fatty acid profiles, each sample consisted of a pool of 10 elaiosomes detached from their seeds and randomly collected on the same plant (but not the same fruit). The analyses were repeated using 10 plants per population in 2002 and 20 plants per population in 2003. Pools of elaiosomes were soaked in 2 ml dichloromethane : methanol (2:1) for 12 h, 3 times consecutively to ensure full lipid extraction. After evaporating the solvent to dryness, triglyceride, diglyceride and free fatty acid fractions were separated using a method adapted from Tserng and Griffin (2003): The lipid residue was dissolved in 500 µl (× 2) isooctane : ethyl acetate (10:1) and applied to an alumina column (4 cm Pasteur pipette filled with flash alumina pre-equilibrated with isooctane : ethyl acetate 10:1). The column was then eluted with 4 ml isooctane : ethyl acetate (10:1) to yield the triglyceride fraction. Elution of the diglyceride fraction was then realized with 5 ml of isooctane : ethyl acetate (3:1). Finally, the free fatty acids were collected after elution with 6 ml of isooctane : ethyl acetate : acetic acid (75:25:2).

The resulting fractions of triglycerides, diglycerides and free fatty acids were dried and dissolved again in 1 ml of dichloro-methane : methanol (2:1). One tenth of this volume (1 elaiosome equivalent) was transferred to a 2 ml vial containing 20 µg of octadecane as an internal standard and dried before transesterification (for tri- and diglyceride fractions) or methylation (for the free fatty acid fractions) and further GC-MS analyses. The remaining volume (9 elaiosome equivalents) was stored at room temperature (20-25 °C) until bioassays.

Trans-esterification was realized by adding 100 µl of methanolic KOH (0.5 M) to the dry triglyceride and diglyceride fractions. After 30 min the reaction was stopped with 100 µl of HCl (1 N). Then 20 µl of hexane was added to the mixture and the organic phase was recuperated and dried. On the other hand, methylation was carried out by treating the free fatty acid fraction with 50 µl of boron trifluoride-methanol complex for 1 h.

All the obtained fractions, composed of fatty acid methyl esters were dried and dissolved again in 500 µl of isooctane, of which 1 µl was injected into a gas chromatograph equipped with a 30 m DB-5 column. Temperature was programmed as follows:

70-150 °C at 15 °C/min, 150-250 °C at 5 °C/min, 250-310 °C at 10°C/min hold 5 min. The eluted compounds were detected by FID and quantified using peak areas. Compound identification was realized on three representative samples by mass spectrometry using similar chromatographic conditions.

### Statistics

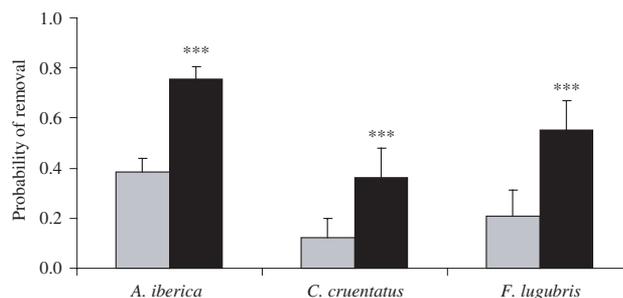
To analyze the results of the bioassays, each offered item (diaspore, elaiosome or dummy) was given a value of 1 if removed and a 0 if not. Data were fitted to generalized linear models with the binomial error distribution and logit link function using the GENMOD procedure from SAS 8.02 (SAS Institute, Inc). For the first series of bioassays (testing entire diaspores), independent models were fitted separately for the three ant species. For *C. cruentatus* and *F. lugubris*, the categorical predictors were the origin of the diaspores (Origin: Caurel vs Cazorla) and the Colony of ant. For *A. iberica*, the categorical predictors were "Origin", "Colony", "Year" (2002 and 2003) and "Origin"×"Year" interaction. For the second series of bioassays (elaiosome only), the categorical predictors were "Colony" and "Origin". Finally, for third series of bioassays, the predictors were "Colony" and "Dummy" (Caurel, Cazorla and Control). The LSMEANS statement was used to detect between-level differences.

Results of elaiosome mass and lipid composition were analyzed using Statistica 6 (Statsoft, Inc.). Mean elaiosome mass was compared between populations and years using factorial analysis of variance with "Year", "Population" and the interaction as fixed effects. The analyses of geographic and temporal variations of fatty acid profile in the triglyceride, diglyceride and free fatty acid fractions were done using the arcsin-transformed data. Three independent multiple analyses of variance were conducted including "Year", "Population" and their interaction as categorical predictors and the concentration of the four major acid as dependent variables. When the result was significant, univariate ANOVA was used to compare fatty acid percentages separately. Then, the Newman-Keuls post hoc test was used to detect differences between the levels of the main effects.

### Results

Entire diaspores of *H. foetidus* were highly attractive for *F. lugubris*, *A. iberica* and *C. cruentatus* (Fig. 1, Table 1). However, the probability of diaspore removal varied greatly between colonies especially among *A. iberica* (range: 0.40 – 0.77) and *F. lugubris* (range: 0.17 – 0.73). When allowance is made for intercolonial variations, diaspores from Caurel were more readily removed by the three ant species than diaspores from Cazorla. During the tests realized in 2003 the probability of removal by *A. iberica* did not change significantly (Table 1) and the preference for Caurel was maintained (probabilities of removal:  $0.47 \pm 0.06$  vs  $0.87 \pm 0.04$  (mean  $\pm$  SE) for Cazorla and Caurel respectively). The preference of this species for diaspores from Caurel was, at least in part, determined by elaiosome traits since it still held when seeds were removed and only the elaiosomes were offered (probability of removal:  $0.25 \pm 0.29$  and  $0.47 \pm 0.27$  (mean  $\pm$  SE) for Cazorla and Caurel respectively; Generalized Linear Model:  $df = 1$ ,  $\chi^2 = 8.11$ ,  $P = 0.004$ ). However, as with entire diaspores, ant preference differed greatly between colonies (range: 0.06 – 0.96; Generalized Linear Model:  $df = 8$ ,  $\chi^2 = 133.60$ ,  $P < 0.001$ ).

Elaiosome characterization revealed that ant preference was accompanied by important morphological and chemical differences between the two populations. Elaiosomes collected in Cazorla were 28 % smaller than those from



**Fig. 1** Mean ( $\pm$  SE) probability of removal of diaspores of *H. foetidus* from Cazorla (grey bars) and Caurel (black bars) by the three major dispersers: *F. lugubris* (from Caurel), *C. cruentatus* and *A. iberica* (both from Cazorla). The represented data were obtained in the tests realized in 2002. Results obtained in 2003 with *A. iberica* are given in the text. Statistical significances are summarized in Table 1.

**Table 1** Results of the 3 generalized linear models fitting the probability of diaspore removal by *Formica lugubris*, *Camponotus cruentatus*, *Aphaenogaster iberica*

Effect	df	$\chi^2$	P
<b>Probability of removal by <i>F. lugubris</i></b>			
Origin	1	18.15	< 0.001
Colony	4	34.12	< 0.001
<b>Probability of removal by <i>C. cruentatus</i></b>			
Origin	1	9.25	0.002
Colony	4	3.12	0.538
<b>Probability of removal by <i>A. iberica</i></b>			
Origin	1	53.09	< 0.001
Colony	8	18.15	0.020
Year	1	3.59	0.058
Origin x Year	1	0.82	0.364

Caurel (mean  $\pm$  SE:  $0.76 \pm 0.02$  mg,  $n = 300$  and  $1.06 \pm 0.02$  mg,  $n = 300$  respectively; ANOVA:  $df = 1$ ,  $F = 19.20$ ,  $P < 0.001$ ). Though elaiosomes from both populations were globally smaller in 2002 than in 2003 (mean  $\pm$  SE:  $0.84 \pm 0.03$  mg,  $n = 200$  and  $0.98 \pm 0.02$  mg,  $n = 400$  respectively; ANOVA:  $df = 1$ ,  $F = 96.55$ ,  $P < 0.001$ ), the difference between Cazorla and Caurel was higher the second year as indicated by a significant "Year" by "Population" interaction (ANOVA:  $df = 1$ ,  $F = 4.81$ ,  $P < 0.029$ ).

The 3 fractions extracted from the elaiosomes were dominated by oleyl- (C18:1), palmityl- (C16:0), stearyl- (C18:0), and linoleyl- (C18:2) lipids. Traces of linolenyl- (C18:3) and arachidonyl- (C20:4) lipids were also detected in the triglycerides but not quantified. Oleic acid was always the major compound representing 51 to 66 % of the total. However, some differences appeared between populations and years (Table 2 and 3). First, the proportion of linoleyl-containing triglycerides was globally higher in Cazorla than in Caurel (Univariate ANOVA:  $F = 9.816$ ,  $P = 0.003$ ) and in 2002 than in 2003 (Univariate ANOVA:  $F = 15.647$ ,  $P < 0.001$ ). Second, both in 2002 and in 2003, the elaiosomes

**Table 2** Results of the multivariate analyses of temporal and geographic variations in the relative fatty acid concentration in the three fractions<sup>a</sup>

Effects	Wilks' statistics	df	F	P
Triglycerides				
Year	0.732	4, 53	4.8	0.002
Population	0.507	4, 53	12.9	< 0.001
Year x Population	0.967	4, 53	0.5	0.772
Diglycerides				
Year	0.911	4, 53	1.26	0.297
Population	0.847	4, 53	2.35	0.066
Year x Population	0.963	4, 53	0.50	0.734
Free Fatty Acids				
Year	0.911	4, 53	1.3	0.282
Population	0.696	4, 53	5.8	0.001
Year x Population	0.897	4, 53	1.5	0.208

<sup>a</sup>MANOVA were conducted on arcsine-transformed percentage of palmitic, stearic, oleic and linoleic acid.

**Table 3** Relative percentages of fatty acid methyl esters within triglycerides, diglycerides and free fatty acids fractions<sup>a</sup>

	Caurel		Cazorla	
	2002	2003	2002	2003
Triglycerides				
Palmitic acid	15.7 ± 2.6 (a)	14.8 ± 1.2 (a)	14.1 ± 1.2 (a)	13.9 ± 0.4 (a)
Stearic acid	11.9 ± 1.8 (a)	11.7 ± 1.2 (a)	13.4 ± 1.8 (a)	13.2 ± 0.5 (a)
Oleic acid	62.9 ± 5.6 (a)	68.3 ± 5.0 (a)	58.8 ± 2.6 (a)	64.5 ± 0.8 (a)
Linoleic acid	9.5 ± 1.8 (a)	5.2 ± 1.8 (b)	13.5 ± 0.3 (c)	8.5 ± 1.8 (a)
Diglycerides				
Palmitic acid	13.9 ± 1.7 (a)	14.9 ± 1.7 (a)	13.7 ± 1.2 (a)	12.7 ± 1.2 (a)
Stearic acid	11.3 ± 1.7 (a)	12.3 ± 1.2 (a)	12.8 ± 1.8 (a)	14.5 ± 1.3 (a)
Oleic acid	66.8 ± 3.9 (a)	67.2 ± 2.7 (a)	62.9 ± 3.8 (a)	62.1 ± 2.8 (a)
Linoleic acid	8.0 ± 2.0 (a)	5.6 ± 1.4 (a)	10.6 ± 2.1 (a)	10.7 ± 1.5 (a)
Free Fatty Acid				
Palmitic acid	13.4 ± 1.5 (a)	15.5 ± 1.0 (ab)	17.8 ± 1.8 (bc)	18.6 ± 1.0 (bc)
Stearic acid	12.0 ± 1.1 (a)	16.3 ± 2.9 (a)	16.6 ± 1.7 (a)	16.7 ± 1.1 (a)
Oleic acid	66.6 ± 1.1 (a)	61.0 ± 1.1 (ab)	56.2 ± 1.1 (bc)	51.4 ± 1.1 (c)
Linoleic acid	8.0 ± 1.2 (a)	9.3 ± 1.1 (a)	7.1 ± 0.7 (a)	13.3 ± 1.2 (b)

<sup>a</sup>Different letters for each line indicate significant differences (Newman-Keuls Post Hoc test,  $P < 0.05$ ) between years and populations. Percentages are given for each fraction independently.

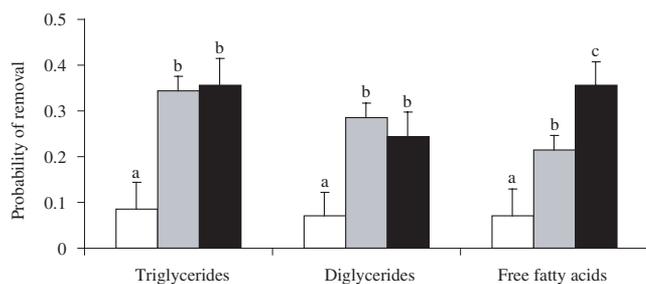
from Caurel were relatively richer in oleic acid than those from Cazorla (Univariate ANOVA:  $F = 15.457$ ,  $P < 0.001$ ), but less concentrated in palmitic and linoleic acids (Univariate ANOVA:  $F = 8.373$ ,  $P = 0.005$  and  $F = 9.863$ ,  $P = 0.003$  respectively). By contrast, no significant difference was detected among the diglycerides.

To test the biological significance of these differences, colonies of *A. iberica* were offered dummies soaked with the collected fractions of triglyceride, diglyceride or free fatty acid fractions from Caurel and Cazorla. The three fractions, irrespective of their geographic origin were systematically more readily removed than the controls soaked with solvent only (Fig. 2, Table 4; Newman-Keuls post hoc test:  $P < 0.05$  in

all pair wise comparisons involving the control group). However, the free fatty acid fractions from Caurel were significantly preferred to those from Cazorla (Newman-Keuls post hoc test:  $P = 0.03$ ). By contrast, the probability of removal did not differ between populations for dummies soaked with triglyceride or diglyceride extracts (Newman-Keuls post hoc test:  $P = 0.85$  and  $P = 0.55$  respectively).

## Discussion

The aim of the present study was to analyze the correspondence between ant behavior and diaspore phenotypes



**Fig. 2** Mean ( $\pm$  SE) probability of removal by *A. iberica* of paper dummies soaked with triglyceride, diglyceride or free fatty acid fractions extracted from the elaiosomes from Cazorla (grey bars) and Caurel (black bars). Controls (white bars) are dummies soaked with solvent only. Different letters indicate significant differences within the series (see Table 4 for the detailed statistics).

from two populations of *H. foetidus* separated by a large geographic distance. In controlled conditions, ants originating from Caurel (*F. lugubris*) and from Cazorla (*C. cruentatus* and *A. iberica*) both preferentially remove seeds from the former locality. Hence, this result indicates that traits important for dispersal differ between both populations and suggests that diaspore phenotype in Caurel better matches dispersers preference than in Cazorla.

This result led us to question what diaspore trait affecting dispersal differs between both populations. The second series of bioassays showed that differences in elaiosome traits were sufficient to explain the preference of *A. iberica* for diaspores from Caurel. Though a similar bioassay could not be reproduced with *F. lugubris* and *C. cruentatus*, we can assume that their preference for entire diaspores was also due to elaiosome differences. Likewise, Mark & Olesen (1996) gave convincing evidences that removal of *Hepatica nobilis* diaspores by *Myrmica ruginodis* was positively related to elaiosome size. This does not exclude that seed traits have additive effects on dispersal as a few interspecific comparisons indicate that small ants tend to avoid plant species producing heavy seeds (Sernander 1906; Hughes & Westoby 1992a; Nakanishi 1994; Gorb & Gorb 1995) but, as far as we know, no evidence really demonstrate that at the intraspecific level, seed weight limits dispersal.

The elaiosomes from Caurel were heavier than those from Cazorla, suggesting they were also richer in rewarding substances. Various authors successively demonstrated that elaiosome diglycerides, free fatty acids and in lower extent triglycerides elicited removal by ants (Marshall *et al.* 1979; Skidmore & Heithaus 1981; Brew *et al.* 1989; Hughes *et al.* 1994). Our results confirmed that the three lipid fractions isolated from *H. foetidus* were attractive for ants. However, only the free fatty acid fraction extracted from the elaiosomes from Caurel was preferred to the same fraction extracted from the elaiosomes from Cazorla while no difference was detected for the tri and diglycerides. Interestingly, chemical analyses revealed that over two consecutive years, elaiosomes from Caurel contained relatively more free oleic acid and less free palmitic and linoleic acids

**Table 4** Results of the 3 generalized linear models fitting the probability of dummy removal by *Aphaenogaster iberica* to treatment (fraction from Caurel, Cazorla and Control) and colony

Effect	df	$\chi^2$	P
<b>Triglycerides</b>			
Treatment	2	20.79	0.006
Colony	6	18.19	<0.001
<b>Diglycerides</b>			
Treatment	2	13.12	0.001
Colony	6	6.00	0.423
<b>Free Fatty Acids</b>			
Treatment	2	15.64	<0.001
Colony	6	1.92	0.925

than elaiosomes from Cazorla. Though our results do not allow sorting out which difference most contribute to the overall preference for Caurel, they are consistent with previous findings that withdrawal behavior is mostly released by oleyl-containing lipids (Marshall *et al.* 1979; Skidmore & Heithaus 1981; Brew *et al.* 1989; Hughes *et al.* 1994). Of course, other non-lipid compounds not analyzed in the present study may also have enhanced ant preferences.

The fatty acid profile found in the elaiosome of *H. foetidus* is relatively common in nature and is typical of most myrmecochorous plants (Mayer *et al.* 2005). Nevertheless, seed fatty acid profiles are generally different and in *H. foetidus* it is dominated by linolenic acid (C18:3) (Aitzetmüller *et al.* 1999). This reinforces the idea that elaiosome lipid composition is not a by-product of seed biosynthesis but evolved specifically in relation with its rewarding function (Hughes *et al.* 1994; Mayer *et al.* 2005). Long-chain unsaturated fatty acids present in invertebrate fat body attract carnivorous insects (Bomar & Lockwood 1994) and release ant necrophoric behavior (Wilson *et al.* 1958; Haskins & Haskins 1974; Howard & Tschinkel 1976). However, ant response to long-chain fatty acids may also depend on the context. For example, the ants *Crematogaster scutellaris*, *Lasius* sp and *Formica cunicularia* are repelled by palmitic, oleic and linoleic acid secreted by paper wasp sternal glands (Dani *et al.* 1996) but occasionally remove *H. foetidus* diaspores (Garrido *et al.* 2002) which contains the same compounds (this study).

Important between-colony variations were also found in the choice tests. While some colonies expressed consistent choice for diaspores, elaiosomes or elaiosome lipid fractions from Caurel, others removed just a few items without marked preference for one or the other population. Our information on colony demography is very limited and though all of them contained a brood, its size was unknown. However, we can hypothesize that part of inter-colony variability may be related to differences in colony size and social context (Gordon 1983). For example, some colonies may tend to remove all diaspores whatever their origins to feed large brood. On the other hand if the colony has small

brood and most ants are engaged in cleaning activities they may reject most food item, independently of their nutritional value. Such variability has been noted in other studies on myrmecochories (e.g. Mayer *et al.* 2005) and would deserve deeper investigation.

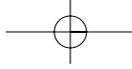
**Concluding remarks.** Our results potentially reflect geographic variations in plant adaptation to dispersal by ants. Though our bioassays were conducted in the laboratory and only lasted 75 minutes, small differences in diaspore attractiveness may have important consequences in the field. Ants are mostly active during the day and, all things being equal, seeds not removed rapidly may suffer high predation by mouse foraging at dusk (Fedriani *et al.* 2004). Other factors such as crop size and the presence of alternative food resources for ants may attenuate or increase the effect of elaiosome quality on plant demography (Boulay *et al.* 2005). In a previous study on *H. foetidus*, Garrido *et al.* (2002) stated that geographic variations in seed mass reflected a geographic mosaic of coevolution (*sensu* Thompson 1994) but this interpretation lacks the demonstration that intraspecific differences in seed mass really affect removal by ants. By contrast, elaiosome traits, especially fatty acid composition, vary between populations and determine diaspore removal by ants. Therefore, these traits are likely selected by ants and should be the focus of further studies on geographic mosaics of coevolution between myrmecochores and their dispersers.

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Vol. 16, 2006

Elaiosome composition of *H. foetidus*

7

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