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FLORAL FRAGRANCE CHEMISTRY IN THE EARLY FLOWERING SHRUB *DAPHNE MEZEREUM*

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Key Word Index—*Daphne mezereum*; Thymelaeaceae; pollination; floral fragrance; (*S*)-(+)-linalool; linalool oxides; ocimene; chirality; enantiomer; *Colletes*; *Andrena*.

Abstract—The floral fragrance of the shrub *Daphne mezereum* in central Sweden was collected by means of the head-space technique and investigated by GC-MS and multi-dimensional GC. (*S*)-(+)-Linalool was the main constituent (95%) of the flower fragrance and its enantiomeric purity exceeded 99% in the samples. The (2*S*, 5*S*)- and (2*R*, 5*S*)-furanoid and the (3*R*, 6*S*)- and (3*S*, 6*S*)-pyranoid linalool oxide isomers constituted 2–5% of the fragrance. The elution order of these compounds on a permethylated β-cyclodextrin column is reported. A fragrance sample of *D. mezereum* as well as (*S*)-(+)-linalool attracted males of the vernal solitary bee species *Colletes cunicularius* and *Andrena cinerea*. A racemic mixture of the two enantiomeric pairs of known furanoid linalool oxides was only weakly attractive to the bees. The role of the fragrance in the pollination specialization of the plant is discussed.

INTRODUCTION

Daphne mezereum L. (Dutch mezereon) is an early-flowering, deciduous, small shrub, widespread in the temperate part of Europe and extending further eastwards to central Siberia with an outpost in the Caucasus mountains [1]. In Sweden, *D. mezereum* grows in soils rich in humus and it is typical of the deciduous forests of the eastern central parts of the country. The shrub flowers in the spring, usually in April but in mild winters as early as in February or March [2]. The light-purple, strongly fragrant flowers form dense cylindrical aggregations consisting of numerous clusters with two to three flowers on the one-year-old branches. As the flowering starts before the leaves develop, the flower-bearing branches, together with the fragrance, seem to form a conspicuous attraction unit for pollinators, especially since all of the flowers on a plant are often open simultaneously. Such a floral display will "increase the proportion of pollination", although attracted pollinators will be less inclined to fly from plant to plant, if the display is combined with nectar rewards [3].

In 1925 Hegi suggested that the floral scent in combination with nectar might attract bees, flies and butterflies to *D. mezereum* [4]. The depth of the floral tube would suggest lepidopterans and/or long-tongued bees as pollinators of the plant. Several authors have reported that these groups are indeed the most common visitors. Kirchner mentioned unspecified bombyliid flies and beetles [5], while Schultz stated that hymenopterans (apids), some 20 species of butterflies and, less frequently, small flies, bettles and parasitic wasps were visitors to *D. mezereum* in the Alps [6]. Observations on the anthecology in *D. mezereum* have recently been made in Sweden [7]. It was found that the flowers were nectarless, and thus—in principle—pollinated by deceit. However, *D. mezereum* was self-compatible, allowing self-pollination (or geitonogamy, i.e. pollen transfer between flowers within the individual plant), and natural fruitset was fairly high. Manktelow concluded that minute insects such as thrips are probably important as within-plant pollinators of this plant species.

The main purpose of the present study was to identify the volatiles giving the flower fragrance, determine their enantiomeric composition, and test their biological functions on potential insect pollinators living in the surroundings of the plant.

RESULTS

The floral fragrance was released from the plant for at least two weeks, e.g. during 17 days in 1994. No rhythmic emission during day or night was observed. The emission

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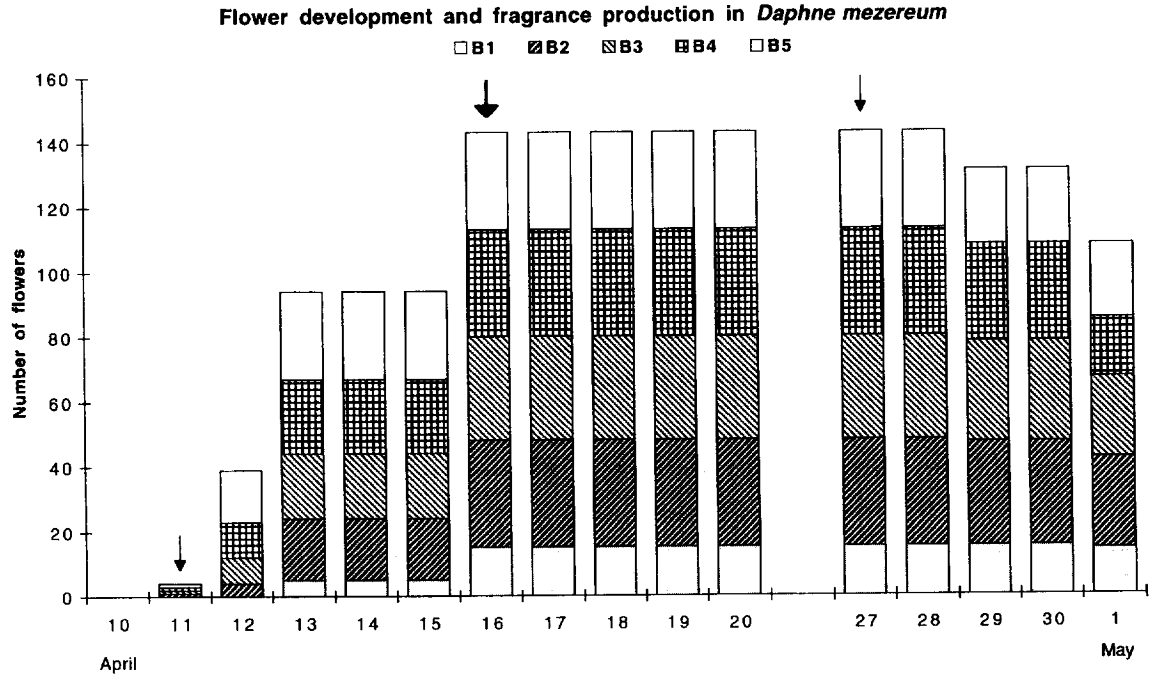


Fig. 1. Flower development and fragrance production (range indicated by arrows, bold arrow indicates peak flowering) during the flowering period of *D. mezereum* in 1994. The column portions B1–B5 represent the numbers of open flowers on each of the branches on one individual at the dates indicated.

of the fragrance started just before the anthesis and ended when the colour of the flowers began to fade (Fig. 1). The flowers at the top of the branches opened 1–3 days earlier than the flowers at the bottom of the clusters.

Chemical composition of the floral fragrance. The major constituents identified in the volatiles of the branches including the flowers were (*S*)-(+)-linalool (main compound) and *trans*-β-ocimene (Fig. 2, Table 1). According to the total ion current on the GC-mass spectrometry 2–4% of the volatiles consisted of the four linalool oxides related to (*S*)-(+)-linalool, i.e. one enantiomer of each of the linalool oxides A, B, C, and D. The full names and the configurations of these enantiomers are given in Table 1 and Fig. 2. Trace amounts of 3,7-dimethyl-1,5-octadiene-3,7-diol and 3,7-dimethyl-1,7-octadiene-3,6-diol, both related to linalool, were found in one of the samples. The stereochemistry of these two compounds could not be determined because of their small amounts. The relative amounts of the constituents were similar among the years, but the highest proportions of the linalool oxides were found in samples taken late in the flowering season. At the end of the flowering period, the relative amount of linalool decreased and *trans*-β-ocimene became the major constituent; thus, *trans*-β-ocimene probably emanated predominantly from the vegetative parts of the plant.

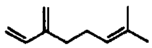
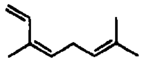
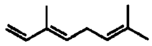
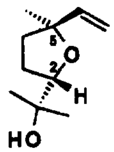
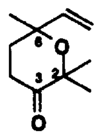
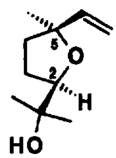
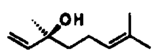
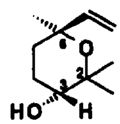
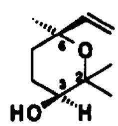
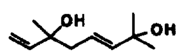
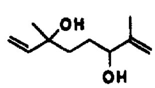
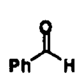
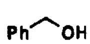
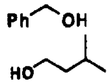
Behaviour tests. There were significant differences in the frequency of high intensity approaches of *Colletes cunicularius* (behaviour type 2) between the (*S*)-(+)-linalool sample as well as the mixture of furanoid linalool oxides and the blank ($p < 0.05$). Males of *Andrena cinerea* and *A. haemorrhoa* were also slightly attracted to (*S*)-(+)-linalool (Table 2), while *A. vaga* did not show any attraction to the test samples. No female bees were attracted to the odour samples.

DISCUSSION

The composition of the floral fragrance of *D. mezereum* was found to be quite simple compared to the compositions of many other flower fragrances [8–10]. Only two major constituents were found and only one of these, (*S*)-(+)-linalool, was present exclusively during the flowering period and greatly contributed to the strong fragrance. However, some minor constituents—less than 5%—such as benzaldehyde, benzyl alcohol and the linalool oxides might well be important contributors to the heavy "note" or "sweet character" of the fragrance. Thus, the conspicuous flower colour of this early-flowering shrub seems to be accompanied by a conspicuous floral fragrance made up largely by the scent of (*S*)-(+)-linalool.

Many species of solitary bees and bumble-bees produce linalool in their mandibular glands [11,12, and Borg-Karlson *et al.*, unpublished results]. The solitary bee *C. cunicularius* is one of the earliest flying hymenopteran insects in Sweden. Both male and female *C. cunicularius* bees produce (*S*)-(+)-linalool in their mandibular glands [Borg-Karlson *et al.*, unpublished results], a pheromone that causes the males to aggregate. There is a clear difference in attraction between the two enantiomers of linalool, the (*S*)-(+)-enantiomer being the most effective [Borg-Karlson *et al.*, unpublished

Table 1. Volatile constituents of the fragrance of *D. mezereum* flowers listed in order of elution from a DB-WAX capillary column

Compounds	Formula	First day of anthesis	Peak of flowering	End of flowering
myrcene			X	
cis-β-ocimene		X	XX	XX
trans-β-ocimene		XXX	XXX	XXX
(2*S*,5*S*)-linalooloxide A = (2*S*,5*S*)-*trans*-5-ethenyltetrahydro-α,α,5-trimethyl-2-furanmethanol		trace	X	X
4,5-dihydro-6-ethenyl-2,2,6-trimethyl -3-pyranone			X	X
(2*R*,5*S*)-linalool oxide B = (*R*,5*S*)-*cis*-5-ethenyltetrahydro-α,α,5-trimethyl-2-furanmethanol		trace	X	X
(*S*)-(+)-linalool		XXX	XXX	trace
(3*R*,6*S*)-linalool oxide C = (3*R*,6*S*)-*trans*-6-ethenyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol		trace	X	X
(3*S*,6*S*)-linalool oxide D = (3*S*,6*S*)-*cis*-6-ethenyl-tetrahydro-2,2,6-trimethyl-2H -pyran-3-ol		trace	X	X
3,7-dimethyl-1,5-octadiene-3,7-diol			X*	
3,7-dimethyl-1,7-octadiene-3,6-diol			X*	
benzaldehyde			X	
benzyl alcohol			X	
3-methyl-1-butanol			X	

*Compound identified on a DB5 fused silica capillary column.

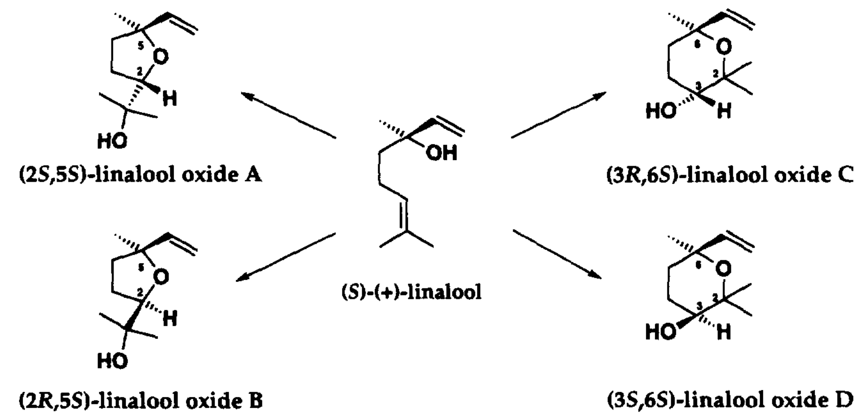


Fig. 2. (*S*)-(+)-Linalool and the corresponding linalool oxides. A = (2*S*,5*S*)-linalool oxide A; B = (2*R*,5*S*)-linalool oxide B; C = (3*R*,6*S*)-linalool oxide C; D = (3*S*,6*S*)-linalool oxide D, following the nomenclature in the Chemical Abstract indices. For full names see Table 1.

Table 2. The attraction of four early flying solitary bee species to scents related to *D. mezereum*

Compound	*Colletes cunicularius* $n = 6$	*Andrena cinerea* $n = 6$	*Andrena haemorrhoa* $n = 6$	*Andrena vaga* $n = 4$
D. mezereum (sorption extract)	X	—	X	—
1% (*S*)-(+)-linalool in hexane	XXX	X	X	—
1% furanoid linalool oxide Mixture in hexane	X	—	—	—
Blank (hexane)	—	—	—	—

n = the number of test occassions. — = no attraction (p in Fig .3), X = weak attraction (1 in Fig. 3), XXX = strong attraction (2 and 3 in Fig. 3).

results]. The bees and the *D. mezereum* shrubs are usually not present in the same, but often in adjacent habitats. However, the insects may fly long distances in their search for food, which may lead *C. cunicularius* to discover and visit the *D. mezereum* populations. These two taxa have (*S*)-(+)-linalool content in common.

The pure (*R*)-(−)-enantiomer of linalool has been found in the floral fragrance of a *Begonia* species from Brazil [13], a taxon which is pollinated by pollen-collecting female bees. Future field studies on hymenopteran insects should focus on the potential differences in attraction between these two enantiomers of linalool, which are present in the fragrances of numerous flowering plants and are possibly formed via partly different biosynthetic pathways.

The observations of flower visitors to *D. mezereum* so far reported are few and scattered and thus it is not clear which insects are its legitimate pollinators [7]. No marked differences between day and night pollination have been observed [7]. This is in agreement with the similarities in day and night scent profiles found in the present study. Thus, no special "chemical" adaptation for example to nocturnal insects such as moths seems to take place. Flower visits by large anthophilous insects such as *Gonepteryx rhamni* and *Bombus* spp. have been reported in several studies [5–7]. Both of these types of insects have long mouthparts suitable for foraging on nectar. Bumble-bee species fly at air temperatures down to 8° but several solitary bee species, such as *Colletes* and *Andrena*, need temperatures above 14° for flying [Borg-Karlson, unpublished results]. Consequently, the possible role of the solitary bees in pollen transfer between *D. mezereum* plants, would be limited to periods of higher air temperature during the flowering season. In addition to lepidopterans and bees, however, many small insects, such as braconid and eucoilid parasitic wasps, have been found as *D. mezereum* flower visitors [7, 14]. Individuals of Heteroptera, Thysanoptera and Acarina have also been found dusted with *D. mezereum* pollen. Most of the pollinations in *D. mezereum* are thus probably the results of geitonogamy via small insects that creep inside the flowers to feed on pollen or simply to use the microclimate for shelter.

A number of early-flowering plants serve as nectar and pollen resources for hibernated bumble-bee queens and newly emerged solitary bees. It has been suggested that the early flowering *Salix* spp. and *D. mezereum* might compete for pollinators [15]. Chemical analyses have revealed the presence of linalool in both female and male flowers in three species of *Salix* [15] and in the fragrance of *D. mezereum* (present work). While *Salix* produces large amounts of nectar and pollen, there is no nectar reward for the visiting insects in the *D. mezereum* flowers in Scandinavia [7, 16]. The light-purple colour of the *D. mezereum* flowers may act as a visual cue for inexperienced bumble-bee queens as found in the pollination of early flowering *Dactylorhiza* species [17]. The floral fragrance of *Salix* contains a complex mixture of aromatic and terpenoid constituents, in which the relative amount of linalool is less than 5% of the total amount measured by GC-mass spectrometry [15]. The female or male insects may visit the *D. mezereum* flowers due to their experience that linalool is associated with nectar-rich plants such as *Salix* spp. However, linalool may also release an innate behaviour of the male insects as it acts as an aggregation pheromone in the nesting areas of bees such as *Colletes cunicularius* [Borg-Karlson *et al.*, unpublished results]. In certain taxa of *Ophrys* (Orchidaceae) the flower scent contains linalool, which acts as a sexual attractant of the males of the pollinating bee species [18]. The summer-flowering Apiaceae such as *Heracleum sibiricum* and *Pastinaca sativa*, which are also frequently visited by solitary bees, bumble-bees and wasps, release linalool in substantial amounts [19].

Linalool seems to be a general attractant of bees [20], acting both as a food attractant and, as regards *Ophrys*, as a sexual attractant of male bees. The linalool oxides do not seem to have an effect in the cross-pollination of *D. mezereum* plants as our topical test sample did not attract the bees. These oxides, however, are major constituents in the fragrance of honeysuckle (*Lonicera* spp.) flowers [Nilsson, unpublished results], which are frequently visited and pollinated by night-flying hawk moths. This suggests a strong biological significance also of the linalool oxides in certain pollination systems.

EXPERIMENTAL

Sorption of flower volatiles. Samples of the flower volatiles of a *Daphne mezereum* plant growing in Österskär (a village situated 30 km north-east of Stockholm, Sweden) were collected during three flowering seasons, 1991, 1992 and 1994. Remaining on the shrub, seven branches carrying 20–30 flowers each were enclosed in oven-proof plastic bags (polyethylene terephthalate, Look, Terinex Ltd, U.K.). The volatiles released from the inflorescences were trapped in glass tubes (5 × 40 mm) filled with Porapak Q (100 mg 80/100 mesh) through suction (60 ml air/min for 8 hr), using a DuPont Sipin 125 battery-driven pump [18]. The outdoor air temperature during the collection varied between 5 and 20°. After the enrichment period, the sorption material was rinsed in two steps, first with 0.5 ml and then with 1.5 ml of pentane (Merck p.a. grade). Each of these solns was analysed without previous concn.

Identification of flower volatiles by GC-MS. The compounds were identified by means of one Finnigan 4500 and one SSQ 7000 GC-MS instrument, both with a Varian 3400 GC, as well as one Fisons MD 800 GC-MS instrument, all three equipped with split/splitless injectors. Fused silica capillary columns—a DB5-ms and a DB-WAX (both 30 m × 0.25 mm, film thickness 0.25 mm, J&W Scientific)—were used. The temperature programme for the DB-WAX column started at 40° for 4 min, followed by a 4° min^{-1} increase to 200°. The programme for the DB5 column started with 1 min at 35°, followed by a rapid increase by 50° min^{-1} to 45°, isothermal for 5 min, followed by an increase of 3° min^{-1} to 220°. The detector temperature was 200°. The MS data were compared with those of authentic compounds or with literature data (GC-MS library).

Determination of the chirality of the fragrance constituent linalool. The enantiomers of racemic linalool were separated by GC using a Pye-Unicam GCV instrument, equipped with a permethylated β-cyclodextrin column (50 m × 0.25 mm id and 0.25 mm film thickness, Chrompack). The sepns were performed under isothermal conditions at 100° with an injector temperature of 180° and a detector temperature of 200°. The elution order of the linalool enantiomers was determined as (*R*)-(−)-; (*S*)-(+)-, using the pure (*R*)-(−)-enantiomer as standard. The chirality of the fragrance constituent linalool in *D. mezereum* was then determined by comparison of the retention times and by co-chromatography with the racemic linalool sample.

Synthesis of furanoid and pyranoid linalool oxides. Racemic linalool was obtained from Dragoco GRB and (*R*)-(−)-linalool from our laboratory. In separate experiments, racemic linalool and (*R*)-(−)-linalool were treated with *m*-chloroperbenzoic acid (MCPBA) in methylene chloride at room temperature [21]. Under the reaction conditions employed, the epoxide ring first formed subsequently underwent ring-opening by the neighbouring hydroxyl group and the linalool oxides were formed. The furanoid oxides were the major products (furanoid: pyranoid ratio = 4:1). The configuration at the chiral centre bearing the hydroxyl group is retained during the ring-closing step, i.e. only the four isomers A, B, C, D, shown in Fig. 2, are formed from (*S*)-(+)-linalool. The antipodes of these four compounds resulted from the reaction of the pure *R*-(−)-linalool. Liquid chromatography (LC), performed according to Baeckström *et al.* [22], was used to separate the furanoid oxides from the pyranoid oxides and the *trans*-forms from the *cis*-forms. Thus, we had the 2*R*,5*R*-,2*S*,5*R*-furanoid and 3*S*,6*R*-,3*R*,6*R*-pyranoid isomers sepd from each other, which enabled us to determine the retention order for the two enantiomers of each linalool oxide separately.

The NMR spectra (400 MHz) of $CDCl_3$ solutions of the furanoid oxides were compared with the thoroughly assigned NMR-spectra published by Askari and Mosandl [23]. The *cis*-form is eluted later than the *trans*-form from the LC. The configurations assigned of the *cis*- and *trans*-pyranoid oxides were then confirmed by means of reference samples kindly supplied by Dr Mikael Lindström, Firmenich SA.

Determination of the chirality of the linalool oxides. The enantiomeric purities of the synthetic linalool oxides were determined on the permethylated β-cyclodextrin column used for the separation of the linalool enantiomers, isothermally at 110°. By injecting four furanoid linalool oxides obtained in the synthesis from racemic linalool and then coinjecting the two furanoid 2*R*,5*R*- and 2*S*,5*R*-isomers obtained from oxidation of (*R*)-(−)-linalool, the elution order of the furanoid linalool oxides could be confirmed as 2*R*,5*R*; 2*S*,5*S*; 2*S*,5*R* and 2*R*,5*S* according to the IUPAC numbering shown in Fig. 2 and Table 1. The elution order agreed with the one published by Askari and Mosandl [23]. The elution order of the linalool oxides of the pyranoid type was determined in the same manner and on the same type of column and was found to be 3*R*,6*S*; 3*S*,6*R*; 3*S*,6*S*; 3*R*,6*R*. The enantiomeric purities of the linalool oxides present in the samples from *D. mezereum* were determined by two-dimensional GC, using a method described earlier [24]. A DB-WAX column was used for the non-chiral sepn with the temperature programme from 40° (2 min) to 220°, rate $10° \min^{-1}$. A Cyclodex B column (30 m × 0.25 mm id, 0.25 mm film thickness, J&W Scientific) was used for the chiral analyses of the linalool oxides in the 2D-GC (80° isothermally for the furanoids and 100° isothermally for the pyranoids). The linalool oxides present in the fragrance from *D. mezereum* were coinjected with those of synthesized reference compounds (see above) and were found to be the ones depicted in Fig. 2.

Behaviour tests. Behaviour tests in the field were performed during the flowering season of *D. mezereum* from mid-April to early May 1992, 1993 and 1994 at Margretelund and Österskär, north-east of Stockholm, Uppland. The following four odour samples were presented to the males of four of the earliest flying solitary bee species in Sweden, *Colletes cunicularius*, *Andrena cinerea*, *A. haemorrhoa*, and *A. vaga*: 1. a sorption extract of *D. mezereum* flowers; 2. a 1% solution of *S*-(+)-linalool in hexane; 3. a 1% solution of a mixture of the racemates of the two furanoid linalool oxides in hexane; 4. a sample of the solvent hexane (as the blank). Samples of each test solution (50 ml) were applied on separate dispensers (5 × 8 mm pieces of black velvet fixed on insect pins) and placed in the mating flight areas of the male bees. The samples were tested one by one in a randomized order. Each test lasted for 5 min. The quantity and quality of the behaviour of the approaching males were classified with respect to the closeness to the odour source, the intensity in flight oscillation and the duration of the behaviour (see Fig. 3) [18,25]. χ^2 tests were used for evaluation of the test data [26].

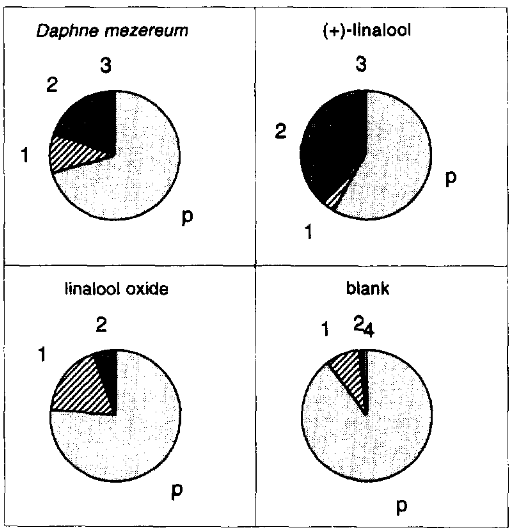


Fig. 3. The attraction of male *C. cunicularius* bees to sorption extract of *D. mezereum*, 1% (*S*)-(+)-linalool in hexane, a mixture of linalool oxides and a blank (the solvent hexane). The behaviour of the bees, in front of the scented dummies, is divided into five categories: p, 1, 2, 3 and 4 listed in increasing order of attraction. p = passage, 1 = quick inspection, 2 = hovering inspection, 3 = circling inspection, 4 = quick visit on the dummy. The cases of behaviour indicating low attraction (1 + p) are summarized and compared with the sum of the case of high attraction (2 + 3 + 4) for each sample and treated statistically by using the X^2-test.

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