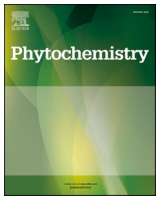
Phytochemistry 224 (2024) 114142

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Chemical imitation of yeast fermentation by the drosophilid-pollinated deceptive trap-flower *Aristolochia baetica* (Aristolochiaceae)

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ARTICLE INFO

Keywords:
Aristolochia baetica
Aristolochiaceae
Drosophilidae
Phoridae
Floral scents
Deceptive pollination
Chemical mimicry
Stereochemistry
Electroantennography
Acetoin

ABSTRACT

Deceptive flowers, unlike in mutualistic pollination systems, mislead their pollinators by advertising rewards which ultimately are not provided. Although our understanding of deceptive pollination systems increased in recent years, the attractive signals and deceptive strategies in the majority of species remain unknown. This is also true for the genus *Aristolochia,* famous for its deceptive and fly-pollinated trap flowers. Representatives of this genus were generally assumed to be oviposition-site mimics, imitating vertebrate carrion or mushrooms. However, recent studies found a broader spectrum of strategies, including kleptomyiophily and imitation of invertebrate carrion. A different deceptive strategy is presented here for the western Mediterranean *Aristolochia baetica* L. We found that this species is mostly pollinated by drosophilid flies (Drosophilidae, mostly *Drosophila* spp.), which typically feed on fermenting fruit infested by yeasts. The flowers of *A. baetica* emitted mostly typical yeast volatiles, predominantly the aliphatic compounds acetoin and 2,3-butandiol, and derived acetates, as well as the aromatic compound 2-phenylethanol. Analyses of the absolute configurations of the chiral volatiles revealed weakly (acetoin, 2,3-butanediol) to strongly (mono- and diacetates) biased stereoisomer-ratios. Electrophysiological (GC-EAD) experiments and lab bioassays demonstrated that most of the floral volatiles, although not all stereoisomers of chiral compounds, were physiologically active and attractive in drosophilid pollinators; a synthetic mixture thereof successfully attracted them in field and lab bioassays. We conclude that *A. baetica* chemically mimics yeast fermentation to deceive its pollinators. This deceptive strategy (scent chemistry, pollinators, trapping function) is also known from more distantly related plants, such as *Arum palaestinum* Boiss. (Araceae) and *Ceropegia* spp. (Apocynaceae), suggesting convergent evolution. In contrast to other studies working on floral scents in plants imitating breeding sites, the present study considered the absolute configuration of chiral compounds.

1. Introduction

Relationships between flowers and pollinators are famous examples for mutualisms in ecology, however, approximately 4–6 % of flowering plant species are deceptive (Renner, 2006). They advertise a reward that they do not provide. Many deceptive flowers have evolved sophisticated

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https://doi.org/10.1016/j.phytochem.2024.114142
Received 6 February 2024; Received in revised form 8 May 2024; Accepted 12 May 2024
Available online 16 May 2024

strategies to target a narrow spectrum of pollinator taxa. This is achieved by mimicking indispensable resources based on a combination of olfactory, visual, and tactile signals, exploiting learned or innate preferences of pollinators (Johnson and Schiestl, 2016).

The most widespread deceptive pollination system is oviposition-site mimicry, which is assumed to occur in thousands of plant species across a wide range of families in different lineages (Johnson and Schiestl, 2016; Jürgens and Shuttleworth, 2015; Urru et al., 2011). It is also the most diverse mimicry strategy in terms of imitated substrates, such as carrion (e.g. Stensmyr et al., 2002; van der Niet et al., 2011; Jürgens et al., 2013), feces (e.g. Johnson and Jürgens, 2010; Johnson et al., 2020; Sayers et al., 2020), mushrooms (e.g. Kaiser, 2006; Policha et al., 2016; Kakishima and Okuyama, 2020), rotting and fermenting fruits (Goodrich et al., 2006; Goodrich and Raguso, 2009; Procheş and Johnson, 2009; Stökl et al., 2010), or a combination of several breeding substrates (Gfrerer et al., 2021). Insects seeking such generally ephemeral substrates mostly rely on olfactory cues to locate them efficiently (Brodie et al., 2014; Cossé and Baker, 1996; Frank et al., 2018; Frederickx et al., 2012; Goodrich and Jürgens, 2018; Keesey et al., 2015; Zito et al., 2014). Those cues are exploited by oviposition-site mimics to dupe typically flies and/or beetles as pollinators (du Plessis et al., 2018; Jürgens et al., 2013; Martos et al., 2015; Stökl et al., 2010).

In recent years, the knowledge about chemical signaling in (supposedly) oviposition-site mimicking systems is constantly increasing (Goodrich and Jürgens, 2018; Jürgens et al., 2013; Kite and Hetterscheid, 2017; Stensmyr et al., 2002), however, the attractive signals and deceptive strategies still largely lack experimental chemo-ecological evidence (but see, e.g. Stökl et al., 2010; Martos et al., 2015).

This is also true for *Aristolochia* (Aristolochiaceae), renowned for their spectacular trap-flowers. So far known, all species are fly-pollinated, including various dipteran families, such as Phoridae, Chloropidae, Muscidae, Drosophilidae and Ceratopogonidae (reviewed by Berjano et al., 2009). As in most fly-pollinated deceptive plants, the pollinator spectra of *Aristolochia* species are largely unexplored at the genus/species level (Woodcock et al., 2014; Karremans and Díaz-Morales, 2019, but see e.g. Bänziger and Disney, 2006; Oelschlägel et al., 2015; Heiduk et al., 2017; Policha et al., 2019). However, knowing the individual pollinators' identities and life histories is essential and a key information for understanding a flower's deceptive strategy. Apart from a few exceptions, where flowers provide true breeding substrates and often lack trap-and-release mechanisms (*Aristolochia inflata* Kunth, *A. labiata* Willd., *A. manshuriensis* Kom., *A. maxima* Jacq.; Disney and Sakai, 2001; Hime and Costa, 1985; Nakonechnaya et al., 2021), *Aristolochia* species are widely regarded to be sapromyiophilous and mimic oviposition-sites of their fly pollinators, such as vertebrate carrion or mushrooms (e.g. Vogel, 1978; Johnson and Jürgens, 2010); however, chemical-ecological evidence is still scarce. To date, floral scents of only seven out of the ca. 500 *Aristolochia* species (*A. bianorii* Sennen & Pau, *A. cymbifera* Mart., *A. fimbriata* Cham., *A. gigantea* Mart. & Zucc., *A. microstoma* Boiss. & Spruner, *A. ringens* Vahl, *A. rotunda* L.) were studied using quantitative chemical analytical techniques (Alpuente et al., 2023; Johnson and Jürgens, 2010; Martin et al., 2017; Oelschlägel et al., 2015; Qin et al., 2021; Rupp et al., 2021; Stashenko et al., 2009). These studies found various scent blends with volatiles characteristic of sapromyiophilous flowers (e.g., dimethyldisulfide) and also larger amounts of e.g., citronella-like compounds (*A. gigantea*), pyrazines (*A. microstoma*) or aliphatic esters (*A. rotunda*, *A. bianorii*), pointing to different deceptive strategies. So far, however, studies experimentally testing the deceptive strategies and determining the attractive signals are restricted to a single species, the Mediterranean *A. rotunda*, where a novel pollination strategy exploiting kleptoparasitic chloropid flies (kleptomyiophly) was discovered (Oelschlägel et al., 2015). Some weakly scented (to the human nose) *Aristolochia* species with strong male sex-bias in pollinators were suggested to mimic female sex pheromones of flies (Hall and Brown, 1993; Rulik et al., 2008). Other species, such as *A. baetica* L., *A. fimbriata*, *A. macrophylla* Lam., and *A. maxima* Jacq. are predominantly pollinated by drosophilids, some of them to a lesser degree additionally by phorids (*Megaselia* spp. in *A. baetica*), which are presumably the most widespread pollinators among *Aristolochia* species worldwide (Vogel, 1965, 1978; Sakai, 2002; review in Berjano et al., 2009). In contrast to phorids, where many species are carrion-associated (Disney, 1994), drosophilids are not typical carrion flies, but most prominently feed on fermenting fruits, yeasts, or mushrooms. Therefore, these flowers are unlikely to be sapromyiophilous, and instead might imitate other fermenting substrates by emitting yeasty scents, as hypothesized for *A. fimbriata* and *A. macrophylla* (Vogel, 1965, 1978). Pollination by drosophilids is generally rare in rewarding systems (Larson et al., 2001), restricted mostly to highly specialized mutualistic systems (Fu et al., 2016; Miyake and Yafuso, 2005; Nakonechnaya et al., 2021; Sultana et al., 2006). In deceptive systems, however, pollination by drosophilids is found in several plant families, and is probably not scarce, especially in the species-rich orchid subtribe Pleurothallidinae (Karremans and Díaz-Morales, 2019). However, plants pollinated by drosophilids have rarely been studied in terms of attractive signals and deceptive strategies. So far, three strategies were identified by chemical-ecological methods among deceptive flowers that target drosophilids as pollinators: 1) mimicry of yeast-fermenting plant material (Araceae: *Anthurium* spp. and *Arum palaestinum* Boiss., Schwerdtfeger et al., 2002; Stökl et al., 2010; Apocynaceae: *Ceropegia* spp., Heiduk et al., 2017; Orchidaceae: *Gastrodia similis* Bosser, Martos et al., 2015); 2) mimicry of mushrooms (Orchidaceae: *Dracula* spp. and *Malaxis monophyllos* (L.) Sw., Policha et al., 2016, 2019; Jermakowicz et al., 2022; Araceae: *Arisaema sikokianum* Franch. & Sav., Kakishima et al., 2019); and 3) mimicry of drosophilid aggregation pheromones (Orchidaceae: *Specklinia* spp., Karremans et al., 2015).

In the present study, we characterized and identified flower visitors and pollinators of the drosophilid-pollinated *A. baetica*. We analysed the floral scents by dynamic headspace methods and (chiral) gas chromatography-mass spectrometry (GC-MS), performed synthetic chemistry, electroantennographic measurements (GC-EAD) as well as bioassays with synthetic floral scents to determine the physiologically and behaviorally active floral scent compounds. Specifically, we asked: 1) Which species and sexes of drosophilids are pollinating *A. baetica*? 2) Which floral volatiles does *A. baetica* emit and how similar is its floral scent bouquet to the scents of potential models mimicked, to other *Aristolochia* species and to brood-site deceptive plants, based on literature data? 3) What is the absolute configuration of chiral compounds of *A. baetica*? 4) Which of the volatile compounds contribute to pollinator attraction? Answering those questions will allow us to determine whether *A. baetica* utilizes a deceptive strategy known from other drosophilid-pollinated flowers or whether it deploys a yet undiscovered strategy.

2. Results

2.1. Flower visitors and pollinators

Across both sites (Aznalcázar and Membrillo, Spain), we collected 2,187 flower visitors, of which 1,325 were found in female-phase, and 862 in male-phase flowers (Supplementary Table S1). The utricles of the flowers harbored a diverse spectrum of visitors, representing taxa from eight different insect orders, as well as occasional spiders, mites, and millipedes.

The overwhelming majority belonged to Diptera (2,065 specimens), mostly Drosophilidae (1,377) and Phoridae (529), and in lower abundances to Sciaridae (32), Scatopsidae (28), and 18 further dipteran families with less than 10 individuals each (Supplementary Table S1).

Among all flower visitors, 363 insects, exclusively Diptera, were found carrying pollen in female-phase flowers, and were thus categorized as pollinators given that *Aristolochia* flowers are proterogynous (Table 1). Pollen loads were typically attached dorsally on the thorax (Fig. 1B). Most of the pollinators were Drosophilidae (93 %), with an

Table 1
Pollinators (specimens that carried pollen in female-phase flowers) of *Aristolochia baetica* at two sites in southern Spain (Aznalcázar; Membrillo). So far identified, the species and sexes are given. For a list of all flower visitors see Supplementary Table S1.

Family	Species	Total	Aznalcázar	Membrillo
Asteiidae	*Asteia amoena* Meigen, 1830	3	2♂, 1♀	
Chloropidae	*Thaumatomyia notata* (Meigen, 1830)	3	1♀, 1	1♂
Drosophilidae	*Drosophila busckii* Coquillett, 1901	8	3♀	4♂, 1♀
	D. hydei Sturtevant, 1921	8	1♂, 3♀	1♂, 3♀
	D. immigrans Sturtevant, 1921	12	1♂, 4♀	4♂, 3♀
	D. melanogaster Meigen, 1830	16	1♂, 7♀	1♂, 7♀
	D. simulans Sturtevant, 1919	118	21♂, 16♀	41♂, 40♀
	D. subobscura Collin in Gordon (1936)	72	20♂, 11♀	28♂, 13♀
	D. suzukii Matsumura, 1931	90	16♂, 24♀	18♂, 32♀
	D. testacea Roser, 1840	1		1♀
	Hirtodrosophila cameraria (Haliday, 1833)	4	2♂, 2♀	
	Phortica variegata (Fallén, 1823)	3		3♀
	Scaptodrosophila rufifrons (Loew, 1873)	1		1♂
	Scaptomyza pallida (Zetterstedt, 1847)	3		1♂, 2♀
Heleomyzidae	*Trixoscelis* sp.	1	1♂	
Milichiidae	*Desmometopa sordida* (Fallén, 1820)	1		1♀
	Neophyllomyza acyglossa (Villeneuve, 1920)	1		1♀
Odiniidae		2		2
Phoridae		15	3♂, 5♀	5♀, 2
Scatopsidae	*Coboldia fuscipes* (Meigen, 1830)	1		1♂

overall balanced sex ratio (Table 1). The most frequent pollinators were *Drosophila* species, mostly *D. simulans*, *D. suzukii* and *D. subobscura*, as well as five further species in lower abundances. The remaining pollinators were drosophilids of the genera *Hirtodrosophila*, *Phortica*, *Scaptodrosophila* and *Scaptomyza*, phorids (9 females, 3 males, 3 unknown sex), and six other fly families in low numbers (Table 1, Supplementary Table S2).

Among the insects collected from male-phase flowers, 471 specimens carried pollen, thus being potential pollinators (Supplementary Table S1). Again, most of them were drosophilids (73.5 %), followed by phorids (16.1 %) and other Diptera (9.5 %).

The proportion of individuals carrying pollen was higher in drosophilids than in phorids, both in female-phase ($\chi^2 = 134.27$, $df = 1$, $P < 0.001$) and in male-phase flowers ($\chi^2 = 59.72$, $df = 1$, $P < 0.001$). However, this difference was more than four times higher in the female (39 % vs. 5 %) than in the male-phase (68 % vs. 37 %) flowers.

2.2. Floral scents

The floral scent of *A. baetica* is perceived as 'yeasty' by the human nose, reminiscent of fermenting fruit. Chemical analyses of the thermal desorption (TD) samples revealed that the absolute amount of scent released by female-phase flowers ranged from 4 to 1,070 ng/h (mean = 251 ng/h). A total of 34 different volatiles (including stereoisomers; Fig. 2) were recorded across the samples (Table 2; Supplementary Table S3), with only two compounds (acetoin acetate, tiglic aldehyde) occurring in all samples. As visualized in Fig. 3, the qualitative scent pattern of *A. baetica* is most similar to yeast-fermenting substrates (e.g. peach, grape, vinegar, yeast), other drosophilid-pollinated deceptive flowers (Araceae: *Arum palaestinum*, *Anthurium hookeri* Kunth; Apocynaceae: *Ceropegia rupicola* Deflers, *C. crassifolia* Schltr.), and the beetle-pollinated *Calycanthus occidentalis* Hook. & Arn. (Calycanthaceae). Characteristic compounds of this group are acetoin, acetoin acetate and 3-methyl-1-butanol.

There was obvious variation in the relative amounts of scent compounds among individuals of *A. baetica* (Table 2), which was due to variation within populations and not between the two populations (ANOSIM: $R = 0.13$, $P = 0.08$). Overall, the most abundant volatiles were acetoin, 2,3-butanediol monoacetate, acetoin acetate, and (in Aznalcázar) 2-phenylethanol. Other compounds that contributed high relative amounts (>10 %) in at least one sample were 2,3-butanedione, ethyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol, tiglic aldehyde and two unknown compounds (unk_1027, unk_1396) (Table 2). Many of these compounds are chiral, generally existing in two (acetoin, acetoin acetate, 2-methyl-1-butanol), three (2,3-butanediol, 2,3-butanediol diacetate) or four (2,3-butanediol monoacetate) stereoisomers. As determined in the solvent acetone (SA) samples by enantioselective GC-MS, the flowers released overall, but not in all samples, all possible

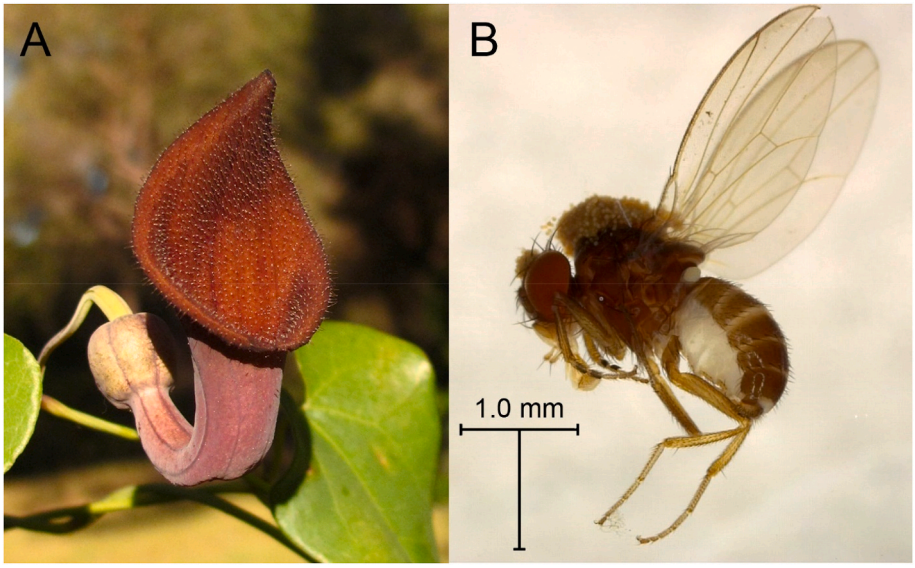

Fig. 1. (A) Trap-flower of *Aristolochia baetica* (Aristolochiaceae) photographed at Aznalcázar, southern Spain, and (B) a male specimen of its frequent pollinator species *Drosophila subobscura* (Diptera: Drosophilidae) collected from a flower utricle, carrying a typical pollen load predominantly on its thorax.

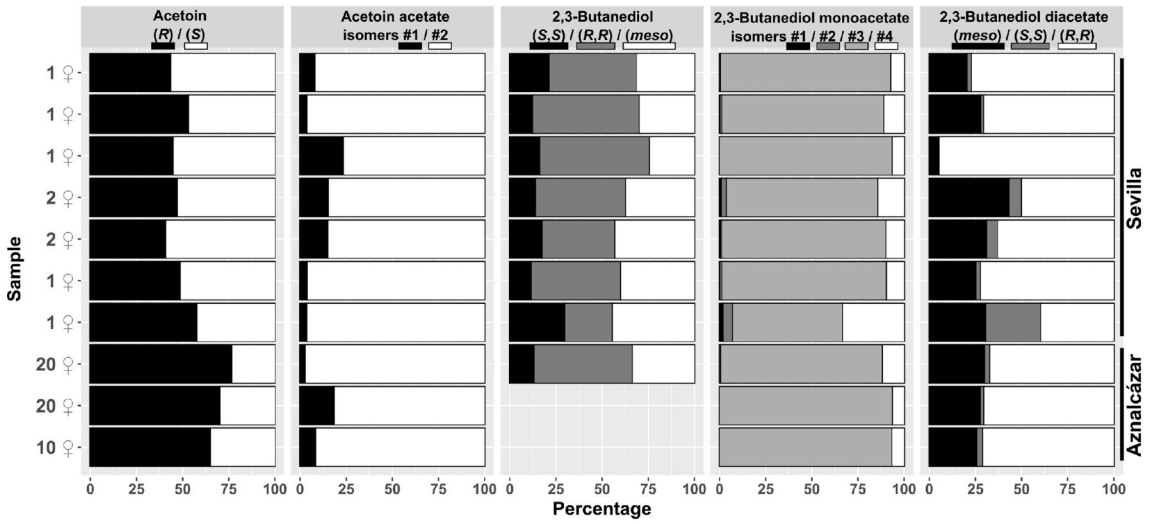

Fig. 2. Absolute configuration (relative amounts in %) of acetoin, 2,3-butanediol (which did not occur in two of the samples) and related acetates in 10 floral scent samples of *Aristolochia baetica*, identified by chiral GC-MS in dynamic headspace samples (solvent acetone; SA). In acetoin acetate and 2,3-butanediol monoacetate, the separated isomers could not be assigned to specific stereoisomers and are therefore numbered and sorted according to their retention times on a chiral fused silica capillary column (30 % DIME-β-CD in 70 % SE-52). Each line represents a sample, with the number of female-phase flowers (♀) pooled to obtain a sample, and the collection site in southern Spain indicated.

stereoisomers of these compounds (Fig. 2). An exception was 2-methyl-1-butanol, as it was only present in the (*S*)-configuration. The absolute configurations of acetoin, 2,3-butanediol, and their related mono- and diacetates were not racemic, but weakly (acetoin) to strongly (other compounds, Fig. 2) biased. Acetoin acetate, 2,3-butanediol monoacetate and 2,3-butanediol diacetate were (strongly) dominated by a single stereoisomer. In 2,3-butanediol, the (2*R*,3*R*)- and (2*S*,3*S*)-stereoisomers, with very few exceptions, were more dominant than the (*meso*)-form.

2.3. GC-EAD

Enantioselective GC-EAD experiments showed that most of the floral scent compounds identified in *A. baetica* elicited physiological responses in the antennae of *Drosophila simulans*, one of the most frequent pollinators (Table 3, Fig. 4). Overall, we found 18 EAD-active compounds, of which six elicited responses in all tested individuals of both sexes [(*S*)-acetoin, acetoin acetate (both stereoisomers), 2,3-butanediol monoacetate stereoisomer #3, 2-phenylethanol, *β*-citronellol (only two tested individuals)]. At least four further compounds were EAD-active in over 50 % of individuals [(2*S*,3*S*)-butanediol, (2*S*,3*S*)- and (2*R*,3*R*)-butanediol diacetate, 2-phenylethyl acetate)]. Some compounds (e.g., 2-methylpropyl acetate, tiglic aldehyde, 2-phenylethyl formate) were only EAD-active in single individuals, and others (ethyl acetate, 3-methylbutyl acetate) only in male, but not female flies. We discovered stereospecific antennal responses in the chiral compounds acetoin, 2,3-butanediol, 2,3-butanediol mono- and -diacetate. Here, the flies responded only to some, but not all of the different stereoisomers. For example, (*S*)-acetoin elicited strong antennal responses in all individuals (Fig. 4), whereas (*R*)-acetoin was never EAD-active (Table 3). In acetoin acetate, in contrast, both stereoisomers triggered strong antennal responses in both sexes (Fig. 4). The (2*S*,3*S*)-stereoisomer of 2,3-butanediol was EAD-active in over 50 % of individuals, but the (2*R*,3*R*)-stereoisomer only in a single female. In 2,3-butanediol monoacetate, all tested flies responded strongly to stereoisomer #3, but never to stereoisomer #4, whereas we could not differentiate between the responses to stereoisomers #1 and #2 as they had very similar retention times. Preliminary tests with four other drosophilid pollinators (*Drosophila* spp., *Scaptomyza pallida*) and a non-pollinating flower-visitor (*Drosophila repleta*) (Supplementary Table S4) suggest that they generally respond similar to the scent compounds of *A. baetica* as *D. simulans*. It seems, however, that female *D. repleta* strongly responds to (*R*)-acetoin (Supplementary Table S4).

2.4. Field bioassays

In Aznalcázar (n = 30 traps) as well as in the Botanical Garden of Salzburg (n = 48 traps) synthetic mixtures of floral scents (Mix2, Mix3; see sections 5.9 and 5.10) very specifically attracted female and male Drosophilidae (Aznalcázar: n = 4; Salzburg: n = 41) and Phoridae (Aznalcázar: n = 3; Salzburg: n = 11), and only exceptionally other insects (Table 4). No drosophilids, but single individuals of Phoridae, Heleomyzidae and Sciaridae responded to acetone negative controls. The attracted drosophilids included the three main pollinator species (*D. simulans*, *D. suzukii*, *D. subobscura*), as well as *D. melanogaster* and *Hirtodrosophila cameraria*. There was no obvious sex-bias in the attracted flies. In the bioassays performed in the natural habitat in Aznalcázar, all attracted drosophilids carried pollen dorsally on their thoraces, resembling *Aristolochia*-pollen in morphology and placement. At the study site in Salzburg, two further *Drosophila* species not recorded from the flowers were attracted to the synthetic scent mixtures (*D. kuntzei*, *D. phalerata*).

2.5. Lab bioassays

Two-choice experiments with custom-made traps (see section 5.11) revealed that the scent of banana, the synthetic complete mixture (Mix4) as well as most single floral scent compounds and combinations thereof were attractive to *Drosophila simulans* flies (Fig. 5). Only 2-phenylethanol, *β*-citronellol, as well as (2*S*,3*S*)- and (2*R*,3*R*)-butanediol diacetate were neutral to the flies. Several compounds were as attractive as the complete mixture, such as acetoin (rac) and the mixture of 2,3-butanediol mono- and diacetate (Fig. 5). Stereoisomer-specific differences in attractiveness were found in 2,3-butanediol, where the (*meso*)- and (3*R*,3*R*)-stereoisomers were less attractive than the complete mixture, whereas the (2*S*,3*S*)-stereoisomer and the racemate were not. Banana (positive control) was more attractive than the complete mixture (Mann-Whitney-U-Test: $Z = 3.73$, $P < 0.001$).

3. Discussion

We found that *A. baetica* is predominantly pollinated by male and female drosophilids (mostly *Drosophila* spp.), and to a lesser extent by

Table 2
Floral scent of *Aristolochia baetica* [dynamic headspace, thermal desorption (TD) samples]. Total absolute (ng/h) and relative (%) amounts of scent (compounds) emitted by single female-phase flowers at two natural sites in Spain (Aznalcázar; Membrillo). The compounds are sorted by chemical class and within class by linear retention index (RI) on a ZB-5 fused silica column. The identities of all identified compounds were verified with authentic standards. The scent compounds found in the single samples and the mass-to-charge ratios (m/z; six most abundant fragments) of the unknown compounds are provided in Supplementary Table S3. Trace values (<0.05 %) are given as 'tr'.

RI		Aznalcázar (n = 7)		Membrillo (n = 9)	
	Compound class/ compound	Median relative amount (min - max) [%]			
	Aliphatic compounds				
576	2,3-Butanedione	0.0	(0.0–23.0)	9.2	(0.0–47.8)
606	Ethyl acetate	0.0	(0.0–0.0)	0.0	(0.0–36.1)
708	Acetoin	39.1	(0.0–51.2)	13.7	(4.8–53.4)
772	2-Methylpropyl acetate	0.0	(0.0 - tr)	0.0	(0.0–0.2)
774	(2*R*,3*R*)-/(2*S*,3*S*)-Butanediol	0.0	(0.0–3.8)	1.5	(tr - 7.9)
785	(*meso*)-2,3-Butanediol	0.0	(0.0–1.8)	tr	(0.0–2.3)
890	Acetoin acetate	8.1	(tr - 13.9)	5.5	(2.3–15.5)
925	2,3-Butanediol monoacetate stereoisomer(s)	10.8	(0.0–28.2)	19.5	(1.8–38.9)
932	2,3-Butanediol monoacetate stereoisomer(s)	0.9	(0.0–1.7)	0.3	(0.0–1.7)
1057	(*meso*)-2,3-Butanediol diacetate	0.0	(0.0–1.0)	0.0	(0.0–0.1)
1070	(2*R*,3*R*)-/(2*S*,3*S*)-Butanediol diacetate	0.0	(0.0–2.2)	0.7	(0.0–1.4)
	C5-branched chain compounds				
731	3-Methyl-1-butanol	3.1	(tr - 18.3)	tr	(0.0–9.9)
735	2-Methyl-1-butanol	3.2	(0.0–23.8)	1.5	(0.0–27.4)
741	Tiglic aldehyde	1.6	(tr - 9.9)	1.9	(0.1–15.5)
876	3-Methylbutyl acetate	0.6	(0.0–18.4)	0.0	(0.0–2.5)
	Aromatic compounds				
1119	2-Phenylethanol	12.6	(0.0–46.1)	0.0	(0.0–14.1)
1183	2-Phenylethyl formate	tr	(0.0–0.6)	0.0	(0.0–0.1)
1263	2-Phenylethyl acetate	0.0	(0.0–10.0)	0.0	(0.0–0.9)
	Terpenoids				
1230	*β*-Citronellol	tr	(0.0–4.9)	tr	(0.0–13.8)
	Unknown compounds				
911	unk_911	0.0	(0.0–0.1)	0.0	(0.0–0.1)
1008	unk_1008	0.0	(0.0–0.2)	tr	(0.0–0.3)
1012	unk_1012	0.0	(0.0–0.4)	0.0	(0.0–0.5)
1027	unk_1027	tr	(0.0–24.6)	0.0	(0.0–0.1)
1154	unk_1154	0.0	(0.0–0.4)	0.0	(0.0–0.1)
1200	unk_1200	0.0	(0.0 - tr)	0.0	(0.0–1.8)
1264	unk_1264	0.0	(0.0–0.0)	0.0	(0.0–9.3)
1396	unk_1396	0.2	(0.0–4.4)	0.0	(0.0–22.0)
1798	unk_1798	0.6	(0.4–5.6)	0.2	(0.0–5.4)
	Total amount of scent per flower (ng/h)	76.7	(15.9–503.2)	76.0	(4.4–1,070.4)

phorids. The flowers emitted a relatively strong scent reminiscent of yeast and fermenting fruit. It was dominated by acetoin, 2,3-butanediol and acetates thereof, as well as by 2-phenylethanol. The absolute configurations of the chiral compounds were weakly to strongly biased. Our electrophysiological and behavioral experiments showed that most of those floral volatiles, but not all stereoisomers of chiral compounds, were physiologically active and attractive to drosophilid pollinators. Altogether, our data evidence that *A. baetica* deceives its pollinators by chemical mimicry of yeast-fermenting fruit.

3.1. Pollinators

We found that the flowers are visited by a diverse assemblage of flies and other arthropod visitors. Thereof, however, they are pollinated by only a small subset of fly taxa, which agrees with studies in other *Aristolochia* species (Berjano et al., 2009; Burgess et al., 2004; Cammerloher, 1933; Hilje, 1984; Rupp et al., 2021). Similar to the results of Berjano et al. (2009), the overall flower visiting fly community in *A. baetica* was strongly dominated by drosophilid flies (Drosophilidae) and to a lesser extent by phorids. Especially phorids, but also drosophilids are known to visit flowers of different *Aristolochia* species around the world, but their contribution to pollination often remains unknown (review in Berjano et al., 2009; Hipólito et al., 2012; Vogel, 1978). In female-phase flowers of *A. baetica* proportionally eight times as many drosophilids carried pollen compared to phorids, but only twice as many in male-phase flowers, when the pollen is released. This suggests that repeated flower visits occur more frequently in drosophilids than in phorids, suggesting that drosophilids are more efficient pollinators. It also indicates that the transfer of pollen to the insect's body is only roughly half as likely in phorids than in drosophilids. As morphological flower traits (i.e. tube diameter and distance between utricle wall to stamens and stigma) define the size of potential pollinators in *Aristolochia* (Brantjes, 1980; Rulik et al., 2008), the generally smaller phorids are probably less effective pollinators than the larger drosophilids in *A. baetica*.

As it was hitherto unknown whether or not the drosophilids (*D. subobscura*, *D. simulans*, *D. phalerata*, and *Scaptomyza pallida*) reported by Berjano (2006) from flowers of *A. baetica* carried pollen, our study for the first time reports confirmed pollinator identities at species level. All the major drosophilid pollinators are cosmopolitan, except for *D. suzukii*, which is a highly invasive, economically important pest introduced to Europe from Southeast Asia (Brake and Bächli, 2008; Cini et al., 2012). Further, both sexes of most of these species are well-known to feed on fermenting fruit and are efficiently attracted by fruit baits (Bächli and Burla, 1985; Otranto et al., 2012). The females of these species additionally oviposit on fermenting or fresh (only *D. suzukii*; Keesey et al., 2015; Cloonan et al., 2018) fruits. Among phorids there are also species in some genera (e.g., *Chonocephalus* and *Megaselia*), whose larvae feed on rotting fruit (Disney, 1994).

Most of the drosophilid pollinator species of *A. baetica* have not been reported from flowers of other *Aristolochia* species, with the exception of *Drosophila simulans* in the mainly phorid-pollinated *A. gigantea* (Hipólito et al., 2012), and *Scaptomyza pallida* in the non-deceptive *A. manshuriensis* (Nakonechnaya et al., 2021). Among other deceptive plants, pollinator species of *A. baetica* are known to be pollinators of the fruit-/fermentation-scented ecotypes of the deceptive Araceae *Arum palaestinum* (discussed in section 3.2) and *Arum orientale* M.Bieb. (*D. subobscura*, *D. busckii*, *D. hydei*), the stapeliad *Orbea schweinfurthii* (A. Berger) Bruyns (*D. immigrans*, *D. simulans*, *D. melanogaster*) (Agnew, 1976; Gibernau et al., 2004), as well as the orchid *Specklinia endotrachys* (Rchb.f.) Pridgeon & M.W.Chase (males and females of *Drosophila hydei, D. immigrans*). This orchid mimics aggregation pheromones of drosophilids (Karremans et al., 2015).

3.2. Floral scents

Most of the floral scent compounds identified in *A. baetica* were not known to occur in *Aristolochia* so far. Only acetoin was reported as a main compound in the floral scent of *A. fimbriata*, also pollinated by drosophilids, without discussing implications for pollination ecology (Qin et al., 2021). A few other compounds occur in minor amounts in *A. microstoma* (3-methyl-1-butanol, 3-methylbutyl acetate), *A. gigantea* (3-methyl-1-butanol, 3-methylbutyl acetate, acetoin, *β*-citronellol) and *A. cymbifera* (2-phenylethanol), all of which are overall dominated by very different compounds associated with different substrates (Johnson and Jürgens, 2010; Martin et al., 2017; Rupp et al., 2021).

All main compounds emitted by *A. baetica* (acetoin, acetoin acetate, 2,3-butanediol monoacetate, 2-phenylethanol), as well as several minor compounds (2,3-butanedione, 2,3-butanediol, 2,3-butanediol diacetate, 2-methylpropyl acetate, ethyl acetate, 3-methyl-1-butanol, 3-methylbutyl acetate, 2-phenylethyl acetate), are characteristic for fermentation

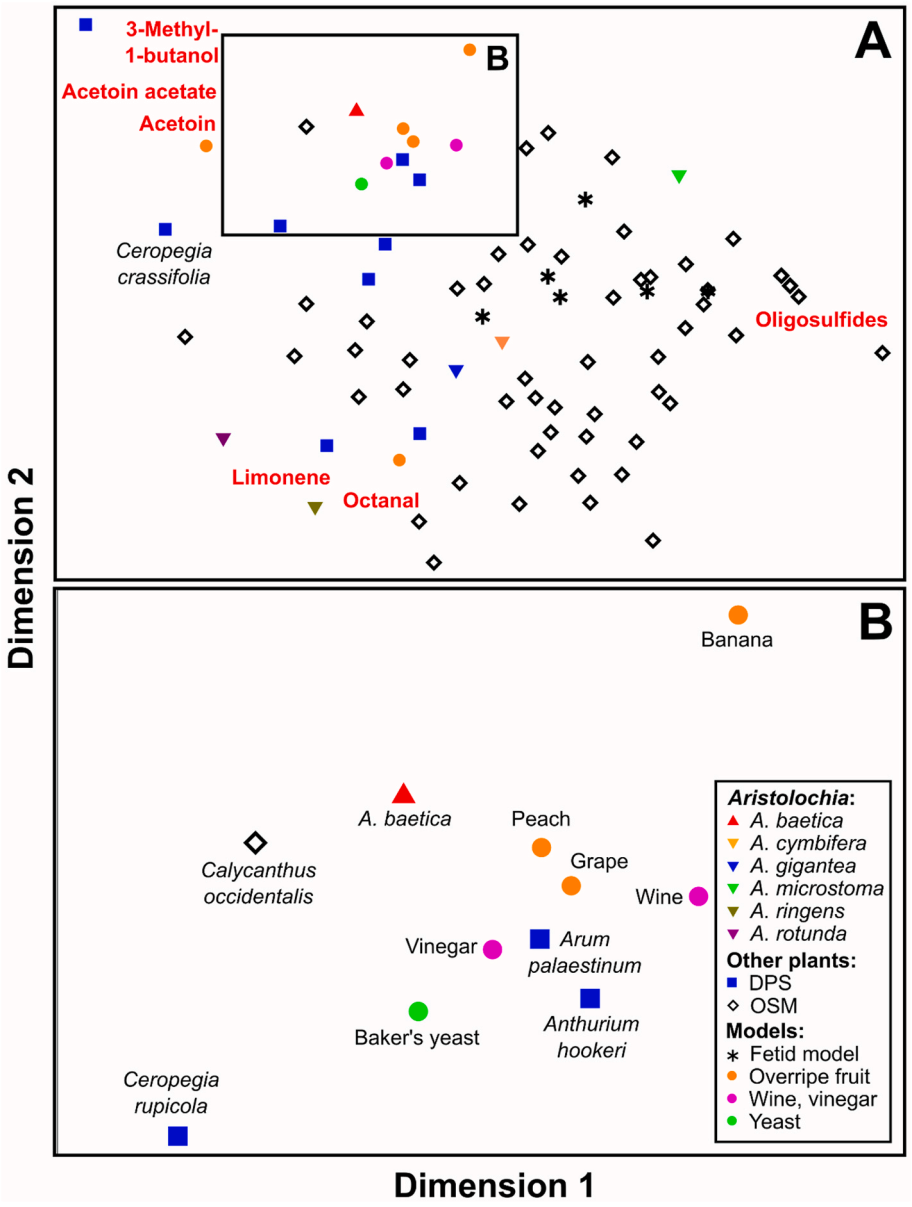

Fig. 3. **A**: Nonmetric multidimensional scaling (NMDS) of the overall scent bouquet of *A. baetica* and of literature data on floral scents in other *Aristolochia* species, other deceptive plants pollinated by drosophilids and other plant species deploying oviposition-site mimicry, and on potential models thereof (fermenting fruit, vinegar and wine, different types of carrion and feces). For more details on the dataset, see section 5.7. Each data point represents a species/model. The compounds most correlating with the NMDS axes are given in red. **B**: Detailed view of the framed section in A. DPS = drosophilid-pollinated deceptive systems; OSM = other oviposition-site-mimicry systems. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of sugar (Xiao and Lu, 2014), known from yeast, fermenting peach, grape, banana, mango and figs, as well as from lambrusco and/or aceto balsamico (Aurore et al., 2011; Bueno et al., 2020; Fischer et al., 2017; Goodrich et al., 2006; Jürgens et al., 2013; Martos et al., 2015; Stökl et al., 2010; Xiao and Lu, 2014). While acetoin and 2,3-butanediol are relatively common in floral scents, their derivatives acetoin acetate, 2, 3-butanediol mono- and diacetates are very rare (Gottsberger et al., 2021; Knudsen et al., 2006; Stökl et al., 2010).

Though we cannot exclude that microorganisms potentially associated with the flowers are responsible for the floral scent emission of *A. baetica*, this is very unlikely given that the yeasty smell of *A. baetica* is only perceived (by the human nose) during the female phase and not anymore during the male phase (Rupp et al., unpublished data).

Many of the aliphatic compounds released by *A. baetica* flowers are chiral, and, for the first time, we determined the absolute stereoisomeric composition of most of those compounds in floral scents. We found that acetoin and 2,3-butanediol have a much less asymmetric stereoisomeric pattern than their acetylated forms, of which especially acetoin acetate and 2,3-butanediol monoacetate were vastly dominated by only one stereoisomer each. This suggests that stereospecific enzymes are involved in the acetylation of acetoin and 2,3-butanediol, whereas the enzymes involved in the production of acetoin and 2,3-butanediol are less stereo-specific. Although generally many floral scent compounds are optically active, only few studies determined the absolute configuration of compounds from floral scents (Dötterl and Gershenzon, 2023). Similar to our study, they found that either only one or few stereoisomers are emitted, or that the flowers release the stereoisomers in similar amounts (Dötterl and Gershenzon, 2023).

Several floral scent volatiles of *A. baetica* are known to attract drosophilid flies feeding on yeast-fermented fruits (e.g., *D. melanogaster, D. suzukii*), including main (acetoin, acetoin acetate, 2,3-butanediol monoacetate, 2-phenylethanol) and minor compounds (2-phenylethyl

Table 3
Antennal responses of male (♂) and female (♀) *Drosophila simulans* (Diptera: Drosophilidae), a frequent pollinator of *Aristolochia baetica*, to floral volatiles of *A. baetica* recorded by enantioselective GC-EAD. The antennae were tested on natural headspace and synthetic scent samples (for details see Supplementary Table S4). Presented is the number of individuals responding to a tested compound, with the number of individuals tested on a specific compound given in superscript. The numbers in the last column refer to the chromatograms (FID) in Fig. 4. The compounds are sorted by chemical class and within class by linear retention index (RI) on a chiral fused silica capillary column (30 % DIME-β-CD in 70 % SE-52). Compounds which elicited antennal responses in at least 50 % of tested individuals are marked in bold. n: total number of individuals tested.

		Drosophila simulans		
		♂	♀	
RI		n = 7	n = 5	no. in Fig. 4
	Aliphatic compounds			
<700	**Ethyl acetate**	$4^{(5)}$	$0^{(3)}$	1
801	2-Methylpropyl acetate	$0^{(2)}$	$1^{(3)}$	–
814	(*R*)-Acetoin	$0^{(7)}$	$0^{(5)}$	2
858	**(*S*)-Acetoin**	$7^{(7)}$	$5^{(5)}$	3
947	**Acetoin acetate #1**	$7^{(7)}$	$5^{(5)}$	4
959	**Acetoin acetate #2**	$7^{(7)}$	$5^{(5)}$	5
1005	**(2*S*,3*S*)-Butanediol**	$3^{(6)}$	$3^{(5)}$	6
1021	(2*R*,3*R*)-Butanediol	$0^{(6)}$	$1^{(5)}$	7
1040	(*meso*)-2,3-Butanediol [a]	$0^{(2)}$	$0^{(2)}$	–
1040 to 1071[a]	**(*meso*)-2,3-Butanediol + 2,3-Butanediol monoacetate #1 + 2,3-Butanediol monoacetate #2 + (*meso*)-2,3-Butanediol diacetate**	$3^{(5)}$	$4^{(5)}$	8
1075	**(2*S*,3*S*)-Butanediol diacetate**	$5^{(6)}$	$2^{(5)}$	9
1105	**(2*R*,3*R*)-Butanediol diacetate**	$6^{(6)}$	$4^{(5)}$	10
1124	**2,3-Butanediol monoacetate #3**	$4^{(4)}$	$5^{(5)}$	11
1130	2,3-Butanediol monoacetate #4	$0^{(4)}$	$0^{(5)}$	12
	C5-branched chain compounds			
789	Tiglic aldehyde	$0^{(2)}$	$1^{(4)}$	13
902	3-Methylbutyl acetate	$3^{(5)}$	$0^{(4)}$	–
906	3-methyl-1-butanol	$0^{(5)}$	$0^{(4)}$	–
909	2-methyl-1-butanol	$0^{(6)}$	$0^{(4)}$	–
	Aromatic compounds			
1276	**2-Phenylethyl formate**	$1^{(2)}$	$1^{(4)}$	14
1291	**2-Phenylethanol**	$7^{(7)}$	$5^{(5)}$	15
1331	**2-Phenylethyl acetate**	$5^{(6)}$	$4^{(5)}$	16
	Terpenoids			
1333	**β-Citronellol** [b]	$1^{(1)}$	$1^{(1)}$	–
	Unknown compounds			
1251	unk_1200	$0^{(2)}$	$1^{(5)}$	17

[a] The RIs of those four compounds varied considerably in the presence/absence of the others and did not allow the assignment of the respective antennal responses to a substance. Responses to (*meso*)-2,3-butanediol could only be analysed in synthetic samples void of co-eluting compounds.

[b] The RI is identical with that of (*S*)-β-citronellol, although we cannot exclude (*R*)-β-citronellol due to the lack of an authentic standard.

acetate, 3-methylbutyl acetate, ethyl acetate) (Bolton et al., 2022; Cha et al., 2013; Feng et al., 2018; Revadi et al., 2015; Stökl et al., 2010). In contrast to other *Drosophila* species (e.g., *D. melanogaster*), females of the frequent pollinator *D. suzukii* rely on yeast- and bacteria-volatiles only for finding substrates for feeding, not for ovipositing, for which fresh-fruit volatiles are utilized (Becher et al., 2012; Bueno et al., 2020; Mori et al., 2017). In *D. melanogaster*, acetoin is the strongest known stimulus of the glomerulus VA2, associated with the close-range attraction to vinegar (Xiao and Lu, 2014).

In our electroantennographic experiments (GC-EAD) with male and female *D. simulans*, a frequent pollinator of *A. baetica*, we found that most floral volatiles are physiologically active. Indeed, many of those compounds were reported as EAD-active in various drosophilid species before, and, together with our results, show that they are widely receivable among these flies (Cloonan et al., 2018; Stökl et al., 2010). However, the stereochemistry of these compounds was neglected in previous EAD studies with flies, and hence it was hitherto unknown whether drosophilids can detect all or only specific stereoisomers. Generally, there are very little data available about the stereoisomeric pattern of chiral floral scent compounds, and even less is known about physiological and behavioral responses of pollinators to different enantiomers (reviewed in Dötterl and Gershenzon 2023). We found differential stereospecific reception, depending on the compounds. Both stereoisomers of acetoin acetate were EAD-active, whereas in acetoin, 2,3-butanediol, 2,3-butanediol mono- and diacetate not all the stereoisomers elicited antennal responses. This highlights the enantioselective olfactory circuitry in drosophilid flies, as it was shown in other insects (e.g. Tolasch et al., 2003; Dötterl et al., 2006; Raguso, 2016). Although only (2*S*,3*S*)- and exceptionally (2*R*,3*R*)-butanediol were EAD-active, all three stereoisomers were attractive in our bioassays, which might be a result of sample size. In contrast, none of the two tested stereoisomers of 2,3-butanediol diacetate were attractive, although both were EAD-active, suggesting that they are not responsible for the attraction of this pollinator species. The presence of the minor compound β-citronellol in the scent of *A. baetica* is surprising, as it was shown to have a repellent effect to *D. suzukii* (Renkema et al., 2017). In our behavioral assays, β-citronellol was neutral to *D. simulans*, and hence probably serves a different purpose in the plant, although we cannot exclude that other drosophilid pollinators than *D. suzukii* and *D. simulans* are attracted by this compound.

Our field bioassays demonstrated that synthetic mixtures that resembled floral scents of *A. baetica* successfully attracted pollinators of this plant species with high specificity, including the main pollinators *D. simulans*, *D. suzukii* and *D. subobscura*, as well as some phorids. The numbers of drosophilids attracted in our field bioassays were much higher in non-native habitats of the plant (Central Europe) compared to the *A. baetica* site in Spain. All four *Drosophila* specimens attracted in Spain were carrying *Aristolochia baetica* pollen, indicating that they had previously visited flowers of *A. baetica*, the only *Aristolochia* species present at that site. This suggests a high competition between our bioassay traps and the flowers, which were abundant during bioassays. Thus, many of the flies were probably inside the flowers and hence unavailable for our bioassay. It also shows that specific fly individuals were attracted to both the flowers and the synthetic mixtures. In Austria we not only attracted drosophilid pollinators, but also two additional *Drosophila* species, of which one (*D. phalerata*) is known to visit flowers of *A. baetica* (Berjano, 2006). Even though the relative ratios of some compounds found in the flowers (2,3-butanediol mono- and diacetates), as well as the stereochemical configurations, could not be well replicated in our experimental setup, the bioassays attracted the same *Drosophila* species, which we found inside of the flowers. As these *Drosophila* species utilize a broad spectrum of different fermenting fruits as brood substrates, which differ significantly in their scent compositions (e.g. Stökl et al., 2010), it is likely that exact qualitative and relative scent compositions of attractive volatiles have comparatively little impact on their attraction. The flies probably are still attracted even in the absence of some of those compounds (Stökl et al., 2010), which would explain the high intraspecific scent variation among flowers of *A. baetica*, where some individuals completely lacked compounds that were main compounds in others. If so, there would be a low selective pressure exerted on the flowers' scent to narrowly fit a specific model, in addition to the classical idea of negative frequency dependent selection that retains variation in scent (Braunschmid and Dötterl, 2020). Overall, our field bioassays confirmed that floral scent alone is capable of attracting pollinators of *A. baetica*. This is in agreement with other mimicry systems targeting flies (Oelschlägel et al., 2015; Johnson and Schiestl, 2016; Dötterl and Gershenzon, 2023).

The findings that *A. baetica* is pollinated by drosophilids associated

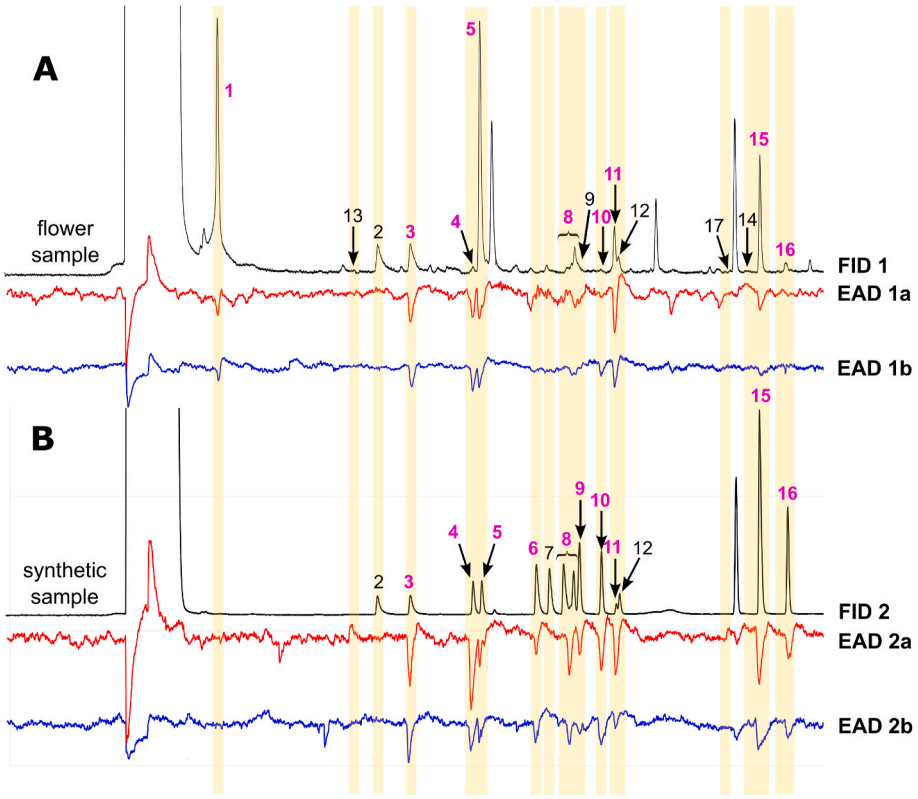

Fig. 4. Representative examples of physiological responses (gas chromatography coupled to electroantennographic detection, GC-EAD) of female (red, EAD 1a, EAD 2a) and male (blue, EAD 1b, EAD 2b) *Drosophila simulans* flies to (**A**) natural headspace (FID 1) and (**B**) synthetic (FID 2) scent samples of female-phase flowers of *Aristolochia baetica*. EAD-active (bold pink) and EAD-inactive (black) compounds are indicated by numbers, which refer to the compounds listed in Table 3. Peaks without numbers are contaminations or green leaf volatiles. All samples were run on a chiral fused silica capillary column (30 % DIME-*β*-CD in 70 % SE-52). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with yeast-fermenting fruit and that these flies are attracted by floral scents resembling the scent of those substrates, allow us to conclude that *A. baetica* deceives its pollinators by chemical mimicry of yeast-fermenting fruit. The flowers exploit the olfactory preference of their pollinators for yeast volatiles in search of food and/or oviposition sites. In *Aristolochia*, mimicry of fermenting fruit was indirectly suggested by Vogel (1965, 1978), who stated that flowers of *A. macrophylla*, *A. tomentosa* Sims and *A. fimbriata* attract Drosophilidae, and sometimes additionally Phoridae, by their fermentation-like ('mostartigem') scent. This hypothesis was, for the first time in *Aristolochia*, tested by analytical chemistry and chemo-ecological experiments in the present study.

Flowers mimicking yeast-fermenting fruit by a similar set of compounds as in *A. baetica* are found in plant species across several plant families and continents, from Cycadopsida (*Stangeria eriopus* (Kunze) Baill.) to various families of angiosperms (e.g., Annonaceae: *Asimina triloba* (L.) Dunal; Araceae: *Arum palaestinum*, *Anthurium hookeri*; Calycanthaceae: *Calycanthus occidentalis*, and Orchidaceae: *Gastrodia similis*). Typically, such plants are pollinated by drosophilid flies and/or beetles (Nitidulidae, Staphylinidae) (Goodrich et al., 2006; Goodrich and Raguso, 2009; Gottsberger et al., 2021; Martos et al., 2015; Procheş and Johnson, 2009; Schwerdtfeger et al., 2002; Stökl et al., 2010). One plant species, *Asarum tamaense* Makino (Asaraceae), releases such compounds in addition to typical carrion-scents (e.g., dimethyldisulfide). This species mimics carrion-scented mushrooms to attract mushroom-associated pollinators (Drosophilidae, Mycetophilidae) (Kakishima et al., 2021; Kakishima and Okuyama, 2020). Overall, *A. baetica* emits a scent bouquet similar to other drosophilid-pollinated deceptive plants from the families Araceae and Apocynaceae, as well as to yeast-fermented substrates (Fig. 3). It emits a different scent than other *Aristolochia* species studied so far – all of which are pollinated by flies others than drosophilids – and plants mimicking other breeding substrates (Fig. 3). Our comparative scent analysis also suggests that the scent of *A. baetica* does not match a specific fermenting model substrate, but generally imitates yeast fermentation.

The floral scent of *A. baetica* most resembles the eastern Mediterranean *Arum palaestinum* (Araceae), which also evolved deceptive trap flowers (Stökl et al., 2010), and the North American *Calycanthus occidentalis* (Calycanthaceae), a species without trapping mechanism (Gottsberger et al., 2021). The scents in all these three species are characterized by acetoin, acetoin acetate, 2,3-butanediol mono- and diacetate. *Aristolochia baetica* furthermore shares 2-phenylethanol and 2-phenylethyl acetate with *A. palaestinum* (Stökl et al., 2010), and ethyl acetate, 2-methylpropyl acetate and 3-methylbutyl acetate with *C. occidentalis* (Gottsberger et al., 2021). *Arum palaestinum* additionally produces quite high amounts of the aliphatic esters hexyl acetate and ethyl hexanoate, both absent in *A. baetica* and *C. occidentalis*. Those additional compounds, but also the compounds shared with *A. baetica*, were attractive to a drosophilid pollinator (*D. melanogaster*) in a lab bioassay in a setup similar to ours (Stökl et al., 2010). While *A. palaestinum* is pollinated by a highly similar spectrum of female and male drosophilid flies as *A. baetica*, sharing *D. simulans* (dominant pollinator), *D. subobscura*, *D. hydei*, *D. melanogaster*, *D. immigrans*, and *D. busckii* (Stökl et al., 2010), *C. occidentalis* is pollinated by small fruit-feeding beetles of the families Nitidulidae and Staphylinidae, regardless of the similar scent profile (Gottsberger et al., 2021). This is partly due to the inability of drosophilid flies, which are also attracted, to enter the flowers of *C. occidentalis*, unlike the beetles, which penetrate to the floral chambers (Gottsberger et al., 2021). It is the reverse scenario to *A. baetica*, where members of these beetle families were found in the floral chambers in lower abundances (Nitidulidae, n = 13;

Table 4
Number of insects attracted in two-choice field bioassays deploying synthetic scent mixtures of floral volatiles of *Aristolochia baetica* solved in acetone against acetone negative controls. The synthetic mixture Mix2 contained acetoin, acetoin acetate, 2,3-butanediol, 2,3-butanediol mono- and diacetate, 2,3-butanedione, 2-methyl-1-butanol, 2-phenylethanol and *β*-citronellol, and Mix3 additionally contained 3-methyl-1-butanol and tiglic aldehyde. The experiments were performed at a natural population during the flowering period of *A. baetica* in Aznalcázar, Spain, and, additionally, in the Botanical Garden of the University of Salzburg, Austria. Bold taxa were identified as pollinators of *A. baetica* in our flower samples (see Table 1). Specimens carrying pollen of *A. baetica* are marked with an asterisk '*'.

Taxa	Aznalcázar, Spain		Salzburg, Austria			
	Mix3	Acetone	Mix2	Acetone	Mix3	Acetone
Diptera						
Drosophilidae						
Drosophila kuntzei Duda, 1924			1♀		2♀, 3♂	
D. melanogaster Meigen, 1830	1♀*					
D. phalerata Meigen, 1830					1♂	
D. simulans Sturtevant, 1919	1♀*					
D. subobscura Collin, 1936	1♂*		1♀		1♂	
D. suzukii (Matsumura, 1931)			12♀, 13♂		4♀, 2♂	
Hirtodrosophila cameraria (Haliday, 1833)	1♂*					
unidentified			2			
Phoridae						
Megaselia giraudii (Egger, 1862)	1♂					
Megaselia spec.	1♂					
unidentified	1	1♂	6♀, 3♂		2♂	
Sciaridae						1
Heleomyzidae		1♀				
Hemiptera (Cicada)			1			
Hymenoptera						
Ceraphronidae	1					

Staphylinidae, n = 7; Supplementary Table S1), but did not pollinate, probably due to morphological constraints. As Gottsberger et al. (2021) state, it would be worth testing whether potential differences in the stereoisomeric patterns of acetoin, 2,3-butanediol and chemically related compounds could explain the bias in attracted drosophilids and/or beetles in the respective plant species. As at least a scarab beetle was shown to be strongly attracted to (*R*)-acetoin, a compound not EAD-active in drosophilid pollinators in the present study (but see preliminary measurements with *D. repleta*, Supplementary Table S4), but not to (*S*)-acetoin (Tolasch et al., 2003), there might also be differential behavioral responses in nitidulids, staphylinids or drosophilids (Gottsberger et al., 2021).

4. Conclusions

Chemical mimicry of yeast-fermenting fruit is identified for the first time in *Aristolochia*. It is a deceptive strategy known from different plant families (e.g., Araceae, Apocynaceae), however, pollinators and scent chemistry in *A. baetica* are particularly similar to that of distantly related Salomon's lily *Arum palaestinum* (Fig. 3). The strategy obviously evolved independently in those lineages as a result of convergent evolution. Whether potential differences in the absolute configuration of chiral compounds (e.g., acetoin, 2,3-butanediol and related acetates) could be responsible for the differential attraction of beetle and drosophilid pollinators in deceptive systems mimicking yeast-fermentation, needs to be tested in future studies.

5. Experimental

5.1. Study system and study sites

Aristolochia baetica L. is an evergreen climber, native to the southernmost Iberian Peninsula and north-western Morocco, common in the understory of southwest-Mediterranean woodlands (Berjano et al., 2009). The plant flowers from October to May, and each shoot typically carries numerous protogynous, dark reddish trap-flowers with a basal chamber (utricle) bearing the gynostemium (Fig. 1A). Pollinators enter in the female flowering-phase, are temporarily retained due to trapping trichomes, and finally released in the male phase, loaded with pollen (Berjano et al., 2009). Our study focused on two sites in Andalusia, southern Spain: Aznalcázar (Sevilla; 37°15′03″N, 06°14′11″W, 20 m a.s.l.) and Membrillo (Hinojos, Huelva; 37°17′48″N, 06°25′16″W, 90 m a.s.l.). Additional floral scent samples were collected at a population in the city of Sevilla (campus of Universidad Pablo de Olavide) (37°21′13″N, 05°56′15″W, 22 m a.s.l.), and some field bioassays were conducted at the Botanical Garden of the Paris-Lodron University of Salzburg, Austria (47°47′12″N, 13°03′34″W, 423 m a.s.l.). Voucher specimens of *A. baetica* from all study sites are deposited at Herbarium Dresdense (DR) (Aznalcázar: DR055641; Membrillo: DR55640, DR55642; Sevilla: DR55639).

5.2. Flower visitors

We randomly collected a total of 2,587 flowers of *A. baetica* (1,332 female phase, 1,255 male phase) at the sites Aznalcázar (n = 1,773) and Membrillo (n = 814). The utricles of collected flowers were opened, the flower phase identified, the trapped arthropods collected and checked for pollen loads under a stereo microscope. Following the most conservative approach, only flower visitors that carried *Aristolochia* pollen in female-phase flowers were treated as pollinators (Oelschlägel et al., 2015; Rulik et al., 2008; Rupp et al., 2021). The so-called 'interphase' (Berjano et al., 2009) was considered as male phase, since the pollen is already released, although the trapping trichomes are still intact. *Aristolochia*-pollen was identified based on the typical positioning on the insects' thorax (Fig. 1B) and the inaperturate exine characteristic for the genus (Berjano et al., 2009; Rupp et al., 2021). We evaluated the flower visitors at population, rather than at plant individual level, as each rhizome of *A. baetica* can produce numerous shoots, and shoots of different individuals often grew intermingled. Hence, we could not reliably differentiate between individuals (Berjano, 2006). At the site Aznalcázar, *A. baetica* was the only *Aristolochia* species present. Therefore, we assumed that all *Aristolochia* pollen carried by drosophilids belonged to *A. baetica*. At Membrillo, *A. baetica* was co-flowering with *A. paucinervis* Pomel; this species has similar pollen characteristics as *A. baetica*, but a different visitor assemblage with only rare visits by Drosophilidae (<1 % of visitors) (Berjano et al., 2009). Other visiting insects, such as Phoridae are frequently shared between both species and thus, the pollen loads on such insects collected from *A. baetica* at this site cannot undoubtedly be determined as *A. baetica* pollen. All collected flower visitors were stored in 80 % isopropanol and identified to insect order; all Diptera were identified to family or species levels (see below). Voucher specimens of the collected arthropods were deposited at the Department of Environment & Biodiversity, Paris-Lodron University of Salzburg and a subset of the Drosophilidae in the collection of the Zoological Museum of the University of Zurich. We tested for differences in the presence of pollen between drosophilid and phorid flower visitors by chi-square tests.

5.3. Morphological identification and molecular characterization of flies

We morphologically identified all Diptera recorded in this study to

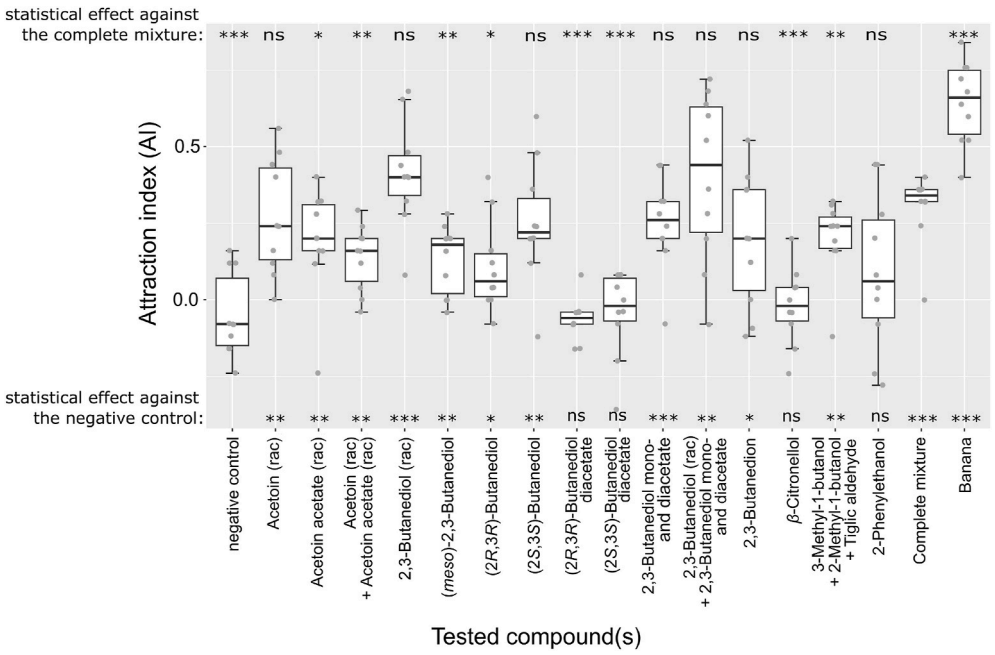

Fig. 5. Lab bioassays testing the attractiveness of overripe banana (positive control) and synthetic floral scent compounds of *Aristolochia baetica* (diluted in H_2O + Tween®20) in *Drosophila simulans* (Diptera, Drosophilidae), a frequent pollinator of this species, against negative controls (H_2O + Tween®20) in two-choice assays (n = 10 replicates each, with 25 flies tested per replicate, see section 5.11). To test for a side bias, we also tested two negative controls against each other. Tested were the complete mixture of available floral compounds (Mix4) and compounds (combinations) thereof, in the same concentration as they were used in the complete mixture (Supplementary Table S5). Attraction index (AI), (flies in test trap – flies in control trap)/all flies. This index ranges from −1 (complete avoidance) to 1 (complete attraction). Significant differences in Mann-Whitey-U-Tests ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***, not significant 'ns') to the negative control (bottom) and to the complete mixture (top) are given.

family level. In Asteiidae, Drosophilidae, Chloropidae, Milichiidae and Scatopsidae, all pollinators and all specimens attracted in field bioassays (see section 5.10) were morphologically identified to species level. Drosophilid pollinators were additionally characterized by molecular barcoding (Supplementary Material S6; Supplementary Table S7).

5.4. Floral scent collection

We focused on female-, rather than male-phase flowers, as pollinators are only attracted during the female phase. Two types of floral scent samples were collected by dynamic headspace methods (Dötterl et al., 2005): Thermal desorption (TD) samples for qualitative and (semi) quantitative analysis of compounds, and solvent acetone (SA) samples for determination of the absolute configuration of chiral compounds and for enantioselective GC-EAD (gas chromatography/electroantennographic detection) experiments (see section 5.6).

TD samples: Female-phase flowers were individually sampled *in situ* at Aznalcázar (n = 7) and Membrillo (n = 9) in April 2019. The plants used for sampling were separated by at least 10 m. Nearly open flower buds were individually wrapped in filter-paper bags to prevent insects from entering the freshly opened flowers. On the first day of anthesis, when the flowers were in female phase, these bags were removed and the flowers inserted into oven bags (10 × 5 cm; Toppits®, Minden, Germany), without damaging the flowers. Scent collection was initiated immediately after bagging, by sucking the air containing the volatiles through an adsorbent tube for 10 min, at a flow rate of 200 ml/min by a membrane pump (G12/01 EB; Rietschle Thomas Inc., Puchheim, Germany). Adsorbent tubes consisted of quartz glass microvials (Hilgenberg GmbH, Maisfeld, Germany: length = 25 mm, inner diameter = 1.8 mm) filled with 3 mg of a 1 : 1 mixture of Tenax-TA (mesh 60–80) and Carbotrap B (mesh 20–40) (both Supelco, Bellefonte, PA, USA) fixed by glass-wool plugs. To control for contaminants and green leaf volatiles, ambient air and leaves of *A. baetica*, respectively, were sampled in a similar way. Samples were stored at 4 °C during fieldwork and at −25 °C in the laboratory until GC-MS analyses (see section 5.5).

SA samples: To obtain solvent scent samples, 1 or 2 pooled flower(s) were sampled *in situ* (n = 7; site Sevilla). In Aznalcázar, 10 or 20 flowers from a single plant individual each were freshly cut and pooled for scent sampling (n = 3). As the flower phase cannot be accurately determined based on external characters, the flowers were dissected after sampling. Samples collected not only from female- but also from male-phase flowers were discarded. Scent collection was performed as described for TD samples, but with larger adsorbent tubes (glass capillaries, length = 8 cm, inner diameter = 2.5 mm) filled with 15 mg Tenax-TA (mesh 60–80) and 15 mg Carbotrap B (mesh 20–40). Sampling lasted between 4 h 23 min and 6 h 25 min. The volatiles trapped in an adsorbent tube were eluted with 100 µl of acetone (Rotisolv, Roth, Germany) and stored at −25 °C until submission to enantioselective GC-MS analyses and/or GC-EAD experiments.

5.5. Gas chromatography coupled to mass spectrometry (GC-MS)

TD samples: The adsorbent tubes containing the trapped volatiles were analysed by gas chromatography coupled to mass spectrometry (GC-MS) using an automatic thermal desorption system (TD-20, Shimadzu, Tokyo, Japan) coupled to a Shimadzu GC-MS (QP2010 Ultra) equipped with a ZB-5 fused silica column (5 % phenyl polysiloxane; length = 60 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Phenomenex), as described by Heiduk et al. (2015). The samples were processed at a split ratio of 1 : 1 and a constant helium carrier gas flow rate of 1.5 ml/min. The GC oven started at an initial temperature of 40 °C, was then increased by 6 °C/min to 250 °C and held for 1 min. The MS interface worked at 250 °C. Mass spectra were measured at 70 eV (EI mode) from m/z 30 to 350.

SA samples: The solvent acetone samples were also analysed using GC-MS (model QP2010 Ultra EI, Shimadzu, Tokyo, Japan), but the GC was equipped with a chiral fused silica column, coated with a 0.23 µm film of 0.4 % heptakis (2,3-di-O-methyl-6-O-tert- butyldimethylsilyl)-

β-cyclodextrin (DIME-β-CD) (30 %) in SE-52 (70 %) (MEGA-DEX DMT Beta SE, 30 m × 0.25 mm ID, MEGA S.r.l., Legnano, Italy), the same as used by Gfrerer et al. (2022). With helium as the carrier gas (flow: 3 ml/min), 1 µl of a sample was injected and run with a split ratio of 1 : 1.

The data of both TD and SA samples were analysed using the software package *GCMSolution version 4.41* (Shimadzu Corporation, Kyoto, Japan, 1999–2015). Compounds were tentatively identified by comparison of linear retention indices (RI, based on a series of commercially available n-alkanes C_7–C_{20}; van den Dool and Kratz, 1963) and a match of mass spectra to spectra available in the databases ADAMS, ESSENTIALOILS-23P, FFNSC 2, and W9N11. All compound identities were verified using retention indices and mass spectra of authentic standards available in the Plant Ecology Lab of the Paris-Lodron University of Salzburg. We performed analyses of similarities (ANOSIM; 10, 000 permutations) to test for differences in floral scent among study sites, using the software *PRIMER 6.1.0.5* (Clarke and Gorley, 2006).

5.6. Enantioselective electrophysiological analyses (GC-EAD)

We performed the electrophysiological measurements with natural headspace (SA samples, see section 5.4) and synthetic scent samples on a gas chromatograph (GC) (Agilent 7890A, Santa Clara, California, USA) equipped with a flame ionization detector (FID) and an electroantennographic detection system (EAD), using a frequent pollinator of *A. baetica* (*Drosophila simulans*, Table 1). The flies were either collected from flowers of *A. baetica* in Sevilla or Aznalcázar (2 males, 2 females), or reared from those flies (Supplementary Table S4). The GC was equipped with the same DIME-β-CD chiral column as described in section 5.5. At the end, the column was split into two capillaries by a µFlow splitter (Gerstel, Mühlheim, Germany), with nitrogen (N_2) as make-up gas (flow rate of 25 ml/min). One of the capillaries (2 m × 0.15 µm inner diameter) led to the FID, the other (1 m × 0.2 µm inner diameter) to the EAD setup, which consisted of a transfer line, heated at 220 °C, and a 2-channel USB acquisition controller (Syntech, Kirchzarten, Germany). The EAD-outlet led to a cleaned, humidified airflow, directed onto a mounted fly antenna. Due to the minute size of the antennae, the entire head was removed (cut) from the specimens under anesthetization with CO_2. The tip of a randomly selected antenna was attached to a recording electrode, while the caudal side of the head was connected to a reference electrode, both via glass micropipettes filled with insect Ringer's solution (8.0 g/l NaCl, 0.4 g/l KCl, 4.0 g/l $CaCl_2$) and connected to silver wires. The FID and antennal responses were recorded and analysed using the software *GcEad V4.6* (Syntech). Only antennal responses unambiguously distinct from background noise and with a characteristic shape were considered. We obtained successful measurements of 6 males and 5 females of *D. simulans*. For additional information, we provide preliminary GC-EAD measurements of four further drosophilid pollinators (*D. busckii*: 1 female; *D. hydei*: 1 male, 1 female; *D. suzukii*: 1 female; *Scaptomyza pallida*: 1 female) and a non-pollinating flower visitor (*D. repleta*: 1 male, 1 female), all obtained from flowers of *A. baetica* in Sevilla or Aznalcázar or reared from those (only *D. repleta*) (Supplementary Table S4). Generally, with each fly individual we performed between 1 and 8 runs with natural headspace and/or synthetic scent samples, depending on the longevity of the prepared antenna/head. As different scent samples (synthetic mixtures and natural headspace floral samples) were tested on different specimens, not all compounds were tested on each individual (Table 3, Supplementary Table S4).

5.7. Comparison of floral scents of A. baetica to literature data

The scent bouquet of *A. baetica* was compared to literature data of 1) other *Aristolochia* species (Johnson and Jürgens, 2010; Martin et al., 2017; Oelschlägel et al., 2015; Rupp et al., 2021; Stashenko et al., 2009), 2) other deceptive plants pollinated by drosophilids (see introduction; Schwerdtfeger et al., 2002; Heiduk et al., 2017; Martos et al., 2015; Jermakowicz et al., 2022; Kakishima et al., 2019; Stökl et al., 2010), 3) fermenting fruit, vinegar and wine (Stökl et al., 2010), and 4) a dataset of 61 plants deploying oviposition-site mimicry and seven potential models therof (different types of carrion and feces, baker's yeast) (Jürgens et al., 2013; Gottsberger et al., 2021). We used presence/absence data of compounds for analyses. Different (stereo)isomers of compounds were pooled and unknown compounds omitted. In *A. baetica*, we included all compounds found in at least one sample. The results were visualized in a NMDS (non-metric multidimensional scaling) using *Primer 6* (stress value = 0.19), calculated on pairwise Sørensen similarities.

5.8. Synthesis of floral volatiles

We synthesized 2,3-butanediol monoacetate and 2,3-butanediol diacetate (stereoisomers) to have them available for bioassays (see sections 5.10 and 5.11) and to identify the absolute configurations of these compounds in the floral scent samples. The compounds were prepared by treating a mixture containing all stereoisomers of 2,3-butanediol with 1 : 1 (reaction I) and 1 : 2 equivalents (reaction II) of acetic anhydride (Sigma-Aldrich) and a catalytic amount of conc. H_2SO_4, as previously reported (Gottsberger et al., 2021; Stökl et al., 2010). Reaction I resulted in a mixture of stereoisomers of 2,3-butanediol mono- and diacetate, and reaction II in 99 % 2,3-butanediol diacetate stereoisomers (resulting compositions see Supplementary Table S8). Similarly, we produced (2*R*,3*R*)- and (2*S*,3*S*)-butanediol diacetate by diacetylating (2*R*,3*R*)- and (2*S*,3*S*)-butanediol, respectively (Supplementary Table S8, reaction IIa and IIb).

5.9. Synthetic scent mixtures

For electroantennographic experiments and bioassays (field, lab) we created synthetic scent mixtures from commercially available and newly synthesized compounds identified in the floral scent samples of *A. baetica*. As not all compounds were available from the beginning, different mixtures were used in the course of our experiments (compositions see Supplementary Table S5). The mixtures included compounds that occurred in at least 50 % of floral samples across populations (acetoin, acetoin acetate, 2,3-butanediol, 2,3-butanediol monoacetate, 2,3-butanediol diacetate, 2,3-butanedione, 3-methyl-1-butanol, 2-methyl-1-butanol, tiglic aldehyde, 2-phenylethanol, β-citronellol). The exception was Mix1 (only used for GC-EAD analyses), which additionally included ethyl acetate, 2-phenylethyl acetate, which were only found in 25 % and 31 % of samples, respectively, and isovaleric acid, a green leaf volatile. We used acetone as a solvent for the scent mixtures in field bioassays and GC-EAD (Mix1, Mix2, Mix3; Supplementary Table S5). For lab bioassays (Mix4; Supplementary Table S5), we used water (following Stökl et al., 2010) instead of acetone as a solvent, as acetone repeatedly attracted *D. simulans* flies in this test setting in preliminary experiments. The detergent Tween®20 was added to increase the solubility of the compounds in water.

As field and lab bioassays lasted for 24 h (see sections 5.10 and 5.11), we sampled (and analysed) the volatiles emitted by the different traps used for the bioassays at different times after applying the mixtures (0, 1, 5, and 24 h), and adjusted their composition to match the range of the natural scent emitted by flowers during the entire experiment. Therefore, we used different mixtures for field and lab bioassays. We finally obtained field and lab mixtures that resembled the absolute and relative amounts (except for 2,3-butanediol mono- and diacetates, due to synthetic constraints; see section 5.8) of the scent of 10 natural flowers of *A. baetica*.

5.10. Field bioassays

Two-choice bioassays with synthetic mixtures of floral scents of *A. baetica* were performed in the field. Using bottle traps, synthetic scent

mixtures (compounds solved in acetone; Supplementary Table S5) were tested against negative controls (acetone). The bottle traps were built from transparent 0.5 l PET water bottles, in which six entrance holes (diameter 4 mm) were drilled circularly 5 cm above the bottom. Each trap contained an open 2 ml glass vial, tangling on a cotton string held in place by the bottle lid. A cotton wick (length 2.5–3 cm, diameter 0.4 cm) was inserted into the glass vial to facilitate scent emission. The cotton wicks were cleaned in four steps before use: sonicated in Millipore H_2O, washed in methanol and then in acetone, and finally heated for 3 h at 150 °C. At the start of the experiment, 0.5 ml of the scent mixture (test) or acetone (negative control) were loaded onto the wick. The traps were offered in the field at a height of about 1 m on branches of shrubs, with a distance between test and negative control traps of ca. 0.5 m, and at least 3 m between different two-choice assays (replicates). The bioassays were performed at the site Aznalcázar in December 2020 and February 2021, when *A. baetica* was flowering (scent mixture Mix3, n = 30, with 10 replicates per day). In Salzburg, tests were performed between August and October 2019 (Mix2, n = 30, with 3 replicates per day) and in August and October 2020 (Mix3, n = 18, with 3 replicates per day). The traps were collected after 24 h and the trapped arthropods stored individually in 80 % isopropanol.

5.11. Lab bioassays

To determine the attractiveness of single floral scent compounds of *A. baetica* and mixtures thereof to drosophilid pollinators, we performed two-choice bioassays in a lab setting with *Drosophila simulans,* a frequent pollinator of the plant (see results). All tested flies were the offspring of specimens collected from flowers of *A. baetica* growing on the campus of Universidad Pablo de Olavide in Sevilla and flower-inexperienced. Flies were reared and cultivated under room conditions on commercial nutrient medium (Formula 4-24 instant, Schlüter Biologie, Germany) in 0.3 l glass jars closed by foamed plastic plugs.

For bioassays, flies were randomly selected from the rearing jars after anesthetization with CO_2.

The experimental setup was similar to that described by Stökl et al. (2010). Two custom-made traps (treatment and control), built from small cylindrical plastic vials (A. Hartenstein, Germany; 3.1 × 4.8 cm, volume = 20 ml) with a cut pipette tip inserted into a drilled hole in the lid were placed in transparent plastic boxes (8.1 × 10.8 × 10.3 cm, width × length × height, volume: 500 ml; Batania, Germany) with five ventilation slits cut in the lid. Each box was equipped with a wet tissue, to create a humid atmosphere. Each trap contained a quarter piece of a filter paper disk (Munktell®, diameter 70 mm, 65 g/m^2) loaded with 200 µl of a watery (distilled water) solution of the tested substance(s) with 0.1 % Tween®20 (Sigma Aldrich, www.sigmaaldrich.com), or with 200 µl of distilled water with 0.1 % Tween®20 as the negative control. As a positive control, we tested 200 mg of overripe banana (following Stökl et al., 2010) with 200 µl of distilled water and 0.1 % Tween®20. The banana was covered by a filter paper and therefore not visible to the flies. To test whether there was a side bias, two traps with water and 0.1 % Tween®20 were offered against each other.

We tested the synthetic scent mixture Mix4 (complete mixture), as well as single compounds and combinations thereof, each used in the same concentrations as in the mixture Mix4, with the volume of the excluded substances substituted by the same volume of water (Supplementary Table S5). Each of these stimuli was tested against a negative control, with 10 replicates each. For a single replicate 25 flies (males and females, sex ratio about 1 : 1) were tested. Each fly individual was only tested once. The experiments were carried out in a climatic chamber (Percival SE-41AR2CLT, CLF PlantClimatics GmbH, Germany) with a 12 h light/12 h dark cycle, at a temp. of 25 °C. The bioassays started between 13:30 and 15:30 h, and 24 h later the flies inside and outside the traps were counted. Following Stökl et al. (2010), data were used to calculate an attraction index (AI) as: AI = (T-C)/(T + C + O), where T is the number of flies in the test trap, C the number of flies in the negative control trap, and O the number of flies outside the traps (no decision). This index ranges from −1 (complete avoidance) to 1 (complete attraction). A neutral scent would be indicated by a value of zero. Mann-Whitney-U-Tests were used to test for differences in the AI between each stimulus and 1) the negative control, and 2) the complete mixture (Mix4), as well as between the complete mixture and the positive control (banana).

Funding

This research was funded in whole or in part by the Austrian Science Fund (FWF) [10.55776/I3722] and the German Research Foundation (DFG, WA 2461/9-1). For open access purposes, the author has applied a CC BY public copyright license to any author-accepted manuscript version arising from this submission.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We thank Bernardo Cañiza (formerly TU Dresden), Francisco Balao Robles (Universidad de Sevilla), Juan José Domínguez Delgado (Sevilla), and Larissa Milde (formerly University of Salzburg) for assistance in the field and/or lab in Spain and Austria, and Roman Fuchs and Andreas Zankl (both University of Salzburg) for lab and technical support, respectively. The staff of the Botanical Garden of the University of Salzburg, especially Elisabeth Egger, supported us during bioassays. Tamara Pokorny and Peter Kreitmeier (both University of Regensburg) provided a sample of synthetic (*R*)-acetoin for chemical analyses. Nikolaus Bresgen (University of Salzburg) provided valuable advice for rearing and handling of drosophilid flies. R. Henry L. Disney (University of Cambridge) identified the Phoridae, Michael von Tschirnhaus (Universität Bielefeld) the Chloropidae, Jean-Paul Haenni (Neuchâtel) the Scatopsidae, Jindřich Roháček (Silesian Museum, Opava) the Asteiidae and Milichiidae, and Tobias Seifert (University of Salzburg) the Coleoptera. We also thank two anonymous reviewers for their critical and constructive comments on an earlier version of the manuscript. The collection

permit was issued by the "Consejería de Medio Ambiente, Junta de Andalucía" (permit number: 64oxu807PFIRMA4b4wM22mq2sUUfSx).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2024.114142.

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