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Smells like debauchery: The chemical composition of semen-like, sweat-like and faintly foetid floral odours in *Xysmalobium* (Apocynaceae: Asclepiadoideae)

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ABSTRACT

Floral volatiles play an important role in plant communication with both pollinators and antagonists, but remain poorly explored for many plant groups. Asclepiads (Apocynaceae: Asclepiadoideae subtribe Asclepiadinae) represent a diverse group in South African grasslands, but the scents of most species remain unexplored and few genera are sufficiently sampled to allow comparisons between congeners. I used dynamic headspace extraction methods and coupled gas chromatography-mass spectrometry (GC—MS) to examine the scent chemistry of three unusually scented asclepiads in the genus *Xysmalobium* and then combined these data with previously published data to explore inter- and intraspecific variation in the genus. A total of 74 compounds (33—44 per species) from various compound classes were detected in the species examined here. The sweet but faintly foetid scent of *Xysmalobium asperum* was dominated by epoxy oxoisophorone in combination with various other terpenoids and aromatics, and small amounts of *p*-cresol. The sweat-like scent of *Xysmalobium tysonianum* was dominated by a few aromatics in combination with isovaleric acid and several aliphatic compounds normally associated with microbial degradation or fermentation. The semen-like scent of *Xysmalobium parviflorum* flowers examined here contained large relative amounts of 1-pyrroline, and comparison with previously published data for dung-scented flowers from a different population revealed clear divergence in the relative amounts of this compound and *p*-cresol. I also detected 25 compounds that were not shared between the two *X. parviflorum* populations. Comparison of scent data for eight *Xysmalobium* species revealed very distinct chemical profiles with limited overlap between species. These results are discussed in relation to the possible roles of these volatiles as pollinator attractants and the evolution of floral scents within the genus.

1. Introduction

Floral volatiles represent an important channel of communication between plants and their pollinators or antagonists (Raguso, 2008; Schiestl, 2015), but remain poorly explored for many plant groups. Members of the subtribe Asclepiadinae (Apocynaceae: Asclepiadoideae: Asclepiadeae *sensu* Endress et al., 2014, commonly called asclepiads) have diversified tremendously in the grasslands of East and southern Africa, and recent investigations in South African grasslands indicate that

many have highly specialized pollination systems (Ollerton et al., 2003; Coombs et al., 2009; Shuttleworth and Johnson, 2008; 2009a,b,c; 2012). Several of these studies have also suggested that floral scent is a particularly important trait for the attraction of pompilid wasps as pollinators (reviewed in Shuttleworth and Johnson, 2012) although the particular volatiles mediating attraction remain poorly understood. Existing investigations of volatiles produced by asclepiad flowers have revealed a diversity of chemical profiles within the group, which have in some cases been putatively associated with particular pollinators (Shuttleworth and Johnson, 2010, 2012). However, the number of studies examining the scent chemistry of asclepiads remains very limited, especially when compared to the diversity of species within the group, and few genera are sufficiently sampled to assess divergence in chemical profiles between congeners. In addition, no studies have examined variation in the volatiles produced by flowers from different populations of the same species.

Intraspecific variation in floral volatile profiles is not often explored, although variation in the floral scents of different populations may influence pollinators and forms the basis for some pollination ecotypes (Dötterl et al., 2005; Johnson et al., 2005; Peter and Johnson, 2014; Van der Niet et al., 2010, 2014). To date, studies of asclepiad scents have only examined single populations or even single plants in cultivation (Jürgens et al., 2008; Shuttleworth and Johnson, 2009a,b; 2010; 2012). In previous studies, we described the pollinators and chemical composition of the odour of *Xysmalobium parviflorum* flowers growing on Gilboa Estate in the Karkloof mountains of KwaZulu-Natal, South Africa (Johnson et al., 2009; Shuttleworth and Johnson, 2009c, 2012). When sampled, the scent of these flowers had a dung-like note and was found to be dominated by several monoterpenes, benzaldehyde and *p*-cresol. The flowers were visited and pollinated by various saprophilous flies in the families Calliphoridae, Muscidae and Scathophagidae. *p*-Cresol is a well-established herbivore dung volatile (Kite, 1995; Schiestl and Dötterl, 2012) and presumably plays an important functional role in attracting these flies. During field-work in a different population of *X. parviflorum* it was noticed that flowers had a powerful semen-like odour (confirmed by three independent observers) which was, to the human nose, very obviously different to the dung-like odour of the Gilboa Estate plants that had previously been examined. Flowers exhibiting a semen-like or spermy odour to the human nose are occasionally encountered (Naef et al., 2002; Kaiser, 2006a,b; Chen et al., 2015), but the chemistry and ecological relevance of this odour remain poorly understood. These flowers were therefore sampled and included in the current study to explore both the chemical basis for this curious odour and also the degree of divergence in the scent chemistry of flowers from these two populations of *X. parviflorum*.

As currently circumscribed, the genus *Xysmalobium* contains 21 species distributed throughout the grasslands of eastern South Africa (Langley, 1980). Pollination systems within the genus are varied, and include specialized pollination by cetoniine chafer beetles (*Xysmalobium involucratum*), *Hemipepsis* spider-hunting wasps (*Xysmalobium orbiculare* and *Xysmalobium stockenstromense*) and saprophilous flies (*X. parviflorum*), as well as bimodal pollination by both *Hemipepsis* wasps and chafer beetles in *Xysmalobium undulatum* var. *undulatum* and generalized insect pollination in *Xysmalobium gerrardii* (Ollerton et al., 2003; Shuttleworth and Johnson, 2008, 2009b,c). The flowers of *Xysmalobium* species studied to date are very strongly scented (most species exhibit total scent emissions over 5000 ng/inflorescence/hour and *X. involucratum* reaches up to 24,000 ng/inflorescence/hour; Shuttleworth and Johnson, 2010, 2012) and show clear qualitative variation between taxa (pers. obs). Previous studies have characterized the volatiles for the six species described above, and suggest that these exhibit distinct and divergent overall scent profiles (Shuttleworth and Johnson, 2010, 2012). In this study, I explore the scent chemistry of another two species of *Xysmalobium* and a second population of the previously studied *X. parviflorum*, all of which exhibit strong and unusual odours to the human observer.

The aims of this study were to expand our knowledge of the diversity of volatiles produced by South African asclepiads by characterising the scent chemistry of three members of the genus *Xysmalobium* and then combine these data with previously published scent data for the genus in order to explore intraspecific variation (in *X. parviflorum*) and interspecific variation in total scent profiles within the genus. The specific objectives were: (1) to characterize the chemical constituents of the scents of *Xysmalobium asperum*, *Xysmalobium tysonianum* and the semen-scented flowers of a second population of *X. parviflorum*; (2) to compare the chemical composition of the scents of flowers from the two *X. parviflorum* populations; and, (3) to summarize the available data on the diversity of total scent profiles within the genus *Xysmalobium*.

2. Materials and methods

2.1. Study species and plant localities

Xysmalobium asperum N.E.Br. occurs in the mid-altitude grasslands of northern South Africa and is a small, multistemmed herb with pale green flowers born in tightly packed umbels at axillary nodes along the stems (Fig. 1A). Flowers exhibit a sweet but faintly foetid odour to the human nose. The pollinators of this species are not known and no visitors were observed in the field. Samples for this species were taken from cut stems (placed immediately in water; see discussion for possible effects of sampling cut stems) collected at Wooden Cross Grassland (27°50′48.7″S; 31°20′13.4″E. 1220 m) on Tygerskloof Farm in the Ngome area, c. 30 km South of Louwsberg, KwaZulu-Natal. Samples 1 and 2 were taken from plants collected on 19 October 2012 and sampled at the nearby Mondi Limited field accommodation. Sample 3 was taken from a plant collected on 20 October 2012 and transported to the University of KwaZulu-Natal, Pietermaritzburg where it was sampled on 21 October 2012.

Xysmalobium parviflorum Harv. ex Scott-Elliot occurs in grasslands from the Eastern Cape Province to Limpopo Province, South Africa and is a multistemmed herb with flowers born in terminal umbels (Fig. 1B). Pollinators and floral scent of this
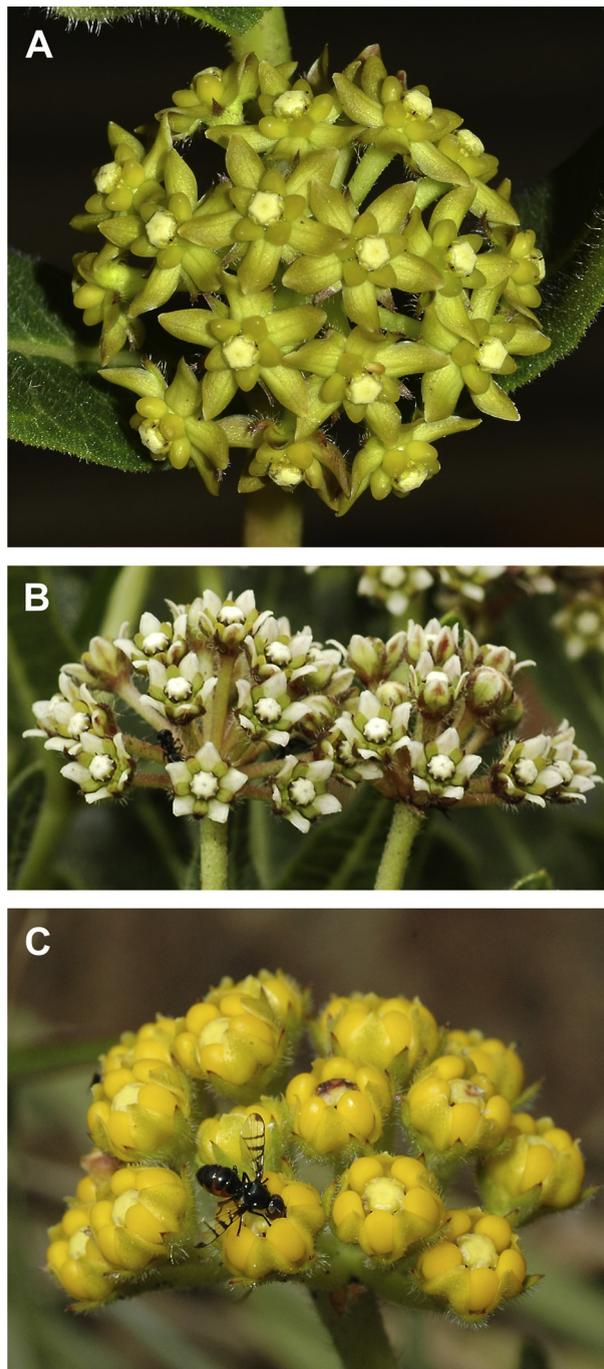
Fig. 1. Inflorescences of the study species. **A**, *Xysmalobium asperum*, Wooden Cross Grassland, Tygerskloof Farm near Ngome. **B**, *Xysmalobium parviflorum*, Gilboa Estate. **C**, *Xysmalobium tysonianaum* being visited by a *Rivellia* sp. (Diptera: Platystomatidae) fly, summit of Mount Gilboa.

species have previously been described from a population growing on Gilboa Estate in the Karkloof mountains of KwaZulu-Natal Province (29°16′30.7″S; 30°16′45.0″E. 1607 m; see Johnson et al., 2009; Shuttleworth and Johnson, 2009c, 2012). A second population growing at Lake Merthley on the outskirts of Greytown, KwaZulu-Natal (29°01′35.5″S; 30°34′56.2″E. 1348 m) was sampled for the current study. This site is approximately 40 km NE of the Gilboa Estate population. Samples were taken from undamaged plants in the field on 3 November 2011. No floral visitors were observed at this site. Scent data presented in Shuttleworth and Johnson (2012) were used for comparison with samples obtained in the current study. 1-Pyrroline was not originally detected in the samples presented in Shuttleworth and Johnson (2012), but closer

examination during the current study revealed small amounts of 1-pyrroline in these samples (relative amount range = 0.3–3.5%, n = 3), and this compound was therefore included in the dataset for this population.

Xysmalobium tysonianum (Schltr.) N.E.Br. occurs in grasslands from the Eastern Cape Province to KwaZulu-Natal Province, South Africa, and is a multistemmed herb with bright yellow flowers born in densely packed terminal umbels (Fig. 1C). Flowers exhibit a foetid odour reminiscent of sweaty socks or French cheeses to the human nose. Pollinators are not known, although I observed visits by *Atrichelaphinis tigrina* (Scarabaeidae: Cetoniinae) and a species of small fly in the genus *Rivellia* (Diptera: Platystomatidae, identified by Dr Ray Miller, University of KwaZulu-Natal) (Fig 1C) at the study site (the latter were also observed to be visiting flowers at the same time that scent samples were being collected). These same insects have been photographed visiting the flowers of this species at a second site in the Ngele mountain range near Kokstad (observations by Graham Grieve, see http://www.ispotnature.org/node/632261, accessed 2 July 2015). The *Rivellia* flies are morphologically more suitable to effect pollination (although none of seven individuals that were collected carried pollinia). This species was sampled in a population of c. 40 plants growing in rocky grassland on the summit of Mount Gilboa (29°17′11.1″S; 30°17′28.4″E. 1763 m). Samples were taken from undamaged plants in the field on 13 December 2010.

Voucher specimens for all study species are deposited in the Bews (NU) Herbarium at the University of KwaZulu-Natal, Pietermaritzburg.

2.2. Scent sampling and GC–MS analysis of volatiles

Volatiles were collected using dynamic headspace extraction methods and analysed by coupled gas chromatography-mass spectrometry (GC–MS) using a Varian CP-3800 gas chromatograph (with a 1079 injector equipped with a ChromatoProbe thermal desorption device) coupled to a Varian 1200 quadrupole mass spectrometer. A detailed description of these methods is given in Shuttleworth and Johnson (2009a), except that an Alltech EC-WAX column was used for *X. parviflorum* and *X. tysonianum* samples and a Bruker BR-Swax column was used for *X. asperum* samples. These column types have similar polarity and would not result in significant differences between samples. All sample sizes and durations of samples are presented in ESM Table 1. Vegetative controls (excluding all floral parts) were taken from *X. asperum* and *X. tysonianum* leaves (one sample each) and used to identify volatiles that may be of non-floral origin. Compounds were identified using the Varian MS Workstation software (version 6.8) with the NIST 2011 mass spectral library and verified, where possible, using published retention indices and synthetic standards. 1-Pyrroline was identified by comparison with the mass spectrum for this compound presented in Chen et al. (2015). Emission rates were estimated by comparison of peak areas from samples with peak area obtained from the injection of a known amount of methyl benzoate (Sigma–Aldrich) into cartridges and thermally desorbed under identical conditions to the samples. We have previously established that on this apparatus, methyl benzoate yields a volume to peak area relationship close to the average obtained from the injection of 200 different synthetic standards representing various compound classes (unpubl. data).

Scent data for an additional six *Xysmalobium* taxa were obtained from Shuttleworth and Johnson (2012) and used for comparison of total odour profiles within the genus (these scent data were analysed on the same GC–MS as used for the current study). Differences between overall fragrance profiles were visualised using non metric-multidimensional scaling (NMDS) implemented in PRIMER 6.1.15. (2012) (Clarke and Warwick, 2001). NMDS was based on Bray–Curtis similarity and data were square-root transformed prior to analysis (Clarke and Warwick, 2001). A one-way Analysis of Similarity (ANOSIM) in PRIMER 6.1.15 was used to test for differences in scent profiles among species. ANOSIM is a non-parametric permutation procedure based on the similarity matrix underlying the ordination, and generates the test statistic R such that values of R close to unity indicate complete separation of groups while values close to zero indicate minimal separation (Clarke and Warwick, 2001). Significance of differences was determined by comparison of the calculated R value to values of R resulting from up to 10,000 random permutations of the sample labels (Clarke and Warwick, 2001). Compounds characterizing the odour of each species (and each population in the case of *X. parviflorum*) were identified using a Similarity-Percentages (SIMPER) analysis in PRIMER 6.1.15. This analysis calculates the percentage contributions of each compound to average overall Bray–Curtis similarity between samples within a group (Clarke and Warwick, 2001), and is useful for identifying compounds that best characterize a group.
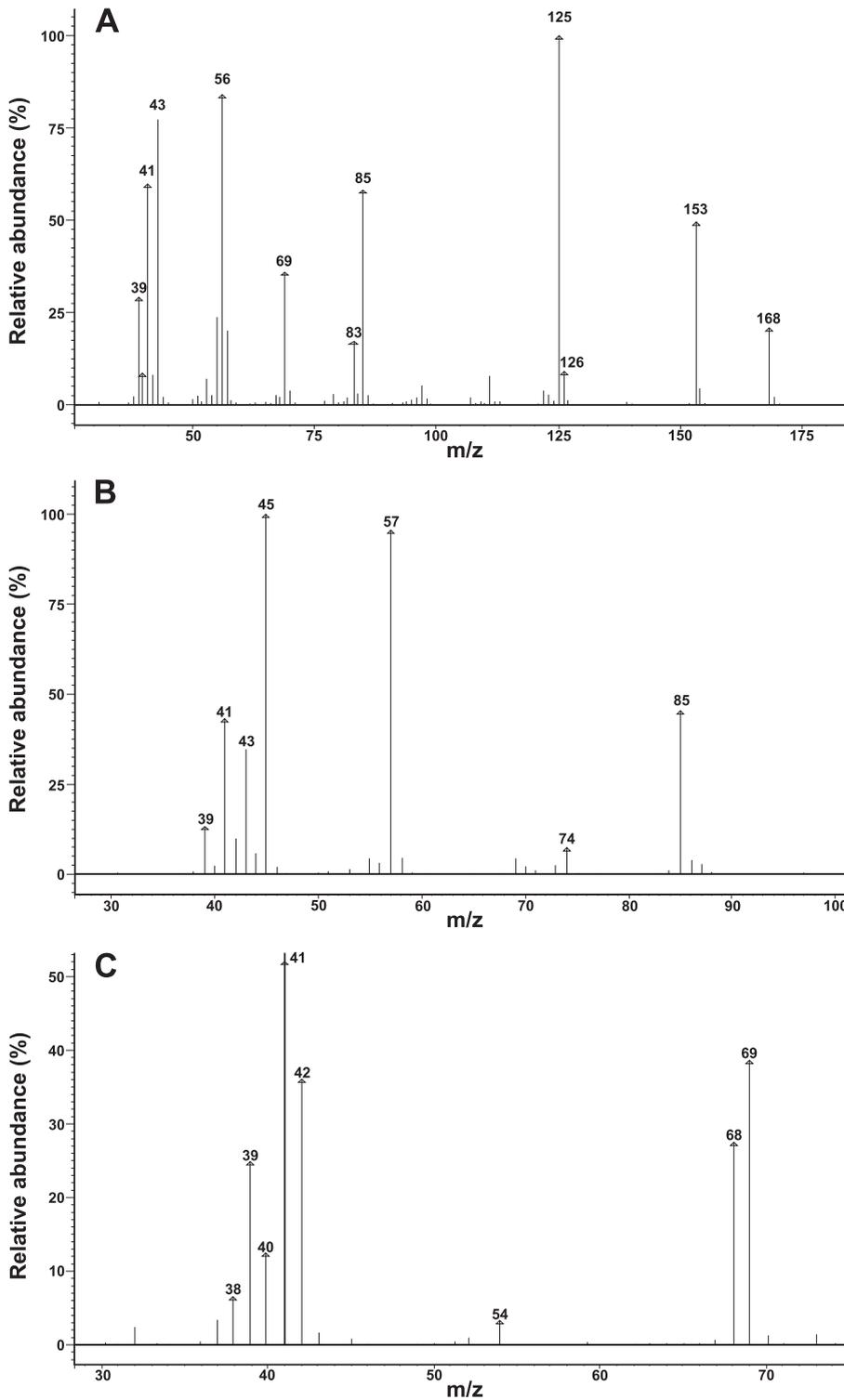
3. Results

Seventy four compounds from various compound classes were identified from headspace samples of the three *Xysmalobium* species examined in this study (Table 1, ESM Table 1). Thirty three compounds were detected from *X. asperum* samples (range = 15–29 across individual samples). The scent of this species was dominated by aromatics (particularly 1-methoxy-4-methylbenzene, benzaldehyde, benzyl alcohol and phenylacetaldehyde) and terpenoids (particularly epoxy oxoisophorone, 4-oxoisophorone, limonene and β-myrcene; see Fig. 2A for the mass spectrum of epoxy oxoisophorone; ESM Table 1). Five of these compounds plus a second unidentified compound (mass fragments: 43,41,56,39,109,71,87) accounted for the first 80% of Bray–Curtis similarity between samples of this species, with epoxy oxoisophorone and 4-oxoisophorone being particularly characteristic based on the SIMPER analysis (Table 1). Some samples also contained small amounts of *p*-cresol (ESM Table 1).

Forty four compounds were detected from *X. tysonianum* samples (range = 25–41 in individual samples). The odour of this species was dominated by isovaleric acid, various aliphatics (particularly isoamyl alcohol, 2,6-dimethylheptan-4-ol and 2,3-heptanedione), aromatics (particularly benzaldehyde, phenylacetaldehyde, benzyl alcohol, 2-phenylethyl alcohol and 4-

Table 1
Compounds characterizing the odour of each species based on a SIMPER analysis. % = contribution to overall Bray–Curtis similarity between samples within each group (species or population). Sim/SD = % contribution divided by standard deviation. High percentage contributions and high sim/SD values would indicate compounds that best characterize a species' scent. Mean relative amount = % of headspace based on peak area. Unknowns are presented with the molecular mass first (if known) indicated by a * followed by the base peak and remaining fragments in decreasing order of abundance. Note that compounds for which values are not presented for a particular species may still have been produced, but were not identified in the SIMPER analysis (see ESM Table 1 and Shuttleworth and Johnson, 2012 for complete scent profiles).

Compound	Xysmalobium asperum		Xysmalobium tysonianum		Xysmalobium parviflorum Lake Mertheley		Xysmalobium parviflorum Gilboa Estate	
	% (Sim/SD)	Mean relative amount (range)	% (Sim/SD)	Mean relative amount (range)	% (Sim/SD)	Mean relative amount (range)	% (Sim/SD)	Mean relative amount (range)
Aliphatics								
Alcohols								
Isoamyl alcohol	–	–	3.2 (2.95)	1.8 (0.40–6.67)	–	–	–	–
2,6-Dimethylheptan-4-ol	–	–	6.5 (4.63)	4.4 (1.50–12.81)	–	–	–	–
Ketones								
2,3-Heptanedione	–	–	9.4 (4.74)	10.5 (2.47–37.48)	–	–	–	–
C-5 Branched chain compounds								
Isovaleric acid	–	–	6.0 (1.29)	6.03 (0.08–12.62)	–	–	–	–
Aromatics								
Benzaldehyde	7.9 (1.77)	3.3 (0.58–6.06)	28.1 (7.08)	50.6 (29.16–70.73)	8.5 (4.51)	5.8 (2.14–10.07)	11.9 (13.35)	13.2 (9.30–17.11)
Phenylacetaldehyde	–	–	5.2 (1.98)	5.5 (0.33–21.93)	–	–	–	–
Benzyl alcohol	–	–	3.9 (6.38)	1.1 (0.52–1.84)	2.9 (12.97)	0.6 (0.36–1.25)	4.7 (24.33)	2.0 (1.60–2.68)
2-Phenylethyl alcohol	–	–	2.4 (3.97)	0.6 (0.16–1.44)	–	–	–	–
p-Creosol	–	–	–	–	–	–	2.4 (2.74)	1.0 (0.27–1.83)
4-Methoxybenzaldehyde	–	–	6.6 (3.19)	5.5 (1.04–15.54)	–	–	–	–
p-Cresol	–	–	–	–	–	–	12.6 (17.38)	17.5 (11.25–28.29)
Terpenoids								
Monoterpenes								
α-Pinene	–	–	–	–	3.4 (2.41)	1.7 (0.29–3.72)	8.5 (10.11)	15.4 (4.88–34.81)
Limonene	7.7 (1.46)	9.7 (0.44–24.94)	–	–	13.0 (2.42)	13.6 (3.15–20.96)	10.2 (17.01)	10.1 (7.50–14.53)
β-Myrcene	5.8 (1.26)	4.8 (0.20–11.85)	–	–	13.8 (1.17)	28.8 (0.42–59.80)	10.5 (7.53)	16.2 (7.18–31.06)
(Z)-β-Ocimene	–	–	–	–	8.4 (5.42)	4.5 (2.85–7.17)	8.1 (15.92)	5.7 (4.68–7.01)
(E)-β-Ocimene	–	–	–	–	15.6 (4.51)	15.7 (7.73–24.57)	11.0 (26.16)	10.3 (8.77–12.85)
Linalool	–	–	–	–	3.2 (11.04)	1.5 (0.42–5.50)	–	–
Irregular terpenes								
Epoxy oxoisophorone	38.1 (13.18)	38.7 (28.03–56.46)	–	–	–	–	–	–
4-Oxoisophorone	18.4 (3.32)	10.4 (4.75–14.24)	–	–	–	–	–	–
Nitrogen-containing compounds								
1-Pyrroline	–	–	–	–	7.6 (1.33)	13.95 (0.25–37.27)	2.6 (2.28)	1.7 (0.28–3.53)
Unknowns								
m/z: 43,41,56,39,109,71,87	4.5 (2.02)	1.1 (0.21–0.98)	–	–	–	–	–	–
m/z: 150*,69,41,81,79,82,53	–	–	–	–	5.9 (2.48)	4.5 (0.74–9.79)	–	–
m/z: 130*,57,45,85,41,43,39	–	–	6.0 (4.72)	3.9 (1.26–12.29)	–	–	–	–
m/z: 162*,91,43,119,65,92	–	–	4.2 (1.45)	4.1 (0.12–12.56)	–	–	–	–
Total	82.4		81.7		82.2		82.4	
Average similarity	48.8		70.3		66.9		76.5	
Number of compounds	6		11		10		10	

Fig. 2. Mass spectra (normalized to the base peak) for some of the dominant compounds present in *Xysmalobium* scent samples which are not included in the NIST 2011 mass spectral library. **A**, Epoxy oxoisophorone (see ESM Table 1 for synonyms) representing between 28 and 31% of the of the headspace odour of *X. asperum*. **B**, Unidentified compound representing between 1 and 12% of the headspace odour of *X. tysonianum* and also present in small amounts in *X. asperum*. **C**, 1-Pyrroline which accounted for up to 37% of the headspace in samples from the Lake Merthley *X. parviflorum* population. Compare this mass spectrum with that of 1-pyrroline presented in Fig. 3 of Chen et al. (2015).

methoxybenzaldehyde) and two unidentified compounds (mass fragments in decreasing order of abundance: 57,45,85,41,43,39 [see Fig. 2B for the mass spectrum of this compound] and 91,43,119,65,92; molecular weights 130 and 162 respectively; ESM Table 1). These 11 compounds accounted for the first 80% of Bray−Curtis similarity between samples (Table 1).

Thirty four compounds were detected from samples of the Lake Merthley *X. parviflorum* population (range = 27−32 in individual samples). The odour of flowers from this population was dominated by benzaldehyde, several common floral monoterpenes (particularly limonene, β-myrcene, both (E)- and (Z)-β-ocimene and α-pinene) and 1-pyrroline (see Fig. 2C for the mass spectrum of the latter; Table 1, ESM Table 1). *p*-Cresol was present in very small amounts and did not make up more than 1.8% of the total odour for any sample. These compounds plus an additional three accounted for the first 80% of Bray−Curtis similarity between samples (Table 1). The dung-scented flowers from the Gilboa Estate population of this species exhibited a similar profile, except that *p*-cresol was a dominant component (range = 11−28%, n = 3) while 1-pyrroline was present in much smaller relative amounts (range = 0.3−3.5%, n = 3) (Table 1; Shuttleworth and Johnson, 2012). Thirteen compounds, mainly aliphatics and aromatics plus 4-oxoisophorone, were present in samples from the Lake Merthley flowers, but were not detected in samples from the Gilboa Estate flowers (Table 2). Twelve compounds, mostly aromatics and sesquiterpenes, were present in samples from the Gilboa Estate flowers but not detected in the samples from the Lake Merthley flowers (Table 2). Scents of flowers from the two populations were significantly different and clearly separated in two-dimensional scent space (Fig. 3A). The SIMPER analysis identified eight compounds, mostly common monoterpenes, which were common to flowers from both populations and particularly characterised the overall scents (Table 1). However, linalool and a single unidentified compound characterized the scents of the Lake Merthley flowers but not the Gilboa Estate flowers, while *p*-cresol and *p*-creosol characterized the scents of the Gilboa Estate flowers but not the Lake Merthley flowers (Table 1).

Table 2
Compounds unique to samples from each of the *Xysmalobium parviflorum* populations. Values represent relative amounts (%) of total headspace, tr = compounds that represent <0.005% of the total odour. See Table 2 for an explanation of the unknowns.

Compound	Lake Merthley					Gilboa Estate		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3
Aliphatics								
Alcohols								
3-Ethyl-4-methylpentan-1-ol	2.21	0.61	0.02	1.16	0.23	–	–	–
Alkanes								
Tricosane	0.45	0.03	0.01	0.18	0.08	–	–	–
Ketones								
2,3-Heptanedione	0.75	tr	–	–	tr	–	–	–
Aromatic compounds								
1-Phenyl-1,2-propanedione	tr	tr	0.27	0.20	0.11	–	–	–
4-Methoxybenzaldehyde	11.13	tr	–	0.10	–	–	–	–
1,4-Dimthoxybenzene	0.44	–	–	–	–	–	–	–
1,3-Dimethoxybenzene	1.92	–	–	–	–	–	–	–
4-Methoxybenzyl alcohol	0.79	–	–	–	–	–	–	–
p-Creosol	–	–	–	–	–	1.83	0.95	0.27
3-Phenylpropanol	–	–	–	–	–	–	0.09	–
(E)-Cinnamaldehyde	–	–	–	–	–	–	0.03	–
Cinnamic alcohol	–	–	–	–	–	–	0.03	–
Terpenoids								
Sesquiterpenes								
β-Bourbonene	–	–	–	–	–	0.02	0.14	tr
α-Cubebene	–	–	–	–	–	0.06	0.08	–
(E)-β-Farnesene	–	–	–	–	–	–	0.04	–
m/z: 204*,120,161,105,93,91,79	–	–	–	–	–	0.01	0.05	tr
m/z: 204*,93,69,41,105,79,94,91	–	–	–	–	–	0.23	0.23	–
m/z: 204*,122,161,107,105,93,81,91	–	–	–	–	–	0.17	0.03	–
m/z: 204*,161,105,93,91,119,79,77,81,92,133	–	–	–	–	–	0.11	–	–
m/z: 204*,161,105,55,189,119,85,83,95,91,133	–	–	–	–	–	0.07	–	–
Irregular terpenes								
4-Oxoisophorone	12.24	0.26	0.24	1.03	0.10	–	–	–
Unknowns								
m/z: 150*,69,41,81,79,82,53	9.79	0.74	3.67	7.04	1.23	–	–	–
m/z: 69,41,119,39,79,53	tr	tr	0.35	0.63	tr	–	–	–
m/z: 57,43,71,85,41,32	0.14	0.01	–	0.25	0.01	–	–	–
m/z: 41,43,39,67,32,69	–	–	0.07	–	–	–	–	–
Number of aliphatics	3	3	2	2	3	0	0	0
Number of aromatics	5	2	1	2	1	1	4	1
Number of terpenoids	1	1	1	1	1	7	6	2
Number of unknowns	3	3	3	3	3	0	0	0
Total number of compounds	12	9	7	8	8	8	10	3
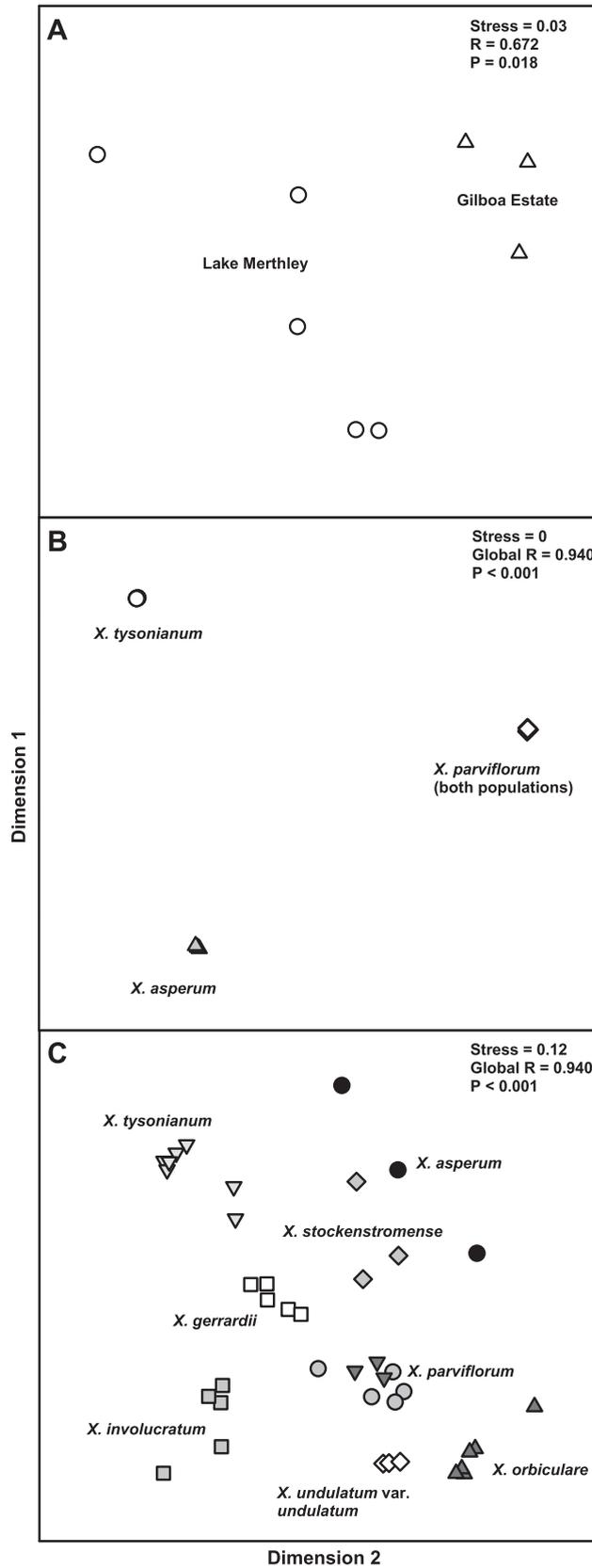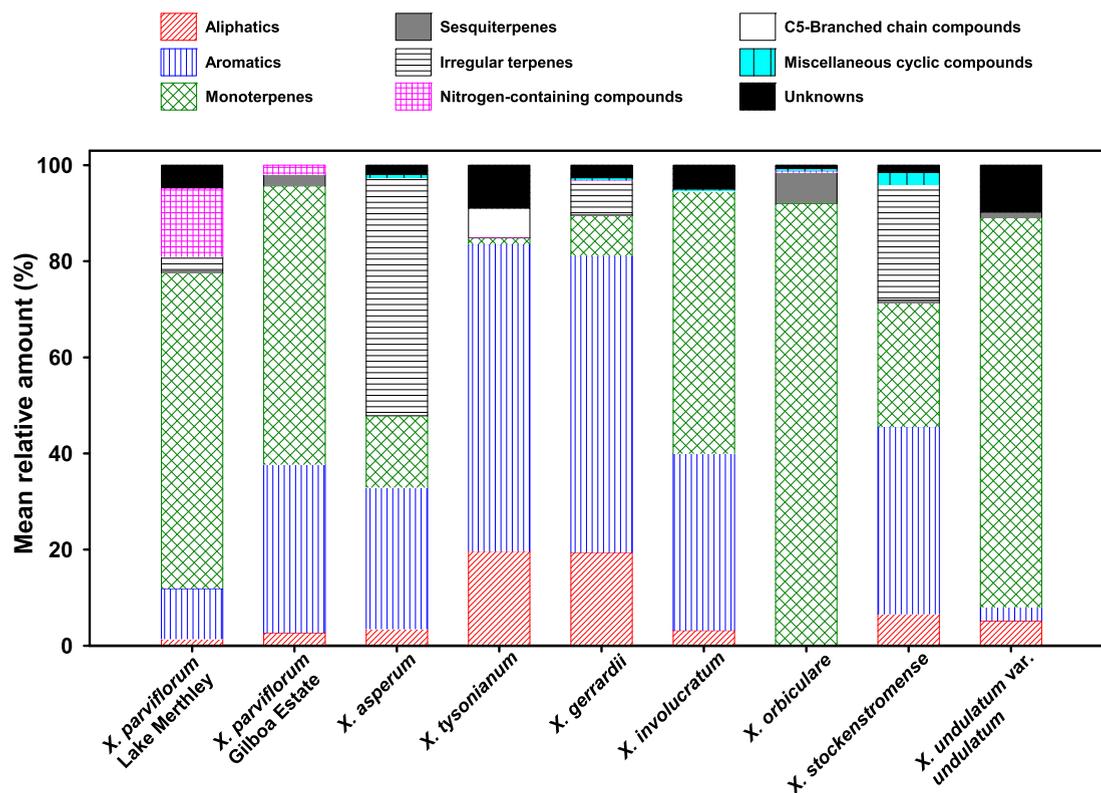
Fig. 4. Mean relative amounts (%) of the total odour profile made up by different compound classes for all *Xysmalobium* species for which scent data are available. Differences in the relative amounts of nitrogen-containing compounds and aromatics in plants from the two populations of *X. parviflorum* are due to the effects of 1-pyrroline and *p*-cresol respectively. The large proportion of irregular terpenes in *X. asperum* is due primarily to the contribution of epoxy oxoisophorone (see Fig. 3A). The same compound is also responsible for the large proportion of irregular terpenes in the scent of *X. stockenstromense* (see Shuttleworth and Johnson, 2012). The large proportion of C5-branched chain compounds in *X. tysonianum* is due solely to isovaleric acid.

Table 3

Test statistics (R) resulting from pairwise ANOSIM contrasts between scent profiles for all *Xysmalobium* species for which scent data are available. See text for sources of additional scent data. * = p < 0.05; ** = p < 0.01; [a] = sample size too small to yield sufficient permutations to establish significance.

	X. asperum	*X. gerrardii*	*X. involucratum*	*X. orbiculare*	*X. parviflorum* Gilboa Estate	*X. parviflorum* Lake Merthley	*X. stockenstromense*	*X. tysonianum*
X. gerrardii	1*	–	–	–	–	–	–	–
X. involucratum	1*	0.780**	–	–	–	–	–	–
X. orbiculare	1*	1**	1**	–	–	–	–	–
X. parviflorum Gilboa Estate	1[a]	1*	0.938*	0.735*	–	–	–	–
X. parviflorum Lake Merthley	1*	1**	1**	0.725**	0.672*	–	–	–
X. stockenstromense	0.741[a]	1*	0.938*	1*	1[a]	1*	–	–
X. tysonianum	1**	1**	1**	1**	1**	1**	1**	–
X. undulatum var. *undulatum*	1[a]	1*	1*	0.642*	1[a]	0.785*	1[a]	1**

The three species examined in this study exhibited very distinct chemical profiles and occupied clearly separated clusters in two-dimensional scent space based on non-metric multidimensional scaling (NMDS; Figs. 3B and 4). A meta-analysis using the scent data for these species combined with previously published scent data for the genus *Xysmalobium* revealed very distinct interspecific chemical profiles, with species occupying clearly separated clusters in two-dimensional scent space

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination of total scent profiles for *Xysmalobium* species. **A**, Lake Merthley and Gilboa Estate populations of *X. parviflorum*. **B**, Three species from the current study (including samples from both *X. parviflorum* populations). **C**, All *Xysmalobium* species for which scent data are currently available. *X. parviflorum* includes both Lake Merthley (grey circles) and Gilboa Estate (dark grey inverted triangles) populations. See Table 2 for the results of pairwise ANOSIM contrasts between taxa.

(Fig. 3C). Pairwise ANOSIM contrasts between species yielded mostly significant differences with high R values, suggesting clear divisions with limited overlap between taxa (Table 3). Comparison of the proportions of the total odour profiles made up by particular compound classes highlighted the broad variation in overall chemical profiles within the genus (Fig. 4). The scents of *X. tysonianum* and *X. gerrardii* were dominated by aliphatics and aromatics. The scents of the remaining taxa were dominated by either aromatics and monoterpenes (*X. parviflorum* from Gilboa Estate, *X. involucratum* and *X. stockenstromense*) or just monoterpenes with small contributions from other compounds classes (*X. orbiculare* and *X. undulatum* var. *undulatum*). *Xysmalobium parviflorum* from Lake Merthley was the only taxon which displayed an appreciable proportion of nitrogen-containing compounds, largely due to the dominance of 1-pyrroline in the scent of flowers from this population (Fig. 4).

4. Discussion

4.1. Intraspecific variation in X. parviflorum and the chemistry of semen-like scent in this species

The clear qualitative differences (to the human observer) between the scents of flowers from each of the *X. parviflorum* populations are supported by the chemistry underlying these scents (Tables 1–3, ESM Table 1; Fig. 3A). Flowers from the semen-scented Lake Merthley population produced larger relative amounts of the nitrogen-containing 1-pyrroline (also known as 3,4-dihydro-2H-pyrrole) and lower amounts of *p*-cresol compared to the dung-scented flowers from Gilboa Estate (Tables 1 and 2). 1-Pyrroline has been identified in other flowers which exhibit a semen-like scent to the human observer (Naef et al., 2002; Kaiser, 2006a,b; Chen et al., 2015) and Chen et al. (2015) provide empirical evidence that this compound is responsible for the strong semen-like scent of *Stemona japonica* (Stemonaceae) flowers. In contrast, the dung-like odour of flowers from the previously studied Gilboa Estate population likely results from the higher relative amount of *p*-cresol in the scents of flowers from this population (Shuttleworth and Johnson, 2012, Table 1). *p*-Cresol is a well-established component of the odour of herbivore dung and is often associated with dung-like floral scents (Kite, 1995; Jürgens et al., 2006; Schiestl and Dötterl, 2012). In addition to these differences in relative amounts, 25 compounds were detected that were not shared between the populations (Table 2). These results highlight the influence of single compounds on the perception of odours by the human olfactory system (humans are, for example, particularly sensitive to *p*-cresol; Leonardos et al., 1969), but it is not clear whether these differences between populations would have a significant effect on the attraction of pollinators.

Relative amounts of different active compounds can play a critical role in the attraction of particular pollinators by sexually deceptive orchids (Schiestl et al., 1999), but it is not clear if this would extend to other systems. In this instance, active compounds remain unknown, although *p*-cresol is frequently produced by oviposition-site mimicking flowers (Jürgens et al., 2013) and has been shown to be attractive to gravid stable flies (*Stomoxys calcitrans*, Muscidae; Jeanbourquin and Guerin, 2007). This suggests that the large relative amounts of *p*-cresol produced by the Gilboa Estate flowers may play an important functional role in the attraction of the calliphorid, muscid and scathophagid fly pollinators recorded at this site (Johnson et al., 2009; Shuttleworth and Johnson, 2009c). However, 1-pyrroline was also produced by flowers from this population, albeit in smaller amounts relative to *p*-cresol, and could also contribute to pollinator attraction.

1-Pyrroline is an uncommon floral volatile and its functional significance as a component of floral odours remains unclear. The association of this compound with flowers pollinated by saprophilous flies is suggestive however (Chen et al., 2015; this study). In carrion, 1-pyrroline is produced through oxidation of the diamine putrescine, a well-established component of decaying vertebrate tissue (Amoore et al., 1975; Paczkowski and Schutz, 2011). Chen et al. (2015) suggest that production of 1-pyrroline may therefore represent a particular form of carrion mimicry for the attraction of carrion-associated insects as pollinators. It is interesting to note that 1-pyrroline has a considerably higher vapour pressure (and therefore volatility) than putrescine (3246.4 Pa versus 310.6 Pa at 25 °C respectively), and could thus represent an indirect, easier-to-detect, signal for the presence of putrescine, and therefore decaying vertebrate tissue. This hypothesis, however, was not supported by bioassays conducted by Chen et al. (2015) using traps baited with pure 1-pyrroline, as these failed to attract any saprophilous flies. Aside from floral scents, 1-pyrroline has also been reported as a major component of the pheromone blend produced by male mediterranean fruit flies (*Ceratitis capitata*, Tephritidae) and is individually attractive to female fruit flies (Baker et al., 1985; Jang et al., 1989). More interesting, however, is that several of these studies have suggested that 1-pyrroline has a synergistic effect on the attractiveness of combinations of other volatiles to mediterranean fruit flies, and its principal function in the pheromone blend appears to be one of enhancing attractiveness of the overall blend rather than direct individual attractiveness (Robacker et al., 1997; Jang et al., 1994). This compound may play a similar role in floral scents and could enhance the attractiveness of other compounds or blends of compounds (such as *p*-cresol and other components of the overall odour blend) to saprophilous flies.

Although semen-like or spermy odour is "frequently found in flower scents" (Kaiser, 2006b, p. 177), 1-pyrroline remains virtually unknown in descriptions of floral volatiles. To my knowledge, this compound has only been reported as a component of floral scent in the studies cited above and is not listed in the comprehensive review of floral volatiles compiled by Knudsen et al. (2006). This may, in part, be due to difficulties associated with identifying this compound as it is not included in the NIST 2011 Mass Spectral Library and a synthetic standard is not readily available. At this stage the importance of 1-pyrroline in floral scents remains speculative and the functional significance of this compound in floral scents, if any, ultimately requires detailed electrophysiology experiments coupled with bioassays testing the attractiveness of this compound singly and in combination with other components of the odour to pollinators.

4.2. Microbial degradation/fermentation volatiles and the sweaty sock scent of X. tysonianum

The strong sweaty odour of *X. tysonianum* flowers is characterized by relatively large amounts of sweet-scented aromatic compounds, combined with foetid volatiles typically associated with microbial degradation or fermentation of organic material (isovaleric acid, 2,3-heptanedione and 2,6-dimethylheptan-4-ol; Tables 1 and 2) which are responsible for the unpleasant odour (to the human nose). Isovaleric acid and 2,3-heptanedione are well-established components of the odours of various cheeses and isovaleric acid is also often described as smelling like sweaty socks (Tavaria et al., 2002; Thierry et al., 2002; Sadecka et al., 2014; Boltar et al., 2015). Isovaleric acid has also been reported from the odours of pig and dog faeces (Johnson and Jürgens, 2010; Jürgens et al., 2013), and decaying vertebrate tissue (Paczkowski and Schutz, 2011). 2,3-Heptanedione has been identified in the odour of spoiled shrimp (Jaffres et al., 2011). 2,6-Dimethylheptan-4-ol is less commonly encountered but has been reported from the odour of fermented milk (Irigoyen et al., 2012). These volatiles have also been reported from other foetid smelling flowers and isovaleric acid, in particular, has often been associated with unpleasant floral odours (Kite and Smith, 1997, Jürgens et al., 2006, 2012, Van der Niet et al., 2010; although isovaleric acid has also been reported as a component of non-foetid floral scent, see Jersáková et al., 2016). 2,3-Heptanedione has been reported as a component of the unpleasant odours of ornamental gentians (Gentianaceae; Lee et al., 2010). 2,6-Dimethylheptan-4-ol has seldom been reported as a floral volatile, but interestingly, has been reported in combination with both isovaleric acid and 2,3-heptanedione in the scents of Hawaiian *Schiedea* species (Caryophyllaceae; Jürgens et al., 2012).

At this stage, the functional significance of the production of sweet aromatic compounds in combination with degradation volatiles is not clear, although this may represent a form of oviposition-site mimicry to attract insects that would normally oviposit in decaying organic matter (Urru et al., 2011). Similar combinations of degradation volatiles with sweet aromatic compounds have occasionally been reported (Erhardt, 1993; Van der Niet et al., 2010), and are usually associated with pollination by saprophilous flies. In addition, Zito et al. (2013) have demonstrated a functional role for sweet terpenoid volatiles in the attraction of houseflies to *Caralluma europaea* suggesting that both sweet and foetid volatiles may influence pollinator behaviour. A hypothesis of oviposition-site mimicry, however, would require more detailed analyses of the ecology and life histories of the plant's pollinators (not yet established for *X. tysonianum*).

4.3. Chemistry of the sweet but faintly foetid scent of X. asperum

The scent of *X. asperum* was dominated by epoxy oxoisophorone (Fig. 3A; see ESM Table 1 for synonyms) in combination with various sweet-scented aromatic and terpenoid volatiles and small amounts of *p*-cresol (Table 1, ESM Table 1). The latter may account for the faintly foetid note in the scent of this species to the human nose. Epoxy oxoisophorone has previously been reported in the scents of four other asclepiad species (Shuttleworth and Johnson, 2010, 2012; described as an unidentified compound with molecular weight 168 and mass fragments 56,85,125,43,41,69,153,83 in these studies) and is also known from the scents of three species of *Buddleja* (Scrophulariaceae) and two species of *Nicotiana* (Solanaceae) (Raguso et al., 2003; Chen et al., 2014; Gong et al., 2014). Although uncommonly encountered in floral scents, when present, epoxy oxoisophorone often accounts for a large proportion of the scent (up to 34% in *X. stockenstromense*, 39% in *Nicotiana bonariensis* and 12% in *Buddleja davidii*; Raguso et al., 2003; Shuttleworth and Johnson, 2012; Chen et al., 2014). This pattern was also observed in the current study, with this compound accounting for up to 56% (mean ± s.d. = 39 ± 15.5%, n = 3) of the scent of *X. asperum* (ESM Table 1). The functional significance of this compound is not clear, although it has been shown to elicit strong antennal responses in butterflies which forage on *B. davidii* flowers (Andersson, 2003).

Samples from *X. asperum* species were somewhat variable in terms of total emission (with sample 3 being considerably stronger than the other samples), number of compounds and composition (ESM Table 1). This variation may be an artefact of sampling flowers on cut stems which were kept in vases for varying lengths of time prior to sampling (Johnson et al., 2005). Interestingly, sample 3 (with the highest total emission rate) was kept in water for the longest time prior to sampling (overnight). However, the flowers for samples 1 and 2 were collected shortly after heavy rain (but sampled several hours later) which may have influenced the composition and strength of the resultant headspace samples.

4.4. Diversity of chemical profiles in the genus Xysmalobium

Total odour profiles are seldom considered in studies examining the evolution of floral scents, although all components of the scent represent traits that are available for selection by interaction partners (Schiestl, 2015). This study showed that members of the genus *Xysmalobium* for which scent data are available exhibit surprisingly distinct, clearly defined, chemical profiles with limited overlap between species (Figs. 2 and 4; Table 3). These broad patterns are difficult to interpret in an ecological context although the importance of scent as a pollinator attractant in many South African asclepiads suggests that some differences may be attributable to selection by divergent pollinators (Shuttleworth and Johnson, 2012). However, this would also suggest some degree of convergence in the scents of species that share similar pollinators, a pattern which is not clearly illustrated by these data. For example, *X. stockenstromense*, *X. orbiculare* and *X. undulatum* var. *undulatum* are all specialised for pollination by the same wasps in the genus *Hemipepsis* (Pompilidae; Shuttleworth and Johnson, 2008, 2009b,c), but are widely separated in two-dimensional scent space (Fig. 3C) and exhibit quite distinct total scent profiles based on compound classes (Fig. 4). Interestingly, scent has been empirically established as a key pollinator attractant for *X. orbiculare* (Shuttleworth and Johnson, 2009b) and is assumed for *X. stockenstromense* and *X. undulatum* var. *undulatum*

(Shuttleworth and Johnson, 2012). In this case, it seems likely that pollinators are attracted by highly specific components of the scent (Shuttleworth and Johnson, 2012), which may have allowed divergence in the production of other (non-active) compounds through either drift (Mant et al., 2005) or selection by factors other than pollinator attraction (Kessler et al., 2013). A similar explanation may be applicable to other members of the genus *Xysmalobium*. Minor components of the total odour blend often have disproportionately large functional roles (Clavijo McCormick et al., 2014) and it seems feasible to expect that specific individual components or combinations of a few components (e.g. *p*-cresol and 1-pyrroline in *X. parviflorum* or isovaleric acid and 2,3-heptanedione in *X. tysonianum*) may be sufficient for pollinator attraction leaving the remainder of the volatile profiles to diverge through drift or selection by non-pollinators. This hypothesis is supported by studies of sexually deceptive *Ophrys* orchids, where non-active (to pollinators) components of the scents are often more variable than active compounds (Ayasse et al., 2000; Mant et al., 2005; Cortis et al., 2009).

Ancestry may also account for part of the patterns observed here. Unpublished morphological studies have suggested that *Xysmalobium* may be polyphyletic (Langley, 1980) and divergent scent profiles may thus be partly due to phylogenetic factors.

Studies over the last 15yr examining particular active compounds for pollinator attraction have greatly enhanced our understanding of the ecology and evolution of floral scents (Schiestl, 2015). However, our understanding of the functions and evolution of total odour bouquets remains limited, although recent studies have pointed to the importance of both mutualists and antagonists in imposing selection on floral scents (Kessler et al., 2013; Schiestl, 2015). The patterns reported in this study highlight the multivariate nature of scent as a component of the floral phenotype and emphasize the need to consider all components of the scent in the context of pollinators, antagonists and phylogeny in order to understand the evolution of total odour bouquets.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2016.03.009.

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