

Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception

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Summary

- Sexually deceptive orchids employ floral volatiles to sexually lure their specific pollinators. How and why this pollination system has evolved independently on multiple continents remains unknown, although preadaptation is considered to have been important. Understanding the chemistry of sexual deception is a crucial first step towards solving this mystery.
- The combination of gas chromatography-electroantennographic detection (GC-EAD), GC-MS, synthesis and field bioassays allowed us to identify the volatiles involved in the interaction between the orchid *Drakaea glyptodon* and its sexually attracted male thynnine wasp pollinator, *Zaspilothynnus trilobatus*.
- Three alkylpyrazines and one novel hydroxymethyl pyrazine were identified as the sex pheromone of *Z. trilobatus* and are also used by *D. glyptodon* for pollinator attraction. Given that our findings revealed a new chemical system for plants, we surveyed widely across representative orchid taxa for the presence of these compounds. With one exception, our chemical survey failed to detect pyrazines in related genera. Collectively, no evidence for preadaptation was found.
- The chemistry of sexual deception is more diverse than previously known. Our results suggest that evolutionary novelty may have played a key role in the evolution of sexual deception and highlight the value of investigating unusual pollination systems for advancing our understanding of the role of chemistry in evolution.

Introduction

As the first step in achieving pollination, plants use a diverse range of visual and olfactory cues to advertise to animal pollinators (Raguso, 2004). Subsequently, food rewards such as nectar and pollen are typically provided to encourage repeat visitation (Whitehead *et al.*, 2012), although some plants achieve pollination by deception (Cozzolino & Widmer, 2005; Vereecken & McNeil, 2010). Floral volatiles are often critical for attracting insect pollinators, with the often complex chemical bouquets acting to attract pollinators, while at the same time filtering out unwanted flower visitors such as herbivores (Pichersky & Gershenzon, 2002; Raguso, 2008; Kessler *et al.*, 2013). Given their importance for plant reproduction, it has long been hypothesized that floral odours may hold the key to understanding flowering plant evolution (Stebbins, 1970), yet much work remains to be done to fully test this hypothesis (Jürgens, 2009; Schiestl, 2010; Schiestl & Dötterl, 2012).

Documenting the diversity of floral volatile components is the first step towards a better understanding of their evolutionary role. Knudsen *et al.* (2006) listed > 1700 compounds found in the headspace of flowers surveyed from across 90 plant families. The diversity of floral volatiles is particularly high in some plant groups and for some pollination systems. For example, in the Annonaceae, which includes pawpaw (*Asimina*), almost 300 floral volatile compounds have been found in just one survey of 11 species spanning two genera (Goodrich & Raguso, 2009). Plants that achieve pollination via the chemical mimicry of insect oviposition sites are also characterized by high chemical diversity in the composition of floral volatiles across five unrelated plant families (Jürgens *et al.*, 2013).

A critical next step is to determine the function of the chemical components and how they act, either singly or synergistically, to achieve pollinator attraction and/or herbivore deterrence. Generally, GC coupled with electroantennography (GC-EAD) is used to determine which of the often many floral volatiles are detected

by the insect antennae (Schiestl & Marion-Poll, 2002). Next, GC-MS is used to aid chemical identification of these compounds. Finally, bioassays in the laboratory or field are used to confirm the biological activity of individual compounds or blends of compounds. Compared with the large number of plant species surveyed for floral volatile composition, far fewer studies have determined the function of individual volatile compounds, with most of these focusing on the more specialized pollination systems, such as nursery pollination.

Nursery pollination systems are often specialized obligate mutualisms in which specific seed predators are also pollinators (Svensson *et al.*, 2010). In *Ficus semicordata*, just one unusual compound, 4-methylanisole, is involved in pollinator attraction (Chen *et al.*, 2009). Other cases of nursery pollination – such as *Silene latifolia* and its moth pollinator *Hadena bicruris* (Dötterl *et al.*, 2006), *Breynia* and its host-specific *Epicephala* moth pollinators (Svensson *et al.*, 2010), and the globe flower *Trollius europaeus* (Ibanez *et al.*, 2010) – appear to achieve pollinator specificity by using combinations of a few common floral compounds. In specialized bee pollination systems, uncommon compounds, such as the spiroacetals in *Campanula* flowers (Milet-Pinheiro *et al.*, 2013) or 1,4-benzoquinone in *Echium* flowers (Burger *et al.*, 2012), provide plant-specific cues. Some highly specialized beetle pollination systems also employ unusual compounds in pollinator attraction. For example, *Philodendron selloum* secures specific beetle pollination by emitting unusual methoxylated aromatic compounds, with just one compound, 4-methoxystyrene (4-vinylanisole), sufficient for pollinator attraction (Dötterl *et al.*, 2012).

Another highly specialized pollination mechanism in which floral volatiles play a key role in pollinator attraction is sexual deception. Within the Orchidaceae, this pollination strategy has evolved independently on at least four continents (Africa, Australia, Europe and South America) and is employed by several hundred orchid species (Schiestl, 2005; Gaskett, 2011). Furthermore, new discoveries of sexual deception continue to be made both within (Phillips *et al.*, 2014a) and beyond the Orchidaceae (e.g. in a daisy (Ellis & Johnson, 2010) and an iris (Vereecken *et al.* 2012)), suggesting it may be even more widespread than currently recognized.

Sexually deceptive orchids lure the males of specific insect species to their flower by emitting volatile semiochemicals that mimic the female sex pheromone (Schiestl *et al.*, 1999, 2003; Franke *et al.*, 2009), with pollination occurring during precopulatory behaviour or attempted copulation with the flower (Peakall, 1990). Although a combination of olfactory, visual and tactile mimicry may be essential to achieve pollination (Peakall, 1990; Schiestl, 2005; Gaskett, 2011), long-range attraction is achieved via floral volatiles (Streinzer *et al.*, 2009; Vereecken & Schiestl, 2009; Peakall *et al.*, 2010; Ayasse *et al.*, 2011). As a by-product of mimicking the specific sex pheromones of insects, these orchids are characterized by highly specific pollination systems that confer strong reproductive isolation (Xu *et al.*, 2011; Peakall & Whitehead, 2014; Whitehead & Peakall, 2014). Therefore, the semiochemicals involved have probably played key roles in enabling rapid

pollinator-driven speciation (Peakall *et al.*, 2010; Xu *et al.*, 2012; Van der Niet *et al.*, 2014).

While to date the semiochemicals used by sexually deceptive orchids have only been confirmed for species from two unrelated genera, a diversity of chemical systems is involved. The specific interaction between *Chiloglottis* orchids and their thynnine wasp pollinators involves one, two or three components from a pool of six related compounds, representing a unique class of natural products, all 2,5-dialkylcyclohexane-1,3-diones or ‘chiloglottes’ (Schiestl *et al.*, 2003; Franke *et al.*, 2009; Peakall *et al.*, 2010). Alternatively, in *Ophrys* sexual attraction of male bee pollinators is achieved by complex mixtures of alkanes and alkenes, with species specificity achieved by unique blends of alkenes (Schiestl *et al.*, 1999; Mant *et al.*, 2005; Stöckl *et al.*, 2007; Ayasse *et al.*, 2011). An exception occurs in *Ophrys speculum*, which is pollinated by the scoliid wasp *Camposcolia ciliata* (Ayasse *et al.*, 2003), where there are 10 active compounds, with 9-hydroxy-decanoic acid essential for eliciting pollinator copulatory behaviour. Similar polar compounds are also involved in the sexual attraction of males of the cuckoo bumblebee *Bombus vestalis*, which is the specific pollinator of two endemic *Ophrys* of Sardinia (Gögler *et al.*, 2009). The diversity of chemical systems indicates that different biosynthetic pathways are involved in the frequent evolution of sexual deception.

Just how and why pollination by sexual deception has evolved repeatedly remains a mystery. In general, it is considered that preadaptations are crucial for the evolution of many plant–animal interactions (Armbruster, 1997; Armbruster *et al.*, 2009). In the most extensive macroevolutionary study of its kind to date, Armbruster *et al.* (2009) concluded from their analyses across the neo- and paleotropical *Dalechampia* that exaptation (the coopting of pre-existing function) played a key role in the evolution of both defence and pollination systems. However, some traits lacking an obvious prior function were implicated as arising by evolutionary novelty (Armbruster *et al.*, 2009).

Preadaptation also provides the best explanation for the evolution of the alkene-based sexual deception in *Ophrys* orchids. A chemical analysis by Schiestl & Cozzolino (2008) of the presence of alkenes across 20 species, representing *Ophrys* and nine related genera, including *Serapias* within the Orchidinae, revealed that alkenes were present in all genera. This chemical analysis, when combined with a phylogenetic overlay, indicated that alkene production evolved earlier than sexual deception. It was further hypothesized that as components of floral cuticular hydrocarbons, alkenes contribute to the crucial function of desiccation resistance (Schiestl & Cozzolino, 2008). More recently, Vereecken *et al.* (2012) reported observations of bee pollination by pseudocopulation in *Serapias lingua*, which they interpreted as an independent evolution of sexual deception enabled by the presence of alkenes as a preadaptation (although we note that confirmation of the biological activity of alkenes in this particular system is lacking).

In this study we investigate the chemical basis of sexual deception in the Australian orchid genus *Drakaea*, the putative sister genus to *Chiloglottis* (Kores *et al.*, 2001; Clements *et al.*, 2002). Both genera sexually exploit male thynnine wasps as pollinators,

yet despite their very close phylogenetic relationship, preliminary findings have indicated that *Drakaea* may use an entirely different chemical system to *Chiloglottis* for securing pollination. We have recently reported that pyrazines, including some new natural products, are present in *Drakaea livida*, as well as in the females of one of its pollinators, *Zaspilothynnus nigripes* (Bohman *et al.*, 2012a,b). GC-EAD has further revealed that these compounds are electrophysiologically active in the pollinator, leading us to propose that pyrazines rather than chiloglottones might be involved in pollinator attraction. However, until now, the field studies required to confirm that pyrazines hold the key to this pollination system have been absent.

The primary subject of this study was *Drakaea glyptodon* and its male thynnine wasp pollinator, *Zaspilothynnus trilobatus* (Peakall, 1990). *Drakaea* orchids exploit the reproductive biology of their thynnine wasp pollinator to the extreme, providing a unique opportunity to investigate the chemistry and evolution of sexual deception. The parasitic thynnine wasps form a diverse component of the Australian wasp fauna (Peakall, 1990; Griffiths *et al.*, 2011). Females spend most of their adult lives underground where they search for beetle larvae on which to lay their eggs. By contrast, the winged adult males live above ground where they routinely make patrolling flights to locate the 'calling' wingless females that emerge periodically from below ground (Supporting Information, Movie S1). Calling females typically perch on low vegetation where they release sex pheromones and are quickly located by males (often within seconds), with the successful male grasping her in flight before carrying her, *in copula*, to a nectar source for feeding and mating (Peakall, 1990).

It is this reproductive behaviour of the pollinator that is fully exploited by *Drakaea* orchids. The flower is highly reduced except for the labellum (the petal that forms the lip), which is remarkably insectiform and bears a close resemblance in size and shape to the flightless female thynnine wasp (Fig. 1a; see also figure in Peakall, 1990). As the male wasp attempts to fly off with the hinged, semiochemical-releasing labellum, the pollinator is tipped upside down and brought into contact with the column (combined stigma and anther of orchids) where pollen removal occurs (Fig. 1c,d; Movie S2). Attempted mating with the flower is frequent, but not necessary for pollinium removal (Peakall, 1990). *Drakaea* are highly efficient at converting pollinator attraction into pollen deposition, resulting in higher pollination rates (Phillips *et al.*, 2013, 2014b).

Here we show that, in *D. glyptodon*, a combination of different pyrazines are both semiochemicals used by the orchid to sexually lure its male pollinator to the flower and components of the sex pheromone of the female of the pollinator. In light of our confirmation that two different chemical systems (chiloglottones and pyrazines) are involved in the sexual attraction of pollinators in two closely related Australian orchid genera, we also conducted an extended chemical analysis of orchid floral volatiles. Our chemical survey across both closely related and more distantly related species allowed us to assess whether or not preadaptation underpins the evolution of the floral volatiles

critical for the operation of sexual deception within Australian orchids.

Materials and Methods

Sample collection

Sampling was conducted during the flowering season of *Drakaea glyptodon* Fitz (September–October) in 2009–2013. Flowers were sourced from multiple populations in southwestern Australia. Male *Zaspilothynnus trilobatus* Turner were obtained for GC-EAD experiments by 'baiting' with *D. glyptodon* flowers in Banksia woodland (Peakall, 1990), while female *Z. trilobatus* were obtained from flowering shrubs. The field bioassays were conducted in October 2012 and 2013, in natural bushland adjacent to Johnston Rd, Yarloop, Western Australia (32.9192S, 115.7555E).

General chemical procedures

For the full details of the extractions, nuclear magnetic resonance (NMR), GC-MS and GC-EAD analyses, and chemical synthesis of five compounds (1–5), see Methods S1. GC-MS and NMR spectroscopic data and GC chromatograms are provided in Notes S1.

Bioassays

Under appropriate conditions, thynnine wasp pollinators respond rapidly to artificially presented flowers (Peakall, 1990; Peakall *et al.*, 2010; Phillips *et al.*, 2013) and synthetic semiochemicals (Schiestl *et al.*, 2003; Schiestl & Peakall, 2005; Franke *et al.*, 2009; Peakall *et al.*, 2010). Peak responses typically occur within the first minute, with responses declining quickly over the next few minutes (Peakall, 1990). However, when the stimulus is moved to another position, new pollinators are rapidly attracted, allowing for repeated trials of their response to flowers and synthetic compounds. Mark-recapture experiments conducted previously with the study species, *Z. trilobatus* (Peakall, 1990), and other thynnine wasps (Peakall & Beattie, 1996; Whitehead & Peakall, 2013) reveal strong short-term avoidance of orchid and/or synthetic stimuli. Furthermore, returns of marked wasps even to relocated baits are rare within the same day. By contrast, pollinators may revisit baits on subsequent days (Whitehead & Peakall, 2013). As is typical of pollinator behaviour at sexually deceptive orchid flowers (Peakall, 1990; Peakall & Beattie, 1996; Vereecken & Schiestl, 2008), the wasps responding to experimentally presented flowers or synthetics display variation in the degree of sexual response (e.g. approach only, land and attempted copulation).

Notwithstanding our ability to work experimentally with thynnine wasps, the opportunities to conduct field bioassays are tightly constrained in this study system as a result of the very short peak flowering period of the orchid (2–3 wk yr⁻¹) and the similarly narrow peak flight time of the pollinator (3–4 wk yr⁻¹). Furthermore, the wasps mostly search for mates during warm

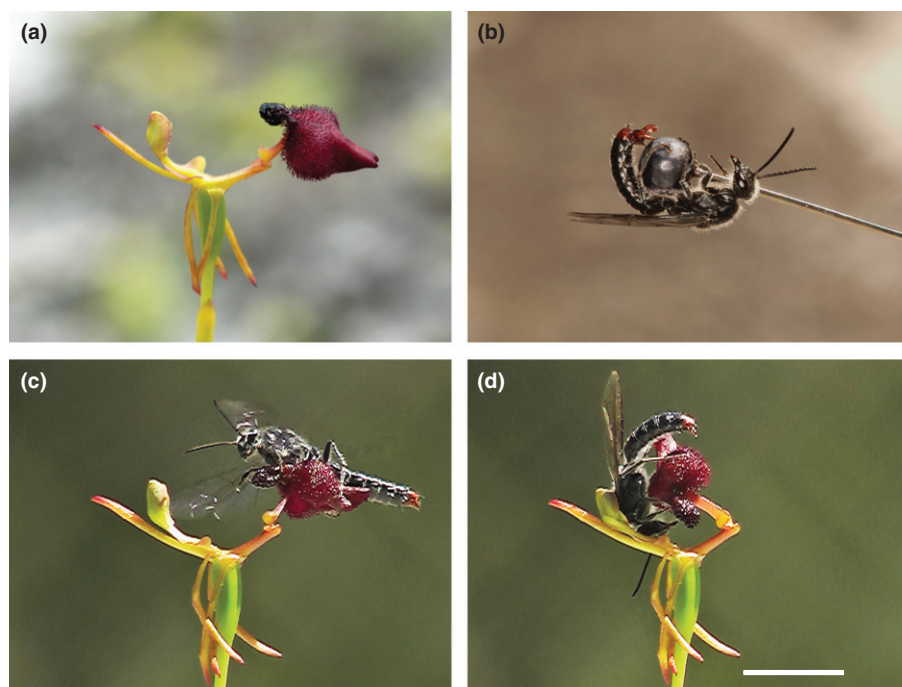


Fig. 1 The floral morphology of *Drakaea glyptodon* and its pollination by male thynnine wasps, *Zaspilothynnus trilobatus*. (a) The flower revealing reduced floral structures and the insectiform labellum. (b) A male wasp attempting to mate with a dummy spiked with a synthetic blend of the pyrazines. Note the extruded genitalia, indicating strong sexual attraction. (c) A male wasp attempting to fly off with the orchid labellum as the first step in pollination. (d) A male wasp still grasping the labellum now tipped upside down and in contact with the orchid column where pollination occurs (see also Movie S2). Bar, 8 mm.

sunny periods, typically between 10:00 and 14:00 h, when temperatures exceed 18°C (Peakall, 1990). This further constrains the number of suitable days available for conducting experimental bioassays in any one season, particularly in the study region where the weather conditions are highly variable during spring. Consequently, in this study we conducted exploratory bioassays as the synthetic compounds (1–5) were progressively identified and synthesized over the field seasons of 2009–2011.

Our field bioassays were undertaken using fresh *D. glyptodon* flowers and synthetic compounds as bait to attract pollinators. For the bioassays with synthetic compounds, we extended the methods of Peakall *et al.* (2010). Briefly, a dummy consisting of a dressmaker's pin with a 4-mm-diameter black plastic head was attached to a bamboo skewer 25 cm above the ground (approximating the height of flowers). The synthetic compound(s) dissolved in dichloromethane were dispensed onto the dummy, with the solvent allowed to evaporate before use. A solvent control was included in initial trials, confirming that the solvent was unattractive. For bioassays involving orchid flowers, the solitary flower on its 15- to 25-cm-long stem was attached to a bamboo skewer with the base of the stem held in water in a 2 ml vial, such that dummy and orchid were presented similarly. In the field, when presenting multiple dummies, or a dummy and a flower, they were positioned perpendicular to the wind direction *c.* 0.5 m apart. Between trials, orchids and dummies were held in a sealed container. All bioassays were performed during optimal weather conditions for pollinator activity (> 18°C and sunny, mostly between 10:00 and 14:00 h).

We adopted a sequential bioassay (see Peakall *et al.*, 2010) that allowed us to determine whether or not a given blend was attractive, or not, to the pollinator without any confounding effects of competing synthetic compounds or other orchid stimuli. This bioassay consisted of two phases. In phase 1, a single synthetic

compound or blend was presented for 3 min. Phase 2 began after 3 min, at which point an orchid flower was presented simultaneously with the dummy for a further 3 min. Phase 2 confirmed the availability of pollinators at that particular location, with trials abandoned if there was no response to either synthetics or flowers, and also provided a choice test between flower and synthetic(s). Given the characteristic rapid decline in pollinator response, noted earlier, the risk of pseudoreplication between phases, and between trials within experiments, is very low in this experimental design.

Each sequential experiment commenced with the preparation of four to six dummies that were loaded with different synthetic combinations (see the Results section for details) and flagged for easy recognition in the field. Several flowers were also prepared for presentation, after a preliminary trial confirmed that they attracted the pollinator. Subsequently, sequential bioassays were performed in a random sequence for each of the synthetic combinations and flowers, and repeated for four to six trials until a minimum of at least 20 wasp responses were obtained for each set. Experiments were replicated two or three times on different days.

Owing to the minute amounts of semiochemicals present in the headspace of the flowers, we were unable to reliably quantitate the amount and ratios. For females calling under laboratory conditions, the amount of compounds detected via solid phase microextraction (SPME) also varied among individual insects. It was therefore impossible to know in advance the optimal combination of compounds (noting that GC-EAD activity does not necessarily equal biological activity), or the ratio or amount of compound(s) for stimulating biological activity. Consequently, we commenced preliminary bioassays starting with equal ratios and working with stock concentrations of 0.1, 1 and 10 $\mu\text{g } \mu\text{l}^{-1}$, which were dispensed on beads in test volumes of 1, 5, 10 and 15 μl . As for other studies (Ayasse *et al.*, 2003; Peakall *et al.*,

2010), we also used the observed ratio of the compounds in solvent extracts of the flowers as a guide in choosing other specific blends to empirically evaluate (natural ratio variation based on six bulk floral dichloromethane extracts of 24 h duration ($n=142$ flowers): 3:5 = 2.9–12.8; S-4:5 = 0.4–6.4). We used this data, mindful that our floral extract measurements reflect the stored composition in the tissue, being extractable under the chosen conditions (solvent and extraction time), not the composition of the headspace (Falara *et al.*, 2013). For the bioassays reported here, an empirically determined maximum of 200 μg for all compounds at the specified ratios was dispensed onto the beads. Beads loaded at this amount remained attractive for >24 h, although in our experiments all beads were discarded within 2 h of being loaded to minimize potential changes in the amount and ratio of compounds released. Importantly, the ratios chosen for the bioassays that we report here (1, 2, 3, S-4 and 5 at 1:1:300:50:100) included a threefold difference between compounds 3 and 5, after preliminary trials indicated that an excess of 3 over 5 increased the sexual attraction, while a sixfold difference did not further enhance the response (outcomes of G -tests for compounds 1, 2, 3 and 5 at 1:1:300:100 vs 1:1:600:100, $G=0.71$, $\text{df}=1$, $P=0.40$, $n=73$; and for compounds 3 and 5 at 300:100 vs 600:100, $G=3.34$, $\text{df}=1$, $P=0.67$, $n=76$ (G -tests applying William's correction comparing the proportion of a and ($l+c$) between treatments)).

In 2013, we extended the bioassays to test the biological activity of S-4. This was the only chiral GC-EAD-active compound and it was found only in the flower. We first performed preliminary tests with S-4 on its own, and subsequently in combination with 1, 2 and 3. On its own, S-4 was barely attractive. With the combination 1, 2, 3 and S-4, approaches were observed, but no landings or attempted copulations ($n=24$), consistent with the behaviour in response to compounds 1–3 only (see Fig. 4b). To further test whether or not the addition of the floral volatile S-4 enhanced pollinator responses to the blend 1, 2, 3 and 5, which was already known to trigger the full repertoire of sexual behaviour (Fig. 4f), we conducted 3 min bioassays in which each of the two treatments (1, 2, 3 and 5 vs 1, 2, 3, S-4 and 5) was alternated. Each treatment was repeated for four to six successful trials, and the experiment repeated twice. For all bioassays, G -tests were performed using GenALEX 6.5 (Peakall & Smouse, 2006, 2012).

Chemical screening of orchid floral volatiles

The purpose of this survey was to screen the floral volatiles of some representative Australian orchids for the presence of known specific pyrazines and chiloglottones (see Table 1 for species list). In 15 cases, we were able to use existing GC-MS data from other published studies (Peakall *et al.*, 2010; Bohman *et al.*, 2012a,b; Peakall & Whitehead, 2014). In 14 other cases, we obtained additional species for new analyses (see Table 1 for full details; and Methods S1 for extraction and GC-MS analysis procedures). In all cases, the floral extracts screened were obtained from solvent extracts made from multiple flowers. For larger flowers, the extracts consisted of separate extracts from orchid labella (the

typical source of chiloglottones and pyrazines) and glandular sepals (when present). In many cases, the remaining flower parts (e.g. petals) were also tested. GC-MS data were screened for the six chiloglottones known as natural products (Peakall *et al.*, 2010) and the 11 known pyrazines from *D. livida* and *D. glyptodon* (Bohman *et al.*, 2012a,b; and this study).

Our survey included representatives of all genera within the Drakaeinae (which contains *Chiloglottis* and *Drakaea*). As all genera in this subtribe secure pollination by sexual deception, floral volatile analysis from alternative pollination systems are not available. We also included sexually deceptive *Leporella fimbriata* and *Cryptostylis ovata*, which are phylogenetically basal to the Drakaeinae (see Kores *et al.*, 2001). Finally, multiple representatives of the genus *Caladenia* (subtribe Caladeniinae) were also included. Although distantly related to the Drakaeinae, this large Australian genus contains both food-deceptive and sexually deceptive species (Phillips *et al.*, 2009) and was known *a priori* to include one species whose floral volatiles contained pyrazines in common with *Drakaea* (Bohman *et al.*, 2013). Inclusion of these samples allowed us to look for possible convergence of floral volatile chemical traits between distantly related, but sexually deceptive species.

Results

GC-EAD of wasp and flower extracts

Solvent extracts were prepared from the labella of *D. glyptodon* flowers and from female *Z. trilobatus* (as crushed whole insects and crushed heads only). These extracts were used in GC-EAD experiments against antennae from males of the pollinator, *Z. trilobatus*. A representative trace is shown in Fig. 2(a). Five compounds (1–5) in the *D. glyptodon* flower extracts were found to be consistently electrophysiologically active in repeated GC-EAD experiments (>20 runs with replication over 4 yr) and were targeted for identification in this study.

Compounds 1–3 and 5, but not compound 4, were also present in the dichloromethane extracts of the heads of female *Z. trilobatus* ($n=10$). Similarly, in SPME analysis of females that were induced to 'call' inside closed vials in the laboratory ($n=10$), compounds 1–3 and 5 were detected, but not compound 4 (Fig. 2b).

Identification of GC-EAD-active compounds

The mass spectra of the GC-EAD-active peaks indicated that all five compounds possessed a pyrazine skeleton (Brophy & Cavill, 1980; Brophy, 1989; Beck *et al.*, 2003; Dickschat *et al.*, 2005a, b). The first three eluting compounds were tentatively identified as alkyl-dimethylpyrazines, with the alkyl group being ethyl-, propyl-, and butyl-, respectively, based on similarities to compounds in mass spectra databases (NIST-05 and Wiley 275 L, 1998). The exact substitution pattern was confirmed by the preparation of all nine possible compounds and comparison of retention times and mass spectra. The synthetic products showing identical retention indices and MS fragmentations were the 2-alkyl-3,5-dimethylpyrazines 1–3 (Fig. 3).

Table 1 Outcomes of floral volatile surveys for specific chiloglottones and pyrazines across representative orchids

Genus ¹	Species ²	Strategy ³	Pollinator ⁴	Compounds		Synonyms ⁷	References ⁸		
				Chiloglottones ⁵	Pyrazines ⁶		Pollination	Chilo	Pyr
Drakaeinae									
<i>Arthrochilus</i>	<i>A. huntiana</i>	SD	<i>Arthrothynnus huntianus</i>	3	N	<i>Thynninorchis huntianus</i>	1		
	<i>A. prolixus</i>	SD	Thynnine wasp?	3	N	<i>Simpliglottis</i>	2	2	
<i>Chiloglottis</i> ('Valida' clade)	<i>C. aff. valida</i>	SD	<i>Neozeleboria</i> sp.	1, 2	N	<i>S. aff. valida</i>	2, 3	2, 4	
	<i>C. aff. jeanesii</i>	SD	<i>N. impatiens</i>	3	N	<i>S. aff. jeanesii</i>	2, 3	2, 4	
	<i>C. pluricallata</i>	SD	<i>N. sp. (impatiens3)</i>	1, 2	N	<i>S. pluricallata</i>	2, 3	2, 4	
	<i>C. turfosa</i>	SD	<i>N. sp. (carinicolis3)</i>	4		<i>S. turfosa</i>	2, 3	2, 5	
	<i>C. valida</i>	SD	<i>N. monticola</i>	1	N	<i>S. valida</i>	2, 3	2, 4	
<i>Chiloglottis</i> ('Formicifera' clade)						<i>Myrmecchila</i>			
	<i>C. trapeziformis</i>	SD	<i>N. cryptoides</i>	1	N	<i>M. trapeziformis</i>	2, 3	2, 6	
	<i>C. formicifera</i>	SD	<i>N. sp. (nitidula5)</i>	1, 4	N	<i>M. formicifera</i>	2, 3	2	
<i>Chiloglottis</i> ('Reflexa' clade)	<i>C. diphylla</i>	SD	<i>Arthrothynnus latus</i>	N	N		2, 3	2	
	<i>C. seminuda</i>	SD	<i>N. sp. (proxima2)</i>	1	N		2, 3	2, 6	
	<i>C. trilabra</i>	SD	<i>N. sp. (proxima3)</i>	1, 3	N		2, 3	2	
<i>Drakaea</i>	<i>D. glyptodon</i>	SD	<i>Zaspilothynnus trilobatus</i>	N	1–5		7		
	<i>D. livida</i> (1)	SD	<i>Z. nigripes</i>	N	6		8, 9		8
	<i>D. livida</i> (2)	SD	<i>Catocheilus</i> sp.	N	7–11		10		10
<i>Caleana</i>	<i>C. major</i>	SD (SF)	<i>Lophyrotoma leachii</i>	N	N		11		
<i>Paracaleana</i>	<i>P. minor</i>	SD	<i>Thynnoturneria</i> sp.	1, 2, 3, 5, 6	N	<i>Sullivania</i>	2	2	
	<i>P. nigrita</i>	SD	?	N	3, 6	<i>S. nigrita</i>			
<i>Spiculaea</i>	<i>S. ciliata</i>	SD	<i>Thynnoturneria</i> sp.	N	N		12		
Megastylidinae									
<i>Leporella</i>	<i>L. fimbriata</i>	SD (MA)	<i>Myrmecia urens</i>	N	N		13		
Cryptostylidinae									
<i>Cryptostylis</i>	<i>C. ovata</i>	SD (IW)	<i>Lissopimpla excelsa</i>	N	N		11, 14		
Caladeniinae									
<i>Caladenia</i> (Subgenus 'Drakonorchis')						<i>Drakonorchis</i>			
	<i>C. barbarossa</i>	SD	<i>Thynnoides</i> sp.	N	6, 12	<i>D. barbarossa</i>	15		16
<i>Caladenia</i> (Subgenus 'Calonema')						<i>Arachnorchis</i>			
	<i>C. attingens</i>	SD	<i>Thynnoides</i> sp.	N	N	<i>A. attingens</i>	15		
	<i>C. plicata</i>	SD	<i>Zeleboria</i> sp.	N	N	<i>A. plicata</i>	15		
	<i>C. startiorum</i>	FD		N	N	<i>A. startiorum</i>			
	<i>C. christineae</i>	FD		N	N	<i>A. christineae</i>			
<i>Caladenia</i> (Subgenus 'Phlebochilus')						<i>Jonesiopsis</i>			
	<i>C. cairnsiana</i>	SD	<i>Phymatothynnus victor</i>	N	N	<i>J. cairnsiana</i>	15		

Table 1 (Continued)

Genus ¹	Species ²	Strategy ³	Pollinator ⁴	Compounds			References ⁸		
				Chiloglottones ⁵	Pyrazines ⁶	Synonyms ⁷	Pollination	Chilo	Pyr
	<i>C. nobilis</i>	FD		N	N	<i>J. nobilis</i>			
	<i>C. polychroma</i>	FD		N	N	<i>J. polychroma</i>			

¹Subtribe classifications follow Kores *et al.* (2001) based on plastid *matK* and *trnL-F*, and Clements *et al.* (2002) based on nuclear internal transcribed spacer. Both classifications are congruent with respect to which genera are included within Drakaeinae, but in neither study are the phylogenetic relationships among the genera within this subtribe fully resolved. Both studies indicate that *Chiloglottis* is sister to *Drakaea*, and *Caleana* is sister to *Paracaleana*. Kores *et al.* (2001) do not specify a subtribe rank for *Leporella*, but both classifications place the genus as basal to the Drakaeinae.

²Species nomenclature is consistent with Kores *et al.* (2001) and Clements *et al.* (2002). For *Chiloglottis* subgeneric clades and species, follow Peakall *et al.* (2010). For *Caladenia*, subgenera and species, follow Hopper & Brown (2004).

³Pollination strategy: SD, sexual deception of male thynnine wasps; SD (SF), sexual deception of male saw flies; SD (IW), sexual deception of male ichneumonid wasps; SD (MA), sexual deception of male winged ants; FD, food deception involving various types of insects.

⁴See the 'References pollination' column for sources on strategy and pollinator identification, if other than this study.

⁵Chiloglottones: N, no chiloglottones found; 1, 2-ethyl-5-propylcyclohexane-1,3-dione; 2, 2-ethyl-5-pentylcyclohexane-1,3-dione; 3, 2-butyl-5-methylcyclohexane-1,3-dione; 4, 5-allyl-2-ethylcyclohexane-1,3-dione; 5, 2-butyl-5-propylcyclohexane-1,3-dione; 6, 2-hexyl-5-methylcyclohexane-1,3-dione. See the 'References chilo' column for sources other than this study.

⁶Pyrazines: N, no pyrazines found; 1, 2-ethyl-3,5-dimethylpyrazine; 2, 2-propyl-3,5-dimethylpyrazine; 3, 2-butyl-3,5-dimethylpyrazine; 4, 2-(1-hydroxyethyl)-3,5-dimethylpyrazine; 5, 2-hydroxymethyl-3,6-diethyl-5-methylpyrazine; 6, 2-hydroxymethyl-3-(3-methylbutyl)-5-methylpyrazine; 7, 2-(3-methylbutyl)-3,5,6-trimethylpyrazine; 8, 2-hydroxymethyl-3,5,6-trimethylpyrazine; 9, (3,6-dimethylpyrazine-2-yl)methyl-3-methylbutanoate; 10, (3,5,6-trimethylpyrazine-2-yl)methyl-(2S)-methylbutanoate; 11, (3,5,6-trimethylpyrazine-2-yl)methyl-3-methylbutanoate; 12, 2-(3-methylbutyl)-3,6-dimethylpyrazine. See the 'References pyr' column for sources other than this study.

⁷Alternative nomenclature is based on Jones (2006), noting that this nomenclature is not universally adopted.

⁸This study is the source of data when no references are listed: 1, Mant *et al.* (2002); 2, Peakall *et al.* (2010); 3, Griffiths *et al.* (2011); 4, Peakall & Whitehead (2014); 5, Poldy *et al.* (2012); 6, Falara *et al.* (2013); 7, Peakall (1990); 8, Bohman *et al.* (2012a); 9, Phillips *et al.* (2013); 10, Bohman *et al.* (2012b); 11, Gaskett (2011); 12, Alcock (2000); 13, Peakall (1989); 14, Schiestl *et al.* (2004); 15, Phillips *et al.* (2009); 16, Bohman *et al.* (2013).

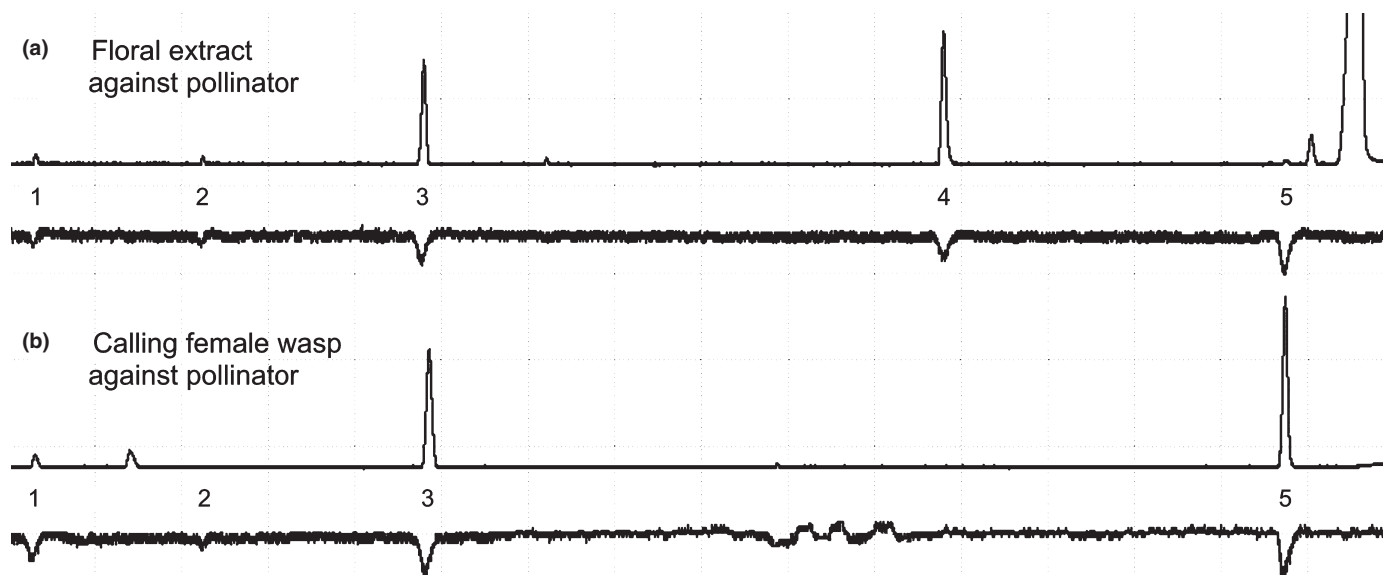
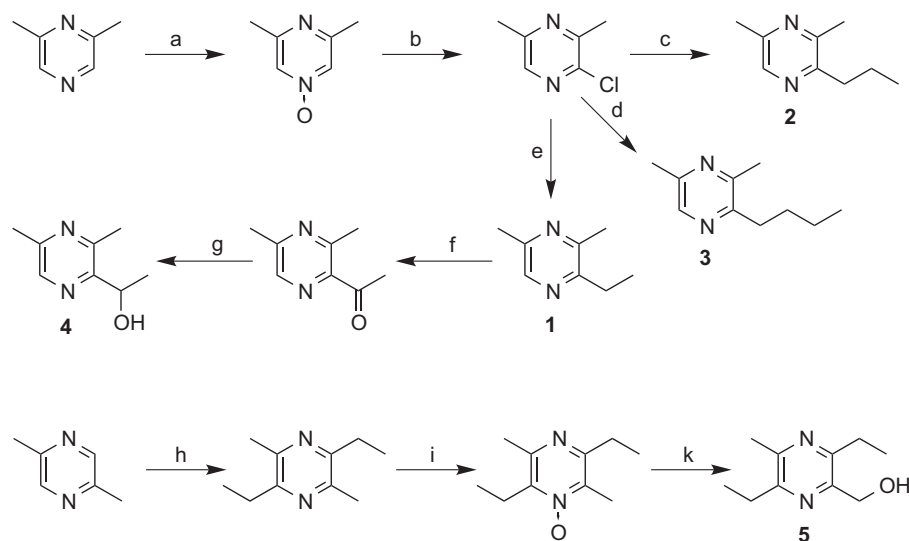


Fig. 2 Gas chromatography with flame ionization detector/ electroantennographic detection (GC-FID/EAD) analyses. GC-FID chromatograms (top traces) of a solvent floral extract of *Drakaea glyptodon* (a) and a solid phase microextraction sample of the headspace of a calling female of the pollinator, *Zaspilothynnus trilobatus* (b). GC-EAD results (lower traces) show the antennal responses of the male pollinator.

The fourth compound (only present in flowers) was tentatively identified based on a mass spectrum that was consistent with a (1-hydroxyethyl)-dimethylpyrazine. The structure was confirmed by comparing the mass spectrum and retention datum of the natural product with the three possible structural isomers, which were synthesized, revealing the compound to be 2-(1-hydroxyethyl)-3,5-dimethylpyrazine (compound 4) (Fig. 3). Enantioselective GC-MS analysis of the floral extracts revealed that both

enantiomers of compound 4 were present in the flowers at an approximate ratio of 90 : 10 *S* : *R*. The enantiomeric configurations were determined using Kauslauskas' empirical rules in the stereospecific transesterification reaction of the secondary alcohol 4 with vinyl acetate by *Candida antarctica* lipase B (Kauslauskas *et al.*, 1991).

The fifth compound appeared to be a novel natural product. Analysis of the base peak, which represented the



(a) HOAc, H₂O₂; (b) POCl₃; (c) PrMgBr, Dppp-NiCl₂; (d) BuMgBr, Dppp-NiCl₂; (e) Et₂Zn, Dppp-NiCl₂; (f) Na₂Cr₂O₇, HOAc; (g) NaBH₄, 50% EtOH; (h) FeSO₄, EtCHO, H₂SO₄, H₂O₂; (i) HOAc, H₂O₂; (k) i: Ac₂O, ii: MeOH, LiOH

Fig. 3 The synthesis of the pyrazine semiochemicals identified from *Drakaea glyptodon* flowers (for more details, see the Methods S1 section on 'Pyrazine synthesis').

quasi-molecular ion, using high-resolution chemical ionization (CI), supported a molecular formula of C₁₀H₁₆N₂O. The strong molecular ion together with the observed fragmentations under electron ionization conditions was consistent with a hydroxymethyl-diethyl-methylpyrazine. All possible isomers were prepared and 2-hydroxymethyl-3,5-diethyl-6-methylpyrazine (compound 5) corresponded to GC and MS data of the natural product (Fig. 3).

All five semiochemicals identified were synthesized using established methods with minor modifications (Fig. 3). Compound 1 was prepared from 2-chloro-3,5-dimethylpyrazine using a Negishi coupling of diethyl zinc facilitated by a Ni-catalyst (Sato & Matsuura, 1996). 2-Chloro-3,5-dimethylpyrazine was prepared from 2,6-dimethylpyrazine via *N*-oxidation and chlorination with phosphoryl oxychloride. Compounds 2 and 3 were synthesized via a Ni-catalysed Kumada coupling of propylmagnesium bromide, and butylmagnesium bromide, respectively, with 2-chloro-3,5-dimethylpyrazine (Seeman *et al.*, 1992). Compound 4 was prepared from compound 1, via chromate oxidation (Wolt, 1975) and sodium borohydride reduction. The two enantiomers were resolved via a *C. antarctica* lipase B-mediated transesterification with vinyl acetate. The novel compound 5 was prepared from 2,5-dimethylpyrazine and propanal via a Fe-catalysed Minisci reaction (Sawada *et al.*, 1991), followed by *N*-oxidation and a Boekelheide rearrangement (Methods S1).

Outcomes of field bioassays

In the 2012 experiments, observations of the pollinator responses to *D. glyptodon* flowers revealed that 76% of the males ($n=101$) closely approached, but did not proceed to land or attempt copulation. Of those that landed on the flower ($n=24$), 79% proceeded to attempt copulation. Sequential bioassays for six combinations of the compounds, 1–3 and 5, were undertaken,

yielding a total of 369 wasp responses (Fig. 4). These bioassays involved first presenting the compound(s) by themselves for 3 min (phase 1), before simultaneously presenting the compounds along with the flowers (phase 2) for an additional 3 min. To facilitate comparisons among treatments, the responses are shown as proportions (of the total within each treatment). All combinations tested attracted wasps in the first phase, confirming the general attractiveness of all treatments. However, only two treatments, 3 and 5 (300 : 100) (Fig. 4e) and 1, 2, 3 and 5 (1 : 1 : 300 : 100) (Fig. 4f), elicited the full repertoire of behavioural responses at the dummy, including close approach (*a*), land (*l*) and attempted copulation (*c*) (Fig. 2d).

Phase 2 of the experiment showed that the pattern of strong sexual response (*l+c*) was similar or exceeded that observed at flowers for treatments 3 and 5, and 1, 2, 3 and 5 ($E: G=0.975$, $df=1$, $P=0.32$, $n=20$; $F: G=8.5$, $df=1$, $P=0.004$, $n=27$; G -tests applying William's correction comparing the proportion of *a* and (*l+c*) at the dummy vs flowers (results at flowers pooled across all treatments, $n=101$)). Collectively, these bioassays provide compelling evidence that the pyrazines, at the blends and concentration tested, behave as sex pheromones.

In 2013, the bioassays involving the addition of *S*-4 to compounds 1, 2 and 3, failed to elicit lands or attempted copulations, consistent with the behaviour with compounds 1–3 only (Fig. 4b). On the other hand, lands and attempted copulation were observed with compounds 1, 2, 3, *S*-4 and 5 (in the ratio 1 : 1 : 300 : 50 : 100). However, there was no significant difference in the degree of sexual response (*l+c*) between this treatment, and the 1, 2, 3 and 5 treatment ($G=0.191$, $df=1$, $P=0.662$, $n=63$, G -test as described earlier), previously shown to be the most sexually attractive combination in 2012 (Fig. 4e). Thus, the addition of the floral volatile *S*-4, which has not been found in the female, neither inhibited nor enhanced the pollinator response.

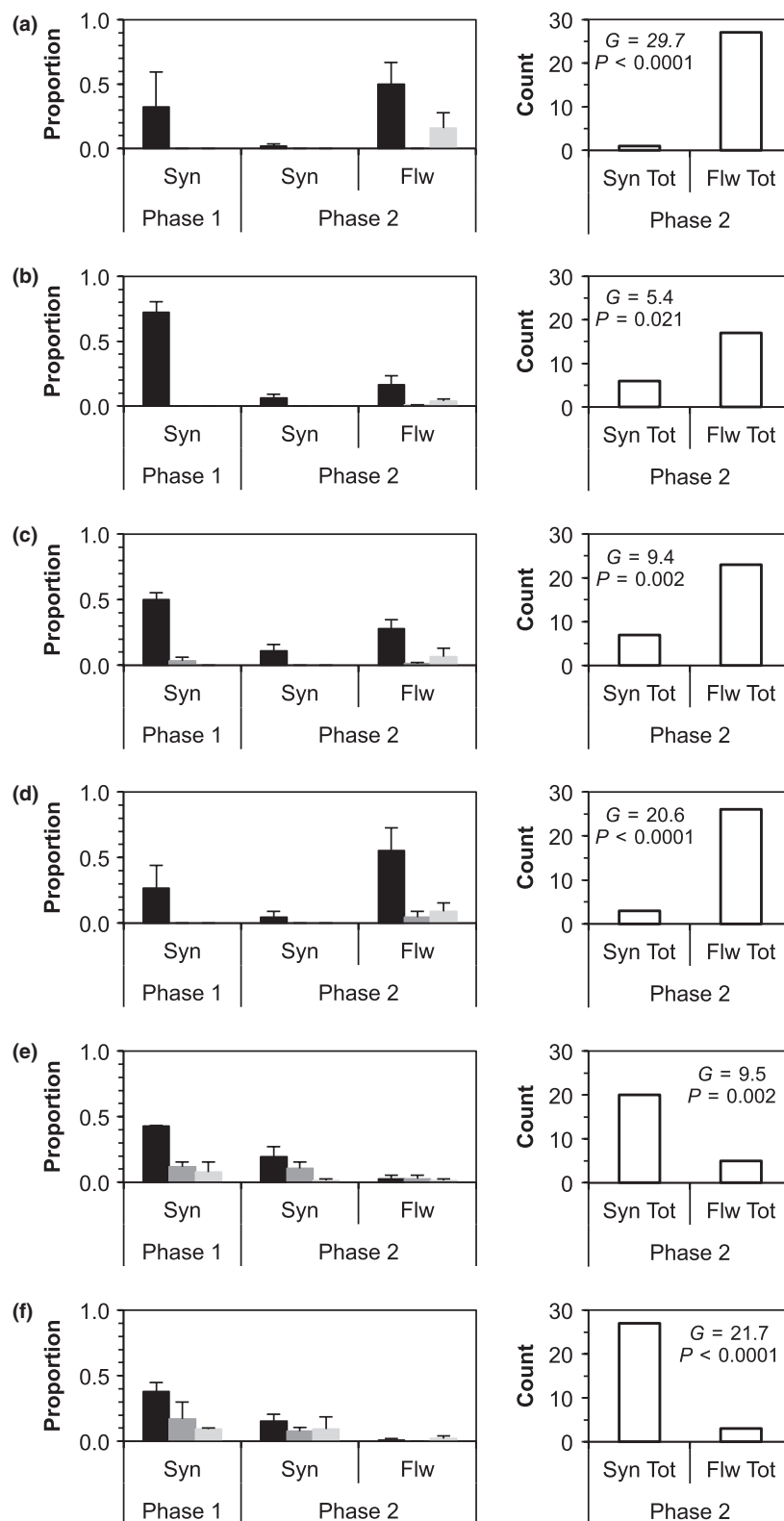


Fig. 4 Outcomes of sequential bioassays to blends of synthetic pyrazines identified in *Zaspilothynnus trilobatus* females and *Drakaea glyptodon* flowers: (a) compounds 1 and 2; (b) compounds 1–3; (c) compound 3 only; (d) compound 5 only; (e) compounds 3 and 5; (f) compounds 1–3 and 5. Phase 1 consisted of the presentation of synthetic compound(s) for 3 min (Syn). This was followed by phase 2, where both synthetic compound(s) (Syn) and orchid flower (Flw) were presented simultaneously for a further 3 min. Pollinator responses are shown as mean proportions of the total ($\pm$ SE), further partitioned into approach (A, black bars), land (L, dark grey bars) and attempted copulation (C, light grey bars) (left panel). The total count across experiments is shown separately for phase 2 (right panel). All bioassays were performed at the base ratio of 1 : 1 : 300 : 100. (1 : 2 : 3 : 5).

Outcomes of chemical screening of orchid floral volatiles

Our survey for chilogluttones and pyrazines spanned 29 taxa (28 species with two chemotypes of *D. livida*) representing nine

genera (Table 1). Within the Drakaeinae, with one exception (*Paracaleana nigrata*), pyrazines were only found in *Drakaea*, none of which also produced chilogluttones. Conversely, chilogluttones were already known to be present across *Chiloglottis*,

and in *Arthrochilus prolixus* and *Paracaleana minor* (Peakall *et al.*, 2010). Re-analysis of the chiloglottone-producing species revealed no case where pyrazines were also present. However, both classes of compounds were found in *Paracaleana*. Pyrazines alone were detected in *P. nigrita* of Western Australia, while five of the six known chiloglottones, but no pyrazines, are known in *P. minor* of eastern Australia (Peakall *et al.*, 2010; Table 1).

Neither chiloglottones nor pyrazines were detected in the sexually deceptive *Leporella* or *Cryptostylis*, basal to the Drakaeinae (Kores *et al.*, 2001). Within the more distantly related Caladeniinae, we surveyed eight species spanning four subgenera of *Caladenia sensu lato* (for taxonomy, see Hopper & Brown, 2004). No chiloglottones were detected in either the food-deceptive or sexually deceptive species. Two specific pyrazines, implicated by GC-EAD but not yet confirmed as biologically active in pollinator attraction in *D. livida*, were found in *Caladenia barbarossa* (see Bohman *et al.*, 2013) and may represent an interesting case of evolutionary convergence.

Discussion

The compounds involved in pollinator attraction

We identified three alkylpyrazines and one hydroxymethyl pyrazine as the key components of the sex pheromone of the thynnine wasp, *Z. trilobatus*. Further, we have shown that these same constituents are used as semiochemicals by the orchid *D. glyptodon* to sexually attract this wasp species to the orchid flower. Although several compounds were GC-EAD-active, only two (but in a specific ratio) are critical for stimulating copulatory behaviour. Such behaviour was not observed in bioassays for either compound on its own, or with other subsets of the compounds (Fig. 4).

While compound 5 is a novel pyrazine, it is similar to the hydroxymethyl pyrazines that we have recently discovered in other *Drakaea* and thynnine wasp pollinators (Bohman *et al.*, 2012a, b). For example, we have detected 2-hydroxymethyl-3-(3-methylbutyl)-5-methylpyrazine in the headspace surrounding *Zaspilothynnus nigripes* females during courtship, and in *D. livida* flowers pollinated by *Z. nigripes* males (Bohman *et al.*, 2012b). Further, we have uncovered chemotype variation in *D. livida*. In this case, some flowers containing five GC-EAD-active compounds, including the novel 2-hydroxymethyl-3,5,6-trimethylpyrazine, attract a species of *Catocheilus* wasp and not *Z. nigripes* (Bohman *et al.*, 2012a). In both these cases, we have so far been unable to confirm the biological activity of the compounds in field bioassays. However, we predict that the hydroxymethyl pyrazines will be critical components for stimulating strong sexual behaviour.

The finding of the EAD-active compound 2-(1-hydroxyethyl)-3,5-dimethylpyrazine (S-4) as an orchid floral volatile, but not a component of the sex pheromone of the female wasp, is of interest in understanding the evolution of the mimicry of sex pheromones. It has been hypothesized that some floral volatiles in *Ophrys*, which are not found in the female of the pollinator, but

are related to components of the sex pheromone, may trigger a stronger sexual response during pollination than the actual sex pheromone (Vereecken & Schiestl, 2008; Vereecken *et al.*, 2010). Indeed, in the case of *Ophrys exaltata* and its bee pollinator *Colletes cunicularius*, the pollinators prefer the floral odour of the orchid mimic, a behaviour that has been explained as receiver bias towards novel signals (Vereecken & Schiestl, 2008). In the case of *D. glyptodon*, our bioassays indicate that the novel floral volatile compound S-4 neither inhibited nor enhanced the degree of sexual response. This compound may therefore be a 'neutral' by-product of the biosynthetic pathway in the orchid that presently plays little or no function in pollinator attraction. However, we cannot rule out a function for this floral compound at other locations across the wide distribution of *D. glyptodon* or during the evolution of pollinator attraction.

Pollinator specificity

Although there are five GC-EAD-active compounds in *D. glyptodon*, bioassays revealed that just two of the compounds in a specific blend were sufficient to elicit the full repertoire of sexual behaviour. In *Chiloglottis* it has already been established that the extreme pollinator specificity is achieved either by the use of different single chiloglottones or by blends of two compounds (Peakall *et al.*, 2010). Pollinator specificity in *Drakaea* is also extreme, with six out of eight species tested using a single unique wasp pollinator (Phillips *et al.*, 2014b). However, a more complete understanding of the chemical basis of pollinator specificity will require the implementation of bioassays, such as those in this present study, in the other species. Nevertheless, while noting that related compounds are involved, the lack of specific compound sharing between *D. glyptodon* and the two chemotypes of *D. livida* (Table 1) suggests that pollinator specificity, at least in part, will be achieved by each species employing different specific compounds for pollinator attraction.

The small number of floral volatile compounds involved in pollinator attraction in *Chiloglottis* and *Drakaea* has parallels with nursery pollination and some other specialized systems, where specific pollinator attraction can be achieved by one (e.g. *Ficus*, Chen *et al.*, 2009; *Annona*, Maia *et al.*, 2012; *Philodendron*, Dötterl *et al.*, 2012) to a few compounds (Dötterl *et al.*, 2006; Ibanez *et al.*, 2010; Svensson *et al.*, 2010). However, the total number of floral volatile compounds appears to be higher in nursery pollination systems. Furthermore, with the exception of *Ficus semicordata*, where an unusual floral volatile compound is employed, many nursery pollination systems appear to achieve pollinator attraction and pollinator specificity by using common floral volatile compounds (Ibanez *et al.*, 2010). Similarly, sexual deception and pollinator specificity in European *Ophrys* is also achieved by employing specific combinations of common compounds, such as alkenes and alkanes (Schiestl & Cozzolino, 2008). By contrast, sexually deceptive Australian orchids have intercepted the unique chemical communication channels of their specific pollinators and employ unusual semiochemicals to achieve pollination by sexual exploitation.

Distribution and function of pyrazines

Pyrazines are regarded as ubiquitous compounds in nature (Mueller & Rappert, 2010) and have been found in a wide array of organisms, including bacteria, fungi, plants, insects, marine organisms and terrestrial vertebrates (Brophy, 1989; Moore *et al.*, 1990; Woolfson & Rothschild, 1990; Dickschat *et al.*, 2005a,b; Leroy *et al.*, 2011). Pyrazines are also well known for their presence in food, where their nonenzymatic formation occurs during heating (Maga, 1992). Consequently, pyrazines contribute important characteristics to food odours and flavours, and are widely used as food additives (Maga, 1992).

Within insects, pyrazines have been found across several orders, including bees, ants, wasps (Hymenoptera), butterflies, moths (Lepidoptera) and flies (Diptera) (Rothschild *et al.*, 1984; Brophy, 1989; Moore *et al.*, 1990; Robacker *et al.*, 2009a). Within the Hymenoptera, the Formicidae (ants) appear to contain the most diverse range of naturally occurring pyrazines (Brophy, 1989). In the few cases where the biological function has been confirmed experimentally, many of the pyrazines appear to function as alarm pheromones (Brophy, 1989; Meer *et al.*, 2010; Showalter *et al.*, 2010). However, a few alkyl pyrazines also act as trail pheromones (Attygalle *et al.*, 1986). Pyrazines have also been reported in a number of wasp families, both solitary and social (Brophy, 1989). In some solitary wasps, they are known as constituents of marking secretions (Borg-Karlson & Tengö, 1980), but their precise function remains elusive.

Despite sex pheromones being particularly well studied in insects (see reviews Francke & Schulz, 1999; Ayasse *et al.*, 2001), the involvement of pyrazines has rarely been studied. Pyrazines have been implicated as possible sex pheromone components in fruit flies (Diptera: Tephritidae) (Baker *et al.*, 1985; Chuman *et al.*, 1987). The pyrazine 2-methyl-6-vinylpyrazine appears to function as a component of the male-produced sex pheromone in papaya fruit fly (*Toxotrypana curvicauda*) (Chuman *et al.*, 1987), and this compound elicits long-range female attraction in wind tunnel experiments. However, the compound is also attractive to mated females and male flies in pheromone traps with appropriate visual cues (Landolt, 1993), suggesting it may also function as an aggregation pheromone. The highly labile pyrazine analogue, dihydrodimethylpyrazine, may be a key component of the male sex pheromone of the sapote fruit fly (*Anastrepha serpentina*) (Robacker *et al.*, 2009a), although the bioassays are indicative but not definitive (Robacker *et al.*, 2009b). Sex-specific pyrazines have also been identified in the wasp *Ammophila urnaria*, but their function also remains unknown (Duffield *et al.*, 1981). Our discovery of pyrazines as confirmed components of the female sex pheromone of a thynnine wasp is the most compelling case across the insects and represents the first confirmed case of pyrazines as sex pheromones.

In plants, aliphatic hydrocarbons, benzenoids, phenylpropa-noids and terpenes are the most common floral odour constituents (Knudsen *et al.*, 1993; Pichersky & Gershenzon, 2002; Dudareva *et al.*, 2004; Pichersky *et al.*, 2006); by contrast, pyrazines appear to be very rare. Despite an extensive survey across > 90 plant families, Knudsen *et al.* (2006) listed only six

unrelated plant families for which pyrazines had been reported (alkylpyrazines: Apiaceae, Araceae, Iridaceae, Oleaceae, Theophrastaceae; methoxy pyrazines: Arecaceae and Orchidaceae). More recent reports of pyrazines in floral volatiles include their rare presence in the Apocynaceae-Asclepiadoideae, where they may attract fly pollinators (Jürgens *et al.*, 2006), and in cycad cones (Suinyuy *et al.*, 2013). Pyrazines are also reported sporadically from nonfloral tissues of plants spanning a diverse range of families (Brophy, 1989; Moore *et al.*, 1990). However, to our knowledge, the only proposed function for pyrazines in plants is that of a 'warning signal'. Moore *et al.* (1990) proposed this function after noting that methoxy-alkylpyrazines were present in some plant hosts of aposematic insects, as well as some common toxic plants. We have been unable to find any experimental tests of this hypothesis. Therefore, our findings of pyrazines within *Drakea* flowers, for which we have been able to experimentally confirm sex pheromone mimicry, appears to be the first confirmed function of pyrazines in plants.

Preadaptation or evolutionary novelty?

In many cases, support for the hypothesis of preadaptation is self-evident, such as the case of *Ophrys* orchids where the alkenes involved in the sexual attraction of the pollinator are widely known outside the genus and a prior function can be easily ascribed (Schiestl & Cozzolino, 2008). By contrast, in the present study, several lines of evidence appear not to support the hypothesis of preadaptation. These include the very sporadic occurrence of pyrazines across plants; the lack of established functions for pyrazines in plants (although this could merely reflect a lack of experimental studies in the few cases where pyrazines are known); our discovery of hydroxymethyl pyrazines as critical components for pollinator attraction, which, to the best of our knowledge, are previously unknown in plants; and the outcomes of our chemical survey, which revealed that pyrazines have a very restricted occurrence, even within Australian sexually deceptive orchids. Interestingly, the chiloglottones represented a new class of natural products when first discovered (Schiestl *et al.*, 2003), and remain known only in orchids and their associated wasp pollinators (Peakall *et al.*, 2010). Furthermore, our chemical survey failed to find any orchid species that produced both chiloglottones and pyrazines. Instead, the presence of either chiloglottones or pyrazines appears to be strictly associated with the exclusive role of specific pollinator attraction.

While the collective evidence does not support a case for either chiloglottones or pyrazines as preadaptations with other functions that were subsequently coopted for pollinator attraction during the evolution of sexual deception, this does not mean that the alternative hypothesis of evolutionary novelty is automatically supported. Building a strong case for the hypothesis of evolutionary innovation is particularly difficult (Cracraft, 1990; Hodges & Arnold, 1995). In our case, the present lack of a fully resolved phylogeny for the Australian terrestrial orchids of interest poses an impediment that prevents meaningful ancestral trait reconstruction. The published phylogenies are based on just two plastid genes (Kores *et al.*, 2001) or the nuclear internal transcribed

spacer region (Clements *et al.*, 2002), and both lack full resolution of the genera, even within the Drakaeinae. A new multigene phylogeny with expanded sampling will be necessary before reliable ancestral trait reconstruction can be completed. Further, since all evolutionary novelties must arise at the molecular genetic level (Cracraft, 1990; Wagner, 2011), confirmation of evolutionary novelty will require a thorough knowledge of the molecular and genetic basis of the traits in question. If evolutionary novelty does prove to be the key to the evolution of sexual deception within Australian orchids, this may stand out as unusual in the evolution of plant–pollinator interactions where preadaptation appears to be the normal evolutionary pathway (Armbruster, 1997; Schiestl & Cozzolino, 2008; Armbruster *et al.*, 2009). Further studies of the chemistry and biochemistry of unconventional pollination systems promise new insights into the evolutionary role of preadaptation vs evolutionary novelty for enabling the diversification of the flowering plants.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Chemical procedures.

Notes S1 Spectroscopic data and chromatograms.

Movie S1 Thynnine wasp mating behaviour showing a female calling for a mate, the pickup of the calling female, and a pair *in copula* feeding on nectar producing flowers.

Movie S2 Pollination of the orchid *Drakaea glyptodon* by sexually attracted male thynnine wasps, *Zaspilothynnus trilobatus*, and the wasp’s response to synthetic pyrazines.

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