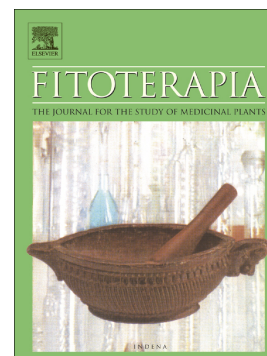


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(Methylthio)phenol semiochemicals are exploited by deceptive orchids as sexual attractants for *Campylothygnus thynnine* wasps

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Abstract

Until recently, (methylthio)phenols as natural products had only been reported from bacteria. Now, four representatives of this class of sulfurous aromatic compounds have been discovered as semiochemicals in the orchid *Caladenia crebra*, which secures pollination by sexual deception. In this case, field bioassays confirmed that a 10:1 blend of 2-(methylthio)benzene-1,4-diol (**1**) and 4-hydroxy-3-(methylthio)benzaldehyde (**2**) sexually attracts the male thynnine wasp *Campylothylnus flavopictus* (Tiphidae:Thynninae), the exclusive pollinator of *C. crebra*. Here we show with field bioassays that another undescribed species of *Campylothylnus* (sp. A) is strongly sexually attracted to a 1:1 blend of compounds **1** and **2**, which elicits very high attempted copulation rates (88%). We also confirm that this *Campylothylnus* species is a pollinator of *Caladenia attingens* subsp. *attingens*. Chemical analysis of the flowers of this orchid revealed two (methylthio)phenols, compound **2** and 2-(methylthio)phenol (**3**), as candidate semiochemicals involved in pollinator attraction. Thus, (methylthio)phenols are likely to be more widely used than presently known. The confirmation of this *Campylothylnus* as a pollinator of *C. attingens* subsp. *attingens* at our study sites was unexpected, since elsewhere this orchid is pollinated by a different thynnine wasp (*Thynnoides* sp). In general, sexually deceptive *Caladenia* only use a single species of pollinator, and as such, this unusual case may offer a tractable study system for understanding the chemical basis of pollinator switching in sexually deceptive orchids.

Keywords: *Caladenia*, semiochemicals, sexual deception, (methylthio)phenols, pollination

1. Introduction

Pollination by sexual deception involves the chemical and/or physical mimicry of female insects [1]. This strategy is known from over 20 genera of orchids, with all cases studied thus far confirmed to use chemicals for long-distance attraction of pollinators [2]. As a by-product of mimicking the highly specific sex pheromone systems of insects, these orchids are highly specialised, typically with a single predominant pollinator species [3-6]. With multiple origins of sexual deception, Australia is a centre of diversity for this pollination strategy, with more than 100 orchid species spanning at least 11 genera exploiting either male Hymenoptera (ants, ichneumonid wasps, scoliid wasps, thynnine wasps, and sawflies) or male Diptera (fungus gnats) ([2, 5, 7].

The chemicals involved in the attraction of male thynnine wasps, the group of insects most widely exploited by Australian sexually deceptive orchids, have now been identified across several genera. Interestingly, in all cases, one or more of the semiochemicals involved have proven to be novel natural products. For example, at least 10 species of Australian *Chiloglottis* orchids use cyclohexanediones (commonly called chiloglottones) [8, 9] representing a new class of compounds when first discovered in these orchids, while related *Drakaea* use previously unknown (hydroxymethyl)pyrazines [10-12]. Some of these unusual pyrazines are also present in *Caladenia barbarossa*, although a role in pollinator attraction has not yet been confirmed [13]. In *Caladenia plicata*, pollinator attraction is achieved by a specific blend of (S)- β -citronellol with a previously undiscovered natural product methyl acetophenone [14]. Finally, in *Caladenia crebra*, which is pollinated by males of the thynnine wasp *Campylothylnus flavopictus* (Tiphidae), we recently discovered that a group of four (methylthio)phenols is involved [15]. Further, we have confirmed that these compounds were present in the females of the pollinator and showed that the two main compounds, 2-(methylthio)benzene-1,4-diol (**1**) and 4-hydroxy-3-(methylthio)benzaldehyde (**2**) (Figure 1A), are essential for eliciting the strong sexual behaviour of the pollinator. Despite the diversity of chemical classes found in sexually deceptive orchids, these findings were particularly unexpected as aromatic sulfurous compounds are rare in nature [15] and sulfur-containing phenols had previously only been identified from bacteria [16].

Caladenia is one of the largest genera of sexually deceptive orchids, with over 360 species currently described [17]. This genus appears to be unique among orchid genera in that it includes examples of orchids that achieve pollination by either rewarding, food deceptive or sexually deceptive strategies [18-20]. Nonetheless, pollination by sexual deception dominates within *Caladenia*, with at least 200 species predicted to use this pollination strategy [6, 19, 21]. Furthermore, these sexually deceptive species exploit male thynnine wasps as pollinators drawn from across at least 34 wasp genera [6, 19], suggesting the potential for a wide diversity of semiochemical systems. In this study, we extend our investigations into the chemical ecology of sexual deception in *Caladenia*.

Given our previous confirmation that the sulfurous compounds found in *C. crebra* are biologically active, we were interested in investigating if these (methylthio)phenols are more widespread as thynnine wasp pheromones and/or orchid semiochemicals. Therefore, in preliminary studies we

exposed a 1:1 mixture (at 10 µg each) of the two main compounds, **1** and **2**, in field bioassays across a range of habitats within diverse communities of thynnine wasps in south-western Australia. At the Ruabon Nature Reserve in the Capel area of Southwestern Australia, an undescribed species of *Campylothylnus* (here after *C. sp. A*) was observed to be regularly and very strongly sexually attracted to these compounds (Figure 2A). Furthermore, some of these wasps were observed carrying the pollen of *Caladenia attingens* subsp. *attingens* (e.g. 6 observations in 2014), confirming *C. sp. A* as an important pollinator of this orchid.

Therefore, the three aims of this study were: 1. To determine whether one or both compounds **1** and **2** are required for the sexual attraction of *Campylothylnus sp. A*. 2. To assess if compounds **1** and **2**, or other known (methylthio)phenols, are also present in the floral tissue of *C. attingens* subsp. *attingens*, and therefore semiochemical candidates. 3. To assess the extent of *Campylothylnus sp. A* involvement as a pollinator of *C. attingens* subsp. *attingens* across the orchid's geographic range.

2. Materials and methods

2.1 Study system

Caladenia attingens is widespread in south-western Australia [17], with this species further subdivided into three mostly allopatric subspecies based on floral morphology. Of these, *C. attingens* subsp. *attingens* is the most abundant, and is the only subspecies occurring in the woodland and forests of the higher rainfall regions (Figure 2A). Prior to our discovery of pollination by *Campylothylnus sp. A* [6], it was thought that *C. attingens* subsp. *attingens* was pollinated solely by sexual deception of an undescribed species of *Thynnoides* [6].

Thynnine wasps are a diverse group of solitary wasps that reach their highest diversity in Australia. Female thynnines are flightless, and release a sex pheromone to attract the volant males, which compete vigorously for the female [22]. The successful male grasps the flightless female and flies off with her *in copula* to a food source, typically nectar or exudates of insects, where feeding and mating take place [23]. This unique mating behaviour is particularly amenable for experimental work. For example, picked orchid flowers pollinated by sexually deceived male thynnine wasps will rapidly attract the wasp pollinator when transferred to a new position in the landscape [24], making it possible to bait for pollinators [20]. Similarly, field bioassays involving synthetic compounds can be readily conducted within appropriate populations of suitable thynnine wasp species [2, 14, 15], with such bioassays representing a critical tool in the chemical elucidation of the sex pheromones/orchid semiochemicals involved in the pollination strategy of sexual deception [2].

2.2 Chemical methods and field bioassays

The semiochemicals used were synthesised as described in Bohman et al. [15]. Bioassays were undertaken between 27th October and 5th November 2014 at Ruabon, Capel district, Western

Australia (33° 38' 36" S; 115° 29' 54"E). Methods for the field bioassays follow those described previously [10]. In short, dummies consisting of dressmaker's pins with round black 4 mm diameter heads were pinned to wooden skewers (25 cm in length) and loaded with the test compound(s) dissolved in dichloromethane at 10 µg/µL. To evaluate whether one or both compounds were required for sexual attraction, experiments consisting of 1 µL each (i.e. A 1:1 blend consisting of 1 µg each) as well as individual compounds (0:1 and 1:0 blends) were tested. Previous dose-dependence studies have shown that the response of male thynnine wasps to bioassays with active compounds (at appropriate blend ratios) is optimal in the range of 0.1 to 10 µg per bead. At these concentrations, similar patterns of pollinator response and sexual attraction can be achieved to those observed at orchid flowers. Furthermore, at these concentrations the bioassay beads remain attractive for at least 24 hr [14, 15].

In general, our field bioassay experiments consist of a series of replicated 3 min trials during which the number of close approaches, lands and attempted copulation with the pin are recorded [10]. However, given that at the study site *C. sp. A* was widespread but at low density, in this study trials were extended to 6 min to allow for insects to approach from a larger distance. All trials with a blend combination that did not attract wasps were followed by a second 3 min phase with the 1:1 blend, known *a priori* to be attractive. Thus, the result of 'no response' to a treatment was only recorded when it was confirmed that wasps were available to respond at a given baiting location. For the bioassay statistical analysis, single factor analysis of variance (ANOVA) was performed in JMP 13 (SAS Institute), while G-tests to compare the proportion of approach versus copulation responses among treatments were performed in GenAlEx 6.5 [25, 26].

Floral volatile extractions from *Caladenia attingens* subsp. *attingens* flowers were prepared as previously reported [15]. Given that the labellum and the terminal glands of the sepals are the source of the pollinator attractant in this and related species (Figure 1D, [27]; RDP unpublished data), floral extracts were prepared by dissecting flowers into the terminal glands of sepals & petals, labellum, and the remaining tissue. Up to ten flowers were pooled in extracts of 100-200 µL, with dichloromethane or ethanol as solvents, and extracted for 24 hours at room temperature. The tissues were removed from the extracts, and stored at -20 °C until subsequently analysed by GC-MS as described in Bohman et al [10].

2.3 Pollinator surveys

Between 2006 and 2012 pollinator surveys were conducted for *C. attingens* subsp. *attingens* at 60 sites using the pollinator baiting method [20, 24]. While *C. attingens* subsp. *attingens* was not necessarily present at these sites, they were all within potentially suitable habitat for this taxon. Baiting was conducted for 6 x 2 minute periods, in a 40 x 20m quadrat with bait positions at each corner of the quadrat and mid-way along its two long sides [6, 7]. Representative pollinator specimens were collected for subsequent identification. Flowers were sourced from populations represented by the vouchers PERTH 08739587 RDP 0343, PERTH 08646058 RDP 0281, PERTH 08646104 RDP

0286 and PERTH 08604436 RDP 0088 in the Western Australian Herbarium. Records of visitation to *C. attingens* subsp. *attingens* opportunistically observed during other fieldwork between 2006 and 2016 have also been included.

3. Results

3.1 Field Bioassays

Preliminary field bioassays in 2013 had revealed that a 1:1 blend of the (methylthio)phenols **1** and **2** were strongly attractive to the *Campylothylnus* sp. A wasp, which was also regularly observed to attempt copulation with the bioassay bead (pin head), and to carry pollen of *Caladenia attingens* subsp. *attingens* (see also Supplementary video 1).

In the 2014 experiments, a total of 75 wasp responses were recorded across the field bioassays, which involved testing the two compounds individually, and as a 1:1 blend, with ANOVA confirming a statistically significant treatment effect (see Figure 1 and caption for statistical details). Compound **1** on its own was not attractive across the 5 x 6 min trials for which in all cases the second control phase secured one or more wasp visits. By contrast, there was on average 0.8 ± 0.3 approaches and 1.2 ± 0.8 attempted copulations per trial with compound **2** (mean \pm SEM, $N=10$ for 5x 6 min trials), with an overall copulation rate of 60%. The 1:1 blend of (methylthio)phenols **1** and **2** was even more sexually attractive than compound **2** on its own, with on average 0.4 ± 0.14 approaches and 2.9 ± 0.4 attempted copulations (mean \pm SEM, $N=65$ for 20x 6 min trials), and an overall copulation rate of 88%. A G-test confirmed a significant difference in the proportion of approaches versus copulation between these two treatments (see Figure 1 caption for statistical details).

Solvent extracts of the floral parts of *Caladenia attingens* subsp. *attingens* responsible for sexual attraction of pollinators (labellum, and the terminal glands of petals and sepals) contained small amounts of two of the compounds previously isolated from *C. crebra*: compound **2** and 2-(methylthio)phenol (**3**). The total amount of these compounds constituted less than 0.1% of the total extract, both in dichloromethane and ethanol, with ethanol being a marginally better extraction solvent.

3.2 Pollinator surveys

During the surveys, pollinators were only detected at five of the 60 sites (Figure 2B). In each of these cases, the pollinator species was *Thynnoides* sp. C (identification and nomenclature follows Phillips et al. [6]), with a total of 23 responses recorded. Additional opportunistic observations were made of this species pollinating *C. attingens* subsp. *attingens* at three other sites (included in Figure 2B). Confirmation that *Campylothylnus* sp. A is a pollinator of *C. attingens* subsp. *attingens* was first made

at one of the Ruabon study sites in 2014. Subsequently, this wasp was recorded at a total of five sites (spanning across approximately 20 km) in the Capel district.

4. Discussion

Here we show that the compound 4-hydroxy-3-(methylthio)benzaldehyde (**2**), is strongly sexually attractive to *Campylothylnus* sp. A, while (methylthio)benzene-1,4-diol (**1**), is not attractive on its own. However, the bioassay results show that a 1:1 blend of **1** and **2** (at the concentration tested) is even more strongly sexually attractive than compound **2**, with this blend securing an extremely high attempted copulation rate of 88%. Indeed, this is the highest rate of attempted copulations we have ever recorded for thynnine wasp males at any field bioassays with semiochemicals [4, 10, 14, 15, 28]. The previously highest recorded average copulation rate is 73% in the case of the male *Zelebora* sp. thynnine wasp pollinator of *Caladenia plicata* [14]. Furthermore, this rate observed for *Campylothylnus* sp. A is much higher than observed at bioassays involving the closely related *C. flavopictus*, the pollinator of *Caladenia crebra* [15], which exhibited average copulation rates of not more than 20%. The exceptionally high degree of sexual excitation in *C. sp. A*, is further evident in the complete absence of the intermediate sexual state of lands only. By contrast, in all other thynnine wasp bioassays that we have reported (see references above), lands only are frequently observed, and often more so, than attempted copulation.

Although the degree of sexual attraction observed in the present study was much greater than in the case of *Campylothylnus flavopictus*, the bioassay results are otherwise similar, in that compound **1** is not attractive on its own to *C. flavopictus*, while compound **2** is partially attractive, but less so, than various blends of (methylthio)phenols [15]. Intriguingly, in *C. flavopictus* a 10:1 blend of **1** and **2** (at equivalent concentrations as in this present study) elicited the strongest sexual behaviour, albeit with much lower copulation rates (~20%). As such, the genus *Campylothylnus* may show conservatism in the class of compounds used as sexual attractants, but with related species using different blends/constituents to achieve attraction. Such a result is well documented in *Neozelebora* wasps that pollinate *Chiloglottis* orchids, where bioassays with synthetic chiloglottones across multiple systems have confirmed that pollinator specificity (and likely sex pheromone specificity) is achieved by both variation in the combination of one to three specific chiloglottones and variation in the blend ratios [4]. While we were not able to locate any females to confirm if this compound or other (methylthio)phenols are part of the female sex pheromone of *Campylothylnus* sp. A, we predict this will be the most likely case based on what has been seen in other sexually deceptive orchids [2], and given the exceptionally strong sexual attraction we observed to these compounds.

The combination of floral visitation, wasps carrying pollen, and the confirmed presence of (methylthio)phenols in floral tissue, suggests that in the Capel district, *Caladenia attingens* subsp. *atingens* achieves pollination by using (methylthio)phenols to sexually deceive *Campylothylnus* sp. A. Based on morphological evidence that the two orchids, *C. crebra* and *C. attingens*, are not closely related, we suggest that sulfurous aromatic compounds are more widely used as orchid pollinator

attractants. Interestingly, although the two pollinators of *C. attingens* subsp. *attingens* do not belong to the same genus, the two genera (*Thynnoides* versus *Campylothynnus*) are quite closely related, at least relative to the wide phylogenetic diversity of thynnine wasp pollinators exploited by *Caladenia* orchids (see Figure 2B which depicts a subset of the full phylogeny by Phillips et al [6]). Numerous other species of *Thynnoides* are known to be pollinators of sexually deceptive orchids, primarily in the *Caladenia dilatata* complex to which *C. attingens* belongs [6, 19], but also in some other *Caladenia* [6], and in the unrelated orchid genus *Drakaea* [7]. As such, there is the potential for (methylthio)phenols to be prevalent semiochemicals across several lineages of Australian orchids as well as multiple lineages of thynnine wasps.

Our survey of pollinators across numerous sites spanning the geographic range of *C. attingens* subsp. *attingens* suggests that *Thynnoides* sp. C is likely to be the primary pollinator, with *Campylothynnus* sp. A. currently only known from the northern margin of the orchid's geographic range (Figure 2A). Thus, this pattern of pollinator switching could potentially arise without a shift in floral semiochemicals, when the orchid encounters different habitats and potentially different pollinator species at its range margin. Alternatively, the Ruabon population may show differences in floral semiochemicals compared with other populations, reflecting local adaptation to differences in the availability of pollinator species (see Peakall and Whitehead [29] for models of pollinator switching). Testing these alternatives will require elucidation of the sexual attractants of *Thynnoides* sp. C, and more detailed population level study of the patterns of floral semiochemicals production across the range of *C. attingens*. As sexually deceptive *Caladenia* orchids generally only use a single species of pollinator, *C. attingens* may offer a tractable study system for understanding the chemical basis of pollinator switching in sexually deceptive orchids more widely.

Finally, it is noteworthy that only very small amounts of (methylthio)phenols **2** and **3** were detected in the orchid flowers. Indeed, without prior knowledge of the relevance of these compounds and the availability of synthetic standards, it would have been very difficult to identify these biologically active compounds in *C. attingens*. Given their presence in such low amounts, and their poor chromatographic behaviour with GC-MS or GC-EAD, it is easy to understand why (methylthio)phenols [15] have only recently been discovered as natural products and sex pheromones. Nonetheless, we predict that with careful scrutiny these compounds may well prove to be widespread and important semiochemicals, particularly as key components of the chemical communication between other sexually deceptive orchids and their pollinators.

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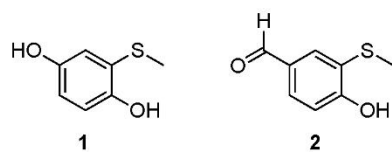
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Figure 1

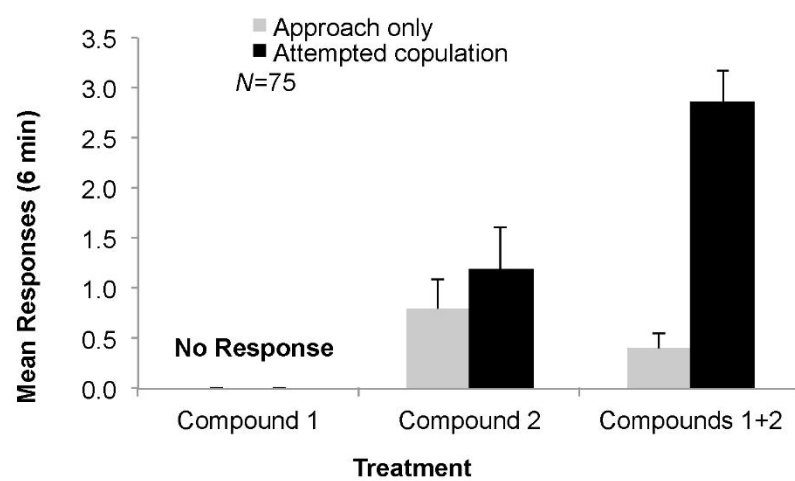
A



B



C



D

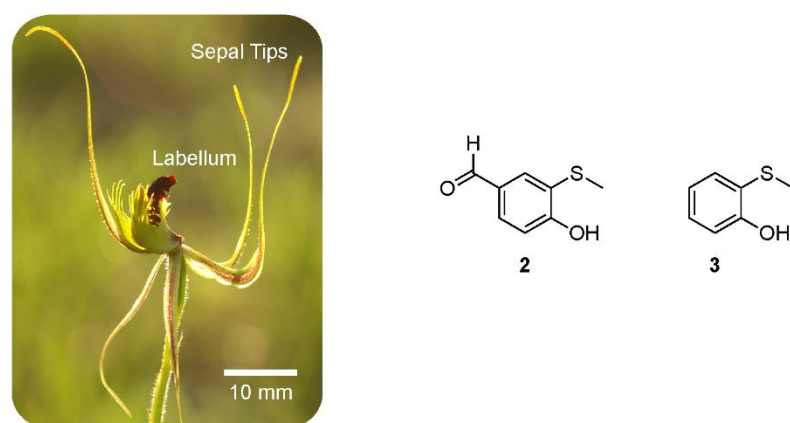
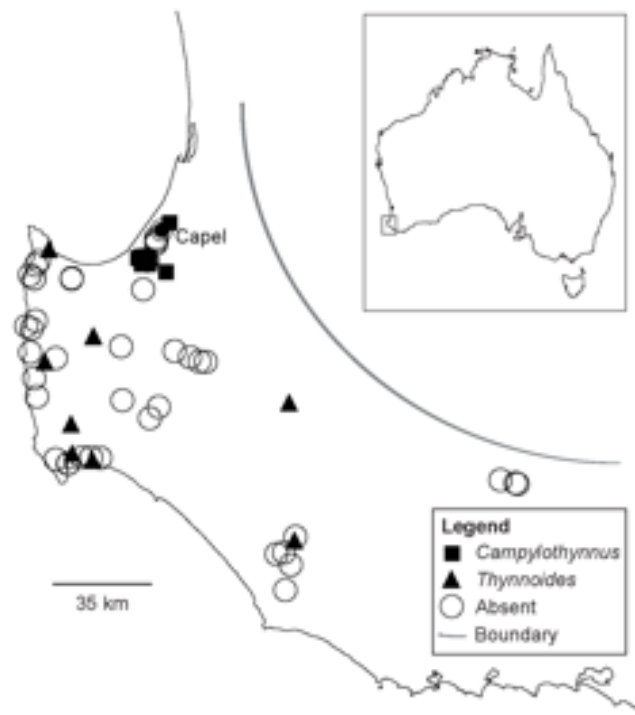


Figure 2

A



B

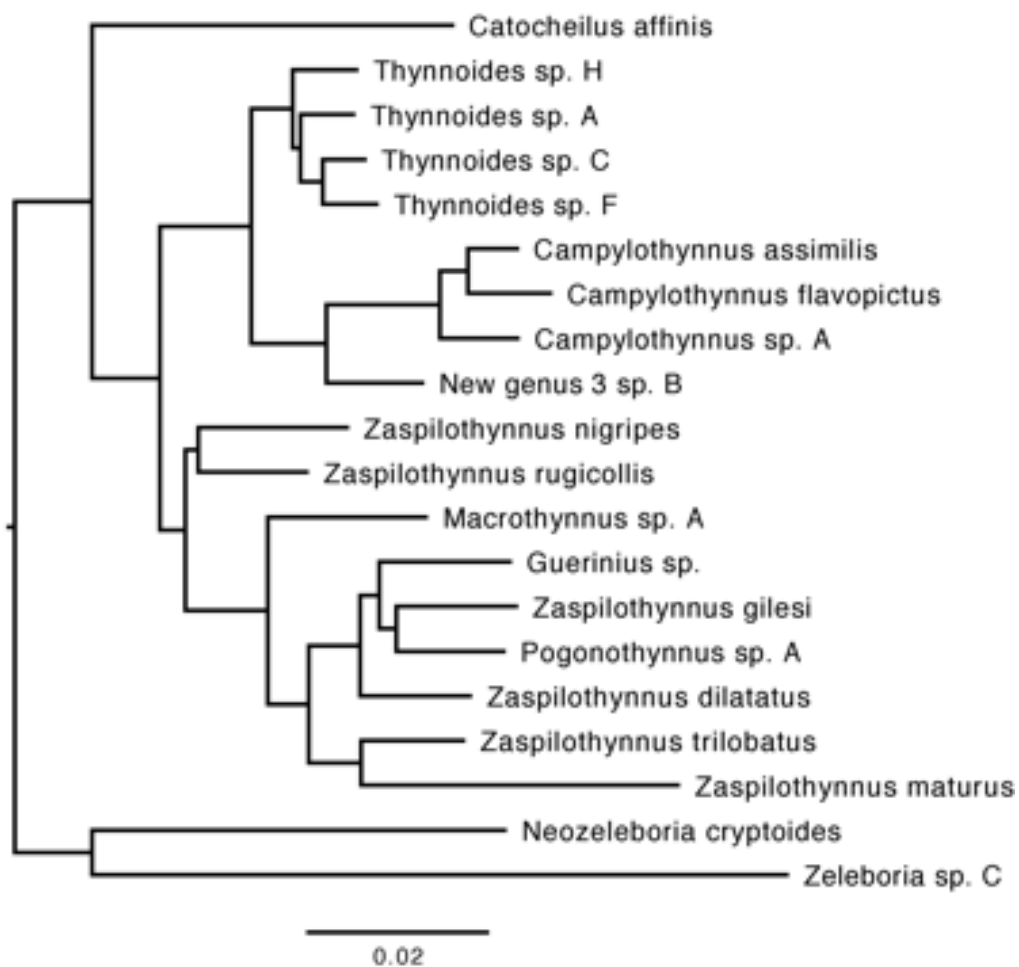


Figure captions

Fig. 1. Study compounds, study wasp and orchid species and outcomes of field bioassays

A. Chemical structures of the two (methylthio)phenol compounds used in the field bioassays: 2-(methylthio)benzene-1,4-diol (**1**) and 4-hydroxy-3-(methylthio)benzaldehyde (**2**).

B. Photographs showing a male thynnine wasp, *Campylothylnus* sp. A, landed (left) and attempting copulation (right) on a bioassay pin spiked with a blend of compounds **1** and **2**.

C. Outcomes of field bioassays showing the mean number of *C. sp. A* wasp responses per 6 min trial for three different treatments: compound **1** only (with no response), compound **2** only, and a 1:1 blend of compounds **1** and **2**. Wasp responses are partitioned into approach only (grey bars), and attempted copulation (black bars).

A single factor analysis of variance (ANOVA) revealed a significant effect of the treatment for the attempted copulation response ($F_{2,27}=5.77$, $P=0.008$), but not the approach only response ($F_{2,27}=1.862$, $P=0.175$). The attempted copulation rates were high for both the Compound **2** (60%) and Compounds **1+2** treatments (88%). However, a G-test revealed a significant difference between these two treatments ($G=3.99$, $P=0.04$, $n=75$).

D. A photograph of the orchid *Caladenia attingens* subsp. *attingens* with sepal tips and labellum structures labelled (left) and the chemical structures of the two (methylthio)phenol compounds found in floral tissues (right): 4-hydroxy-3-(methylthio)benzaldehyde (**2**) and 2-(methylthio)phenol (**3**).

Fig. 2. Orchid distribution boundary and wasp pollinator distributions and phylogeny

A. A map of the lower southwest of Western Australia showing the approximate northern boundary of the distribution of the orchid *Caladenia attingens* subsp. *attingens*. Also shown are the outcomes of the orchid pollinator survey, including sites where pollinators were absent (open circles), sites where the wasp pollinators *Thynnoides* sp. C (closed triangles) and *Campylothylnus* sp. A (closed squares) were present.

B. The results of a phylogenetic analysis showing the relationship of the genera *Thynnoides* and *Campylothylnus*. The phylogeny shows a subset of representative thynnine wasp genera, extracted from the larger phylogeny of thynnine wasps involved in orchid pollination and outgroups (presented in [6]). Please see this paper for full details of the phylogenetic analysis.

Graphical abstract

