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(Methylthio)phenol semiochemicals are exploited by deceptive orchids as sexual attractants for *Campylothynnus* 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 *Campylothynnus flavopictus* (Tiphiidae:Thynninae), the exclusive pollinator of *C. crebra*. Here we show with field bioassays that another undescribed species of *Campylothynnus* (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 *Campylothynnus* 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 *Campylothynnus* 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.

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 specialized, 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 floral volatile methyl acetophenone [14]. Finally, in *Caladenia crebra*, which is pollinated by males of the thynnine wasp *Campylothynnus flavopictus* (Tiphiidae), 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**) (Fig. 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

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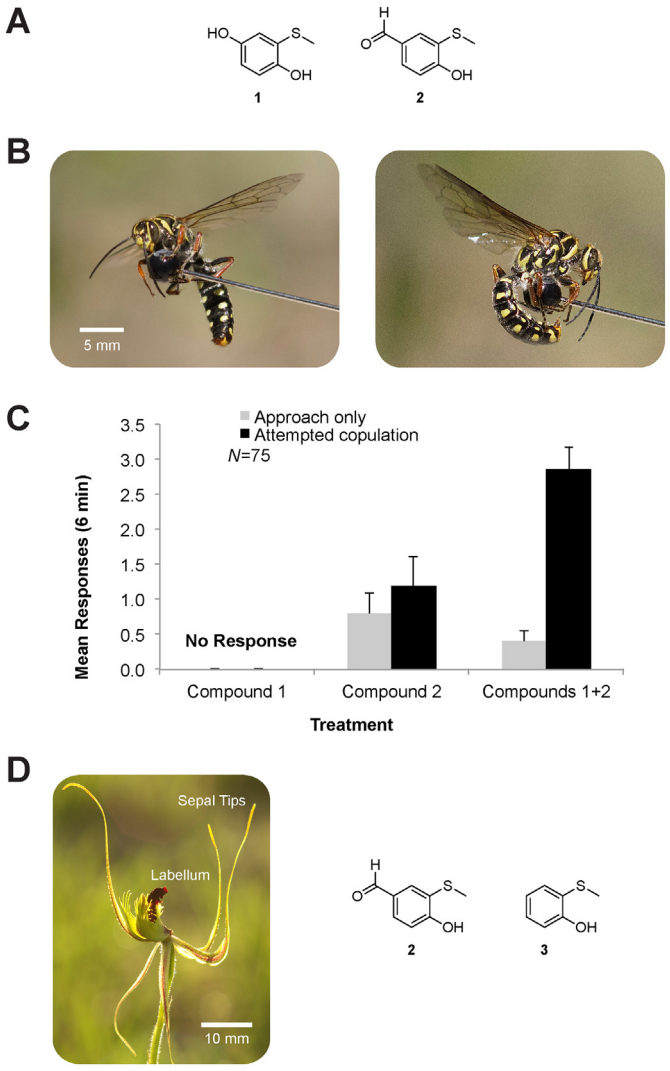

Fig. 1. Study compounds, study wasp and orchid species and outcomes of field bioassays. A. Chemical structures of the two (methylthio)phenol compounds used in the field bioassays: 2-(methylthio)benzene-1,4-diol (**1**) and 4-hydroxy-3-(methylthio)benzaldehyde (**2**). B. Photographs showing a male thynnine wasp, *Campylothynnus* 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**).
Photography by Rod Peakall.

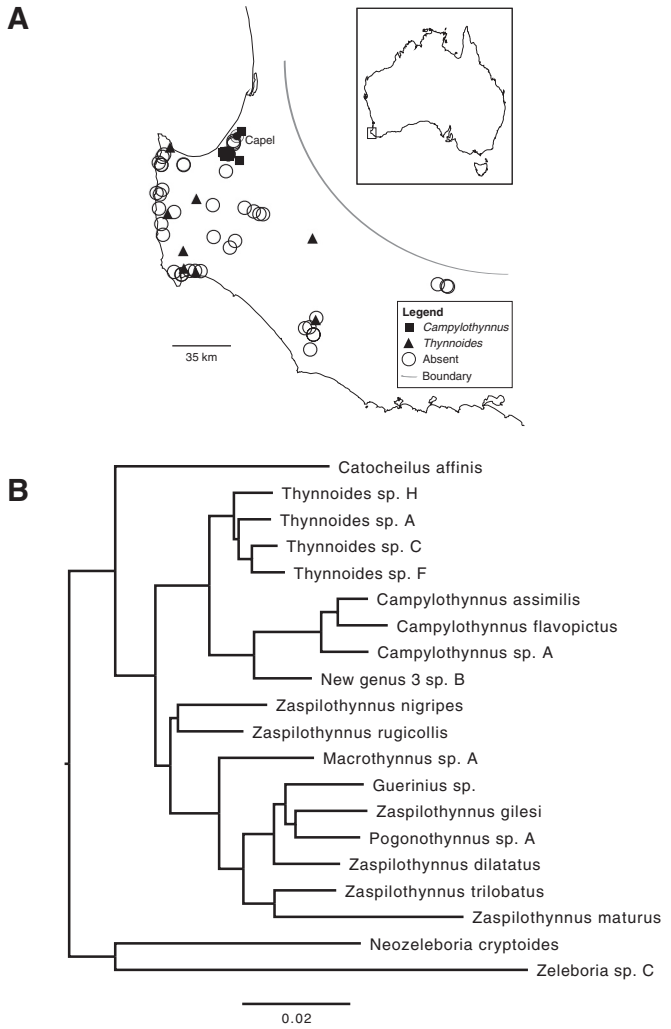

Fig. 2. Orchid distribution boundary and wasp pollinator distributions and phylogeny. A. A map of the lower southwest of Western Australia showing the approximate northern boundary of the distribution of the orchid *Caladenia attingens* subsp. *attingens*. Also shown are the outcomes of the orchid pollinator survey, including sites where pollinators were absent (open circles), sites where the wasp pollinators *Thynnoides* sp. C (closed triangles) and *Campylothynnus* sp. A (closed squares) were present. B. The results of a phylogenetic analysis showing the relationship of the genera *Thynnoides* and *Campylothynnus*. 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.

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 *Campylothynnus* (here after C. sp. A) was observed to be regularly and very strongly sexually attracted to these compounds (Fig. 2A). Furthermore, some of these wasps were observed carrying the pollen of *Caladenia attingens*