Pollinator-mediated selection, reproductive isolation and floral evolution in *Ophrys* orchids

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Pollination by sexual deception is arguably one of the most intriguing pollination syndromes among orchids. In this system, patrolling male insects, mostly hymenopterans, bring about cross-pollination while attempting copulation with the female decoys of orchid flowers, a phenomenon known as ‘pseudo-copulation’. In this study we investigate female sex pheromones of bees and wasps and floral odours of a range of European orchids in a comparative approach, along with bioassays performed with male bees in situ, in an attempt at unlocking the basis and specificity of orchid-pollinator interactions.

**Keywords:** pollination, floral odours, sexual deception, bees, orchids

**Mate choice and sex pheromones in the model: Colletes cunicularius**

*Colletes cunicularius* L. (Hymenoptera, Colletidae) is a vernal solitary bee that nests underground in dense, conspicuous aggregations (Michener 1974, Hefetz et al. 1979b, Westrich 1990, Borg-Karlson et al. 2003, Pouvreau 2004). As frequently observed in wild bees and wasps, males hatch before females and devote much of their time and energy to patrolling over nesting sites where conspecific mates are soon to emerge (Alcock et al. 1978). In *C. cunicularius*, females are presumably monandrous and not limited in their reproductive success by access to males, since sexually active males typically outnumber virgin, emerging females during the reproductive period (Fig. 1). Males are therefore thought to be under strong selection pressure to be first at finding and inseminating freshly emerged female partners (Alcock et al. 1978) and they frequently engage in intense scrambles to access pre-emerging females (Borg-Karlson et al. 2003, Vereecken et al. 2006).

Early studies on the chemical communication in *C. cunicularius* have shown that olfactory cues are primarily responsible for releasing the males’ overall exci-
tation and in directing them towards virgin females (Bergström & Tengö 1978). These works have mostly focused on linalool, the dominant odour compound in mandibular secretions of attractive females (Bergström & Tengö 1978, Hefetz et al. 1979a, Cane & Tengö 1981). Cane & Tengö (1981) have specifically reported on the mate-attractant function of linalool, yet their bioassays carried out with C. cunicularius males using linalool-scented dummies failed to release copulation attempts or pouncing behaviour. These authors showed that head extracts of C. cunicularius females elicited more searching flights and more copulation attempts in patrolling males than linalool alone. More recently, Borg-Karlson et al. (2003) showed that the (S)-(+) -linalool enantiomer was the key compound for mate attraction in mandibular secretions of C. cunicularius females. These authors also pointed out the occurrence of a dramatic decrease of the amount of this compound in virgin vs. mated females, which they suggested to be responsible for the loss of attractiveness of mated females to patrolling males.

During our research project on the chemical communication in C. cunicularius, we have performed a combination of chemical analyses by gas chromatography and electro-antennography (GC and GC-EAD) and we have been able to

Figure 1. Two males of C. cunicularius attempting copulation with a conspecific, emerging female (Photo NJ Vereecken).
perform bioassays \textit{in situ} with patrolling males of \textit{C. cunicularius}. A first step in our analyses has been to investigate the level of attractiveness of head versus cuticle extracts of virgin \textit{C. cunicularius} females to patrolling males and to test whether any of these blends could release a stereotyped mating behaviour. Our preliminary results showed that, contrary to head extracts, cuticle extracts contained a mixture of fatty acids (cuticular hydrocarbons) and their derivatives. By assaying the relative attractiveness of head vs. cuticle extracts, we have been able to demonstrate that, contrary to the head extracts, the cuticle extracts triggered a significant number of pounces and copulation attempts of patrolling males of \textit{C. cunicularius} with the scented dummies (Figs 2 & 3). These results confirmed earlier studies by Cane & Tengö (1981) and Borg-Karlson \textit{et al.} (2003) on the attractiveness of linalool, which indicated the long-range mate attractant function of linalool without however stating which cues are involved in short-range stimulation of male mating behaviour. Considering that (i) linalool is a highly volatile compound secreted by mandibular glands in females and that (ii) long chain hydrocarbons are less volatile compounds secreted on the female body cuticle, the sex pheromone in \textit{C. cunicularius} might be considered as multi-component, with linalool acting as long-range mate attractant and epicuticular compounds eliciting male mating behaviour at short range (Fig. 4) (Vereecken 2004, Mant \textit{et al.} 2005a).
We have then performed another series of bioassays that have helped identifying a set of three compounds in the cuticle extracts as the key compounds for mate attraction. No significant difference was found when testing these three compounds vs. the full blend of cuticular hydrocarbons and their derivatives with patrolling *C. cunicularius* males. The three key compounds of the female sex pheromone are mono-unsaturated fatty acids, namely (Z)-7-heneicosene (= C21ene), (Z)-7-tricosene (= C23ene) and (Z)-7-pentacosene (= C25ene) (Mant et al. 2005a).

We then sat out to investigate whether females from distant populations of *C. cunicularius* used the same chemical communication channel or if there was evidence for population differences in sex pheromone blends. This new project has led us to sample virgin females of *C. cunicularius* from 15 populations across Western Europe, in different countries such as Austria, Belgium, England, France, Germany, Italy Switzerland and the United Kingdom. Our first results were published this year (Vereecken et al. 2007) and they show that distant populations are characterised by sex pheromone ‘dialects’, i.e. the females of different sites use population-specific ratios of the key compounds for the attraction

![Figure 3. Patrolling male of *C. cunicularius* landing on a dummy (plastic bead mounted on a pin) scented with a cuticle extract of a conspecific, virgin female (Photo NJ Vereecken).](image-url)
of their mates. We have also been able to provide evidence for a positive fit between the chemical differences among populations and the geographic distance among the study sites, i.e. the more closely-located the populations are, the more likely they are to share a very similar sex pheromone ‘dialect’ (Vereecken et al. 2007). These studies are very encouraging and they have allowed pushing investigations on sex pheromones in wild bees to a level never attained before, notably (i) by pinpointing a minimum set of compounds as the key odour signal and (ii) by evaluating the variation in this signal among populations.

Since females from different populations used different ‘dialects’ of sex pheromones, we have set out to investigate whether males of a give population were more attracted by their ‘local’ females or if ‘exotic’ females (i.e. from allopatric populations) were preferred by patrolling males. All these behavioural bioassays and the results are described by Vereecken et al. (2007) (see references list). The recordings of behavioural responses performed with C. cunicularius males using synthetic copies of sex pheromones indicate that male bees are able to discriminate among the three synthetic copies of sex pheromones derived geographically-distant populations. Our results showed differences in the intensity of the sexual stimulation released by the synthetic blends and indicate that synthetic

Figure 4. Schematic sketch of mate attraction by multi-component female sex pheromone in Colletes cunicularius. Linalool is secreted by mandibular glands in C. cunicularius females and acts as longrange mate attractant (large circle). Contact aphrodisiacs (i.e. the contact sex pheromone) consist of cuticular hydrocarbons located on the female body surface and stimulate the sexual arousal of patrolling C. cunicularius males at close range (small circle), leading to copulatory behaviour (Vereecken 2004).
blends of the ‘local’ sex pheromones elicited significantly less approaching flight and less contacts with the odour source than blends of ‘exotic’ sex pheromones. As suggested in our original study, these preferences are likely to be the direct outcome of *C. cunicularius* populations experiencing significant levels of inbreeding. This assumption rests upon reports of males patrolling restricted regions of their emergence/reproductive site (Peakall & Schiestl 2004), and observations of females initiating the construction of their nest in the near vicinity of their emergence points early in their reproductive period. Populations of *C. cunicularius* are therefore expected to experience high degrees of philopatry, and inbreeding as an immediate consequence.

A forthcoming step in our studies on the bees’ side will be to sample more populations of *C. cunicularius* in Western Europe to test the hypothesis that the correlation found between ‘chemistry’ and ‘geography’ can also be highlighted at the genetic level, e.g. by using microsatellite markers. More populations are therefore desirable, especially from countries that have not been sampled so far (Denmark, Germany, The Netherlands, Sweden, etc.) where large colonies are present to make sure that the females could be sampled without putting the persistence of these bees in jeopardy locally.

**Pollinator attraction and floral odour in the mimic: Ophrys exaltata**

Quite the most remarkable pollination mechanism to be found in any flowering plants is that of the ‘insect orchids’ of the genus *Ophrys*. The flowers of these orchids are well-known for their striking resemblance to various insects. What function this resemblance serves has remained a mystery for a long time (Proctor *et al.* 1996). The remarkable similarity between some orchids and insects has long been discussed and many explanations were given for this resemblance, including the belief that the likeness to insects had nothing to do with pollination, but served to frighten away browsing cows (Van der Pijl & Dodson 1966). Even though Darwin’s (1862) treatise provides a remarkable contribution in the field of orchid pollination, many of the mechanisms and phenomena that are now part of the scientific background have been discovered and understood only over the past two decades (Dafni 1983, 1984, Ackerman 1986, Peakall 1990, Nilsson 1992, Schiestl *et al.* 1999).

It was not until the early decades of the twentieth century that it was discovered that pollination in most of what we now know as sexually deceptive orchid species is brought about by insects attempting copulation or a pre-copulatory routine on the orchid labellum (Correvon & Pouyanne 1916, Pouyanne 1917, Kullenberg 1961, Proctor *et al.* 1996, Peakall & Beattie 1996, Schiestl *et al.* 1999). This procedure, often called *pseudo-copulation* (when the insect attempts to copulate with the flower) (Fig. 5) or *sexual stimulation* (when the insect goes through part only of its mating behaviour during the pollination process) was first discovered by Pouyanne (Correvon & Pouyanne 1916, 1923, Pouyanne 1917) after
some 20 years of observations carried out on the field in Algeria. His works have revealed that the flowers of the ‘mirror orchid’, \textit{Ophrys ciliata} (= \textit{Ophrys speculum}) (Fig. 6), attract a male wasp (namely \textit{Dasyscolia ciliata} – Hymenoptera: Scoliidae) by imitating its female partner (Fig. 7). In the course of a century, many controversies have arisen around sexual deception. Pouyanne’s observations, perhaps because he was not a member of the scientific community, were met with disbelief by laboratory biologists who denounced his findings as the ‘nonsensical ideas of an amateur ecologist’ (Van der Pijl & Dodson 1966, Van der Cingel 1995, Proctor \textit{et al.} 1996). Later, Godfrey (1922, 1929) and Wolf (1950) corroborated Pouyanne’s botanical field observations and deductions, while Kullenberg (1948, 1952a, 1952b, 1956, 1961, 1973) analysed the phenomenon from the zoological, physiological, and chemical perspective.

The attractiveness of a flower to its pollinating agents can be attributed to three major categories of cues, namely (i) the olfactory stimulus; (ii) the optical appearance; and (iii) the tactile stimulus (hairiness or surface villi) (Proctor \textit{et al.} 1996). These cues act on the senses of the pollinator (Van der Pijl & Dodson 1966), which associates them with an expected reward (Nilsson 1983, 1992, Dafni 1984, Vogel 1993, Roy & Widmer 1999). Even though the three categories of floral cues are commonly interdependent (\textit{i.e.} appearance combines with odour to attract the pollinator), some of these stimuli may be prevailing in attracting...
Ophrys pollinators (Van der Pijl & Dodson 1966). For many decades since Pouyanne’s first reports (1917), the key issue in Ophrys pollination has undoubtedly concerned the nature of the exact floral cues that have the ability to sexually stimulate male Hymenopterans in search for a mate (Barth 1991, Nilsson 1992).

Of the three main types of stimuli and their respective role in attracting male Hymenopterans, it seems that the visual cue is the least important (Borg-Karlson 1990). Being the least important does not mean that visual attraction does not take place. Correvon & Pouyanne (1916, 1923) reported striking decrease male visitation when Ophrys ciliata labella are placed upside down and on back to front: male insects seemed to spend more time finding the labella because the brown hairs and the central blue patch (‘speculum’) of the orchid labellum (which was suggested to mimic the female’s crossed wings in the sunshine) were no longer visible. It is not really surprising that olfactory cues are the most important means of insect attraction in sexually deceptive orchids (in Ophrys as in other non-European orchid genera) since, as mentioned above, olfactory cues are key stimuli in the sexual behaviour of numerous insect species including Aculeate Hymenopterans (Kullenberg & Bergström 1973, 1976, Alcock et al. 1978, Bergström 1978, Eickwort & Ginsberg 1980, Ayasse et al. 2001). Most investigations on the chemistry of Ophrys pollination have stressed the similarity bet-

Figure 6. Detail of an individual flower of the mirror orchid, Ophrys ciliata (= O. speculum) (Photo NJ Vereecken).

Chemical mimicry in Ophrys involves cuticular hydrocarbons similar to those found in the attracting female Hymenopterans. These compounds mainly consist of long-chain fatty acids and their derivates similar to those found in the respective females’ cuticle extract, and trigger a mating behaviour in the targeted males (Borg-Karlson 1990, Schiestl et al. 2000, Schiestl & Ayasse 2002). The Ophrys floral odour bouquet is sometimes composed of up to 100 volatile compounds of which only a small proportion takes part actively in insect attraction (Schiestl et al. 1999, Ayasse et al. 2000, Schiestl & Marion-Poll 2002). Qualitative and quantitative differences in floral odour and the numerous combinations in these volatile compounds represent a considerable source of chemodiversity which enhance possibilities to attract pollinators on a species-specific basis.

Recent investigations have demonstrated that O. exaltata attracts patrolling males of C. cunicularius by releasing the same set of key odour compounds as C. cunicularius females (Mant et al. 2005a), thereby providing further evidence for chemical mimicry in Ophrys pollination. Further studies on this species in the south of Italy have shown that in the orchid too, the pollinator-attracting odour signals vary across populations (Mant et al. 2005b). In short, orchids belonging

Figure 7. A female of the scoliid wasp Dasyscolia ciliata showing the red hairs covering its abdomen (Photo NJ Vereecken).
to the same species but occupying different populations are characterised by different proportions of their key odour compounds, a situation identical to what was observed on the bees’ side.

**Predictions for the evolution of the Ophrys-pollinator mimicry system**

After we had discovered that the signals mediating the attraction of *C. cunicularius* males varies among populations both in the model (female bees) and in the mimic (the orchid) of this plant-pollinator interaction, we have started wondering how accurate the mimicry was in terms of the relative proportions of the key odour compounds. Do the orchids imitate the local ‘dialect’ of their model species? Or are there differences in odour between the orchids and the bees when sampled in sympatry? From the bioassays we have performed with *C. cunicularius* males and the available data on floral odour variation in *O. exaltata*, we have predicted that selection should favour orchids that emit odour blends that would slightly differ from the local females’ mating signals, which would (i) allow the attraction of *C. cunicularius* and (ii) make the orchids more attractive than their local models to patrolling *C. cunicularius* males (Vereecken et al. 2007). We are currently in the process of analysing our data from multiple populations where the bees and the orchids were sampled in sympatry, and our results indicate that the predictions made prove to be accurate. More details on the results and the bioassays that followed will be given during the keynote presentation.

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