COMMENT

Integrating past and present studies on *Ophrys* pollination – a comment on Bradshaw *et al.*

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In a recent research article in the *Botanical Journal of the Linnean Society* Bradshaw *et al.* (2010) provided a detailed and richly illustrated comparative analysis of labellum micromorphology in *Ophrys* L. They described the variety of epidermal cell types and the different patterns of structural complexity observed in representative species in *Ophrys*. We agree with Bradshaw *et al.* that the study of the labellum micromorphology, besides its general significance for plant morphology, is also of interest in the framework of pollinator attraction, as it might influence the optical properties of the labellum and play an important role in directing male insects to the right position for collection or delivery of pollen masses during pseudocopulation. The latter aspect was discovered some 50 years ago by Kullenberg (1956a, b, 1961) and investigated further by Ågren, Kullenberg & Sensenbaugh (1984) (a key reference overlooked by Bradshaw *et al.*), and the detailed descriptions by the authors shed light on possible directions of phenotypic evolution in *Ophrys*. However, we think that Bradshaw *et al.* drew largely unsupported conclusions and overlooked a considerable body of literature when they discussed the role and evolution of other floral traits, including pollinator-attracting signals, patterns of reproductive isolation and speciation in this genus of sexually deceptive orchids. Their view is notably summarized in the abstract of their article, in which they argued that 'The relative contributions of olfactory, visual and tactile cues to the sophisticated pseudocopulatory pollination mechanism that characterizes *Ophrys* remain unclear, but the degree of reproductive isolation achieved, and thus the speciation rate, have certainly been greatly exaggerated by most observers'. This statement echoes similar conclusions in other papers (see, for example, Bateman *et al.*, 2003; Pedersen & Faurholdt, 2007; Devey *et al.*, 2008, 2009). Based on a long series of independent past and present studies, we advocate that the view expressed in the last section of their article and in the other works cited below is too simplistic and speculative and misrepresents the current state of understanding of *Ophrys* ecology and evolution.

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In this comment on the article by Bradshaw *et al.*, we first challenge their views on the nature of *Ophrys*–pollinator interactions. Second, we review pollinator-attracting signals based on the available evidence. Finally, we discuss reproductive isolation in *Ophrys* and its evolutionary consequences, because this issue has been central to our research during the past decade.

OPHRYS POLLINATION: CO-EVOLUTION OR UNILATERAL ADAPTATION?

The fact that Bradshaw *et al.* based their introduction and part of their discussion on the 'co-evolutionary features of *Ophrys* orchids' demonstrates that the authors have misunderstood the widely accepted nature of the interactions between sexually deceptive orchids and their pollinators. 'Co-evolution' in the strict sense is defined as 'an evolutionary change in a trait of the individuals in one population in response to a trait of the individuals of a second population, followed by an evolutionary response by the second population to the change in the first' (Janzen, 1980). There is a clear component of reciprocity in this concept, which is unlikely to be applied to the case of sexual deception, where the orchids are completely dependent upon their pollinators that are unwittingly exploited to secure pollination of the orchid. This is a typically one-sided interaction, an adaptation of the plants to the insects and not vice versa (Mant *et al.*, 2002 demonstrated this nicely using a phylogenetic approach; see also Paulus, 1988). There is no evidence so far that any change in the floral odour chemistry of *Ophrys* flowers could trigger changes in the female sex pheromone of the associated male insects, which in turn could affect the evolution of the orchid floral scent in a reciprocal fashion. The male insects are exploited and our data suggest that there is no co-evolutionary pattern of any kind to be found in this parasitic plant–insect interaction (see Vereecken, 2009).

FLORAL ODOURS ATTRACTING POLLINATORS IN *OPHRYS*

Since the description of insect pollination in *Ophrys* by Pouyanne (1917; see also Correvon & Pouyanne, 1916, 1923) and the subsequent works of Kullenberg (1956a, b, 1961, 1973), it is a demonstrated fact that *Ophrys* orchids lack nectar, that they do not provide collectible pollen and that their exclusive pollinators are male insects, primarily wild bees, solitary wasps and sometimes beetles (Paulus & Gack, 1990; Paulus, 2006). These insects visit *Ophrys* flowers during their patrolling flights for mates, and they pollinate the flowers during attempted copulation (i.e. pseudocopulation) with the female decoys on the flowers (Kullenberg, 1961). The male insects regularly approach the flowers from downwind in a stereotyped zig-zagging fashion that is observed in several other groups of insects approaching odour sources once they enter the concentration gradient of volatile compounds of the odour plume. This behaviour was described at length by Kullenberg (1956b, 1961, 1973; see also Kullenberg, Borg-Karlson & Kullenberg, 1984), and several field experiments carried out by Pouyanne and Kullenberg (see references above) showed that the male insects kept on approaching flowers that had been hidden inside a mesh or a piece of velvet permeable to the air and therefore to the floral scent emitted by the labellum. Our own behavioural bioassays performed under natural conditions have shown that male bees regularly attempt copulation with small plastic beads scented with synthetic mixtures of their female sex pheromones and synthetic copies of the floral scent extracts of *Ophrys* flowers that contain the same odour compounds (reviewed by Schiestl, 2005; Ayasse, 2006; Vereecken, Mant & Schiestl, 2007; Stökl *et al.*, 2008; Vereecken & Schiestl, 2008, 2009; Ayasse *et al.*, 2010). Collectively, these experiments show that the key to the attraction of male insects as pollinators is the floral scent in *Ophrys* orchids, particularly mixtures of *n*-alkanes and *n*-alkenes emitted by the labellum, but also [omitted by Bradshaw *et al.* (2010: 536)] oxygenated acids in the case of the mirror orchid (*O. ciliata* Biv. = *O. speculum* Bertol.) (Ayasse *et al.*, 2003) or a mixture of aldehydes, alcohols, fatty acids and corresponding esters in the Sardinian endemics *O. chestermanii* (J.J. Wood) Gölz & H.R.Reinhard and *O. normanii* J.J.Wood (pro hybr.), both pollinated by males of the cuckoo bumblebee *Bombus vestalis* (Gögler *et al.*, 2009). The primacy of floral scent for pollinator attraction is also evident among Australian sexually deceptive orchids (Schiestl *et al.*, 2003; Franke *et al.*, 2009; Peakall *et al.*, 2010).

Bradshaw *et al.* (2010: 536) stated that: 'Given the micromorphological and apparent ultrastructural complexity of the labellum, it seems highly desirable to determine more conclusively which regions and cell types on the labellum are osmophores, and whether particular pseudopheromones are preferentially generated from particular regions of the labellum'. Uncovering osmophores on the *Ophrys* labellum is certainly of general interest and Vogel (1954) previously illustrated them in detail. Other authors, including Ascensão *et al.* (2005), also took up the challenge of locating and describing osmophores in *Ophrys*, but, like the working hypothesis of Bradshaw *et al.*, their quest was almost bound to reach an impasse. Indeed, the idea that osmophores may be key for pollinator attraction in *Ophrys* reflects the

authors' lack of knowledge about the chemical nature of the behaviourally active 'scent' compounds. The key pollinator attractants in *Ophrys* pollinated by *Andrena* and *Colletes* males are primarily alkenes, a class of unsaturated, long-chain cuticular hydrocarbons, that are not produced by osmophores, but by subcuticular cells in the epidermis of the plant tissue (Samuels, Kunst & Jetter, 2008). Alkanes and alkenes are major constituents of the wax layer of all plant tissues, serving as a waterproof barrier that helps regulate tissue dehydration, etc. (Riederer & Müller, 2006 and references therein). They are also found in significant amounts on the surface of leaves, stems and sometimes even sepals, albeit with different compositions. Hence, the production of pollinator-attracting compounds on the labella of *Ophrys* may indeed differ between different regions of the labellum, but this may not strictly be linked to osmophores in a classical sense.

VISUAL DISPLAYS OF *OPHRYS* FLOWERS

The spectacular visual displays of *Ophrys* flowers have long intrigued pollination biologists who set out, from the early years, to quantify the relative role of floral scent and floral colour (among other traits) in pollinator attraction. From these studies, it appears that, as we have explained above, the floral scent holds the key to pollinator attraction in most cases investigated so far (practically all the species investigated by Bradshaw *et al*.), although the colour of the perianth or of the labellum can in some instances increase pollinator attraction or the detectability of flowers against their background (Kullenberg, 1956a, 1961; Spaethe, Moser & Paulus, 2007; Vereecken & Schiestl, 2009; Streinzer, Paulus & Spaethe, 2009; Streinzer *et al*., 2010; Spaethe, Streinzer & Paulus, 2010). Why, then, Bradshaw *et al*. claim that '. . . although each of the three categories of cue has tended to be researched separately, making it difficult to determine their relative importance' remains a mystery as the hierarchical importance of the floral traits in pollinator attraction has been established for many years (see references above).

Bradshaw *et al*. focused on the structural colour and the physical nature of iridescence of *Ophrys* flowers at length in their introduction and discussion, and they suggest that the *Ophrys* labellum patterns (or 'speculum') could represent a novel model system for the study of these morphological traits. Unfortunately, they fail to provide any data on the iridescent nature of the speculum of some *Ophrys* other than stating that 'iridescence [is] readily detectable to the human eye' (Bradshaw *et al*., 2010: 533). The transmission electron microscopy (TEM) pictures presented might allow speculation about iridescent properties, but they are, in our view, insufficient to provide evidence for iridescence.

To demonstrate iridescence, it is necessary to perform reflection measurements of the flower labellum from different angles to show that reflection changes depending on the angle. Subsequent model calculations based on the visual system of any animal in question (in the case of *O. speculum*, the scoliid wasp *Dasyscolia ciliata*) are a crucial prerequisite to show that the colour shift between different view angles could be theoretically perceived by the male wasps and thus might act as a signal. Our own results (M. Streinzer, H. F. Paulus & J. Spaethe, unpubl. data) suggest that there is no apparent iridescence on the labellum of *O. speculum*.

We agree with Bradshaw *et al*. that the visual system and colour vision, in particular, differ between humans and bees, although we think that more appropriate references could have been cited in their article, and we wonder why the authors did not present reflection measurements (as stated in their methods) instead of referring to the reflectivity of different structures (especially the speculum) by characterizing them as 'reflective' or 'more reflective'. This approach overlooks the potential ultraviolet (UV)-reflectivity that is often high, especially for the speculum region [for *O. insectifera* L. see Kullenberg 1961; *contra* Bradshaw *et al*. (2010: 533)].

REPRODUCTIVE ISOLATION AND SPECIATION IN *OPHRYS*

Bradshaw *et al*., 2010: 504) stated that '. . . the degree of reproductive isolation achieved [has] certainly been greatly exaggerated by most observers', but this statement was not backed up by any evidence and, more importantly, it deliberately ignored and contradicted existing studies from the earliest until the more recent. Although some *Ophrys* spp. growing in sympatry occasionally hybridize (Stebbins & Ferlan, 1956; Danesch & Danesch, 1972; Souche, 2008; Stökl *et al*., 2008, 2009; Cortis *et al*., 2009; Vereecken, Cozzolino & Schiestl, 2010a), this phenomenon should be regarded as the result of infrequent interspecific pollen transfers mediated by the pollinators. Any such transfer of pollen masses from one species of *Ophrys* to another has the potential to create several thousands of seeds, which in turn can result in hybrid individuals growing intermixed with their parents for several years and creating the illusion that the production of hybrids is a continuous process. From our studies and the data gathered, we argue that it is premature to conclude from the occasional hybrid zones or overlaps in molecular markers that floral isolation is weak. We have measured intra- and inter-

specific pollen flow in several closely related *Ophrys* spp. in the field using stained pollinia and found high levels of intraspecific transfer, but not a single transfer among species, suggesting that floral isolation is indeed strong among those species (*O. exaltata* Ten., *O. sphegodes* Mill. and *O. garganica* E.Nelson ex O.Danesch & E.Danesch) (Xu *et al.*, 2011; see also Schlüter *et al.*, 2007, 2009).

Measuring floral isolation involves designing and conducting specific pollinator or pollen flow experiments using molecular markers or stained pollinia before statements about pollinator-mediated reproductive isolation be made. From a lack of phylogenetic resolution, or patterns of overlap in molecular markers alone, it is not possible either to infer gene flow between species or to discuss reproductive isolation and the specific taxonomic status of certain taxa (contra Devey *et al.*, 2008, 2009). Although molecular markers may often show overlapping patterns between closely related species, floral traits under selection, such as the floral scent in *Ophrys*, may still be distinct (Mant, Peakall & Schiestl, 2005; Stökl *et al.*, 2009). Hence, it appears that one can get a different picture by using a more integrative perspective with floral scent analyses, behavioural bioassays with the pollinators under natural conditions and molecular markers (see, for example, Mant *et al.*, 2005; Cortis *et al.*, 2009; Gögler *et al.*, 2009; Stökl *et al.*, 2009; Peakall *et al.*, 2010; Vereecken *et al.*, 2010a). From our recent multidisciplinary studies on different groups of *Ophrys* spp., we found that molecular markers (AFLP and microsatellites) often revealed considerable overlap, contrasting markedly with patterns of pollinator attraction, floral scent differentiation and gene flow measured *in situ* among the same species (Soliva & Widmer, 2003; Mant *et al.*, 2005; Xu *et al.*, 2011). For example, individuals of *O. bilunulata* Risso and *O. fabrella* Paulus & Ayasse ex P.Delforge occurring in sympatry on the island of Majorca had the same genotype based on AFLP markers (Stökl *et al.*, 2009), whereas chemical analyses revealed that the two taxa had distinct floral odours and morphology, and behavioural bioassays showed that they attracted different pollinators. From what we know up to now, this is largely consistent with high levels of floral isolation (pollinator isolation). This is in marked contrast to the claims by Bradshaw *et al.* that 'microspecies of *Ophrys* are not reliably identifiable by any known method'. Therefore, we are determined to resist adopting the view that the genus *Ophrys* is exclusively limited to a relatively small number of 'macro-species', each comprising a number of subspecies (Bateman *et al.*, 2003; Pedersen & Faurholdt, 2007; Devey *et al.*, 2008, 2009).

Furthermore, we think that the largely unsupported statement that 'Many of the supposed micro-species, particularly those showing local distributions, are likely to be hybrid swarms' is overstated. First, because the apparent high variability in floral morphology sometimes renders the identification of *Ophrys* spp. challenging in the field (see also Vereecken, Dafni & Cozzolino, 2010b), one needs to be careful in assigning individual plants the status of hybrids. (see, for example, the discussion by Schlüter *et al.*, 2007 on *O. fleichmanii* Hayek). Sorting out ancestral polymorphism from ongoing gene flow or hybridization/introgression using molecular markers can be challenging in groups of organisms that are apparently of recent origin or that show little genetic differentiation (see, for example, Muir & Schlötterer, 2005). Floral and total reproductive isolation and patterns of hybridization can and must be measured using standardized methods (see, for example, Ramsey, Bradshaw & Schemske, 2003) before such conclusions can be drawn and a more thorough understanding of the taxonomic status of many forms can be reached. More work should be carried out towards some sort of compromise relating to the number of species recognized, which probably lies somewhere between the extremes of 19 species recognized by Pedersen & Faurholdt (2007) and the 250+ species by Delforge (2005), among others. It is likely that not all the narrow endemic species have a long history and, in some cases, they might be of recent hybrid origin.

Finally, we anticipate the criticism that we defend a dogma of extremely high levels of specificity in *Ophrys*-pollinator interactions which, as Bradshaw *et al.* noted, would constrain any further diversification in the orchid genus. Our observations from the field in different regions of the Mediterranean over the years strongly support the fact that these interactions are highly specific (although we agree that they are not systematically the extreme case of one orchid/one pollinator). They are therefore in agreement with results from the studies by Kullenberg (see references above), who showed that *Ophrys* flowers are pollinated by a narrow taxonomic range of pollinators, from a single species to up to five closely related species. For example, *O. lutea* Cav. *sensu stricto* is known to be pollinated by several species of mining bees (genus *Andrena*) in the subgenera *Chlorandrena* and *Chrysandrena* all around the Mediterranean basin (Paulus & Gack, 1990; Paulus, 2006). Further reports in agreement with the lack of a strict one-to-one relationship were provided by Lorella, Mahé & Séité (2002), who showed, over a period of several years, that *O. sulcata* Devillers-Tersch. & Devillers and *O. sphegodes* are each pollinated by several *Andrena* spp. in western France. These last two examples provide evidence that, contrary to the generalization of Bradshaw *et al.* that pollinator records are 'highly geographically, temporally and/or

numerically restricted', there are repeated and consistent *Ophrys* pollinator records that support the view that *Ophrys*-pollinator interactions are flexible in time and space, unlike the stereotyped one-to-one relationship upon which Bradshaw *et al*. based their criticism. Bradshaw *et al*. referred to Bateman *et al*. [2010, in press in *L'Orchidophile*, but this was subsequently published as Bateman *et al*. (2011) in another journal], as supporting their views, but the backbone of this companion article aimed at a general audience (orchid enthusiasts mostly) echoes essentially the same line of arguments as those of Bradshaw *et al*. (2010) on topics detailed above, such as co-evolution, one-to-one relationships and reproductive isolation.

In conclusion, if indeed 'More general lessons could be learned regarding the evolution, adaptive significance and genetic control of complex floral traits', a first lesson to learn would be for researchers in general to widen their perspectives by integrating past and present studies from all research groups and performing studies in a multidisciplinary fashion.

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