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Ecologie et Evolution des odeurs florales chez Antirrhinum Majus

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« Le but de l'évolution
est de rendre la vie sur terre
plus consciente »

Barry Long,
Intuitions sur l'origine

Remerciements

Toute petite déjà, dans ma contrée champenoise, je passais des heures à observer, expérimenter et comprendre … comment les dytiques reformaient leur bulles d'air, les escargots de Bourgogne pondaient, les vairons frayaient, les fourmis amassaient des proies bien plus grosses qu'elles… et aussi ce que pouvaient bien pouvoir se raconter ces fameuses gueule-de-loup que je pinçais dans le jardin de mamie Aimée !

Ce n'est bien plus tard que j'ai appris que ces questions avivaient aussi parfois l'esprit des adultes ; on pouvait même en faire son métier ! Jamais je n'aurais cru que cela me serait accessible, et pourtant, me voilà à écrire le point final de ma thèse en écologie.

Aussi, je tiens à remercier Jérôme et Christophe T. qui, avec la complicité de Christine et Valérie, ce jour là, m'ont offert une thèse sur un plateau d'argent parce que je collais des arbres phylogénétiques géants sur les murs du labo en stage de master... ça valait bien son pesant de champagne de début de thèse.

Rapidement, j'ai commencé à m'intégrer grâce à l'équipe *Antirrhinum* : Christophe A., Monique, Emmanuelle et Aurélie, plus tard Benoit ; et grâce aux thésards de l'époque qui nous apprenaient, à nous, les nouveaux, combines et astuces. Je pense à Maylin, Erwan, Jean, Suzanna, Fabian, Charles, Julien.

Au labo de chimie, « m'intégrer », je froisserais les blouses blanches ambulantes de Fabien, Mathieu, Lupita, Sylvain, Emilie, Louise et… _vous êtes décidément trop nombreux_ si je leur disais que je m'y suis intégrée. Effectivement, je remercie sincèrement la patience que Christine, mais aussi Valérie, Géraldine, Céline et Laure ont eu à me former aux techniques analytiques.

Le printemps arrive. Il me faut apprendre à cultiver les plantes fétiches avec l'aide précieuse des mains vertes de Jean-Luc, Serge, Colette et Dominique à l'humour inébranlable. Sur le terrain, je trouve les savoirs d'Enrico Coen, Pablo Vargas et Pierre Rasmont pour dénicher, observer et capturer. L'échantillonnage n'aurait pas été possible sans l'adresse de Julie, l'habileté de Pascal, la vivacité de Mathias et l'efficacité de Benjamin, les quatre stagiaires qui m'ont accompagné. Un clin d'œil particulier à Pascal qui m'a gratieusement initié à la guitare malgré les longues, très longues, heures d'observation de pollinisateurs.

Les premiers résultats apparaissent et l'émulsion intellectuelle est notamment animée par la rencontre de Bertrand et Laurent, aux GDR d'écologie chimique, qui deviendront plus que des collaborateurs, mais de véritables conseillers avec qui j'échangerai en toute liberté. Un grand merci à vous deux.

Merci également à Martin Giurfa de m'avoir accueilli dans son laboratoire et de m'avoir guidé avec pertinence dans ce nouveau monde qu'était pour moi la cognition animale.

Je n'oublierai certainement pas le clin d'œil qui leur est dû … aux thésards avec qui j'ai partagé mon quotidien « d'apprenti chercheur », à savoir, Elodie, Chloé, Aurélie, Juliette, Sarah, Marion, sans oublier mes très chers Fanou et Marius en direct de Paris et Bordeaux.

Enfin une infinie et chaleureuse pensée à « la fratrie Suchet » et à ma petite mamounette qui ont su ranimer la flamme dans mes baisses de moral sans se poser de questions, et sans me juger. Vous pouvez être fiers de vous ; c'est aussi grâce à vous.

Peut-être que oui, peut-être que non, j'espère que oui… cette pensée, comme toutes les autres, te parviendra papa.

Sébastien, tu ne peux plus l'entendre LE mot, mais je vais te le redire une dernière fois pour que tu comprennes à quel point l'accomplissement de ma « thèse » je te la dois aussi. Tu ne voudrais pas soutenir à ma place ?! Tu la connais par cœur…

Merci à tous pour cette belle aventure, au combien enrichissante, qui m'a permis de réaliser un des mes rêves de vie du haut de mes 26 ans !

Sommaire

Publications

Mes travaux de thèse ont abouti à cinq articles scientifiques qui constituent le cœur des trois chapitres de ma thèse.

CHAPITRE 1 :

Article 1 (En re-soumission dans *Journal of Chemical Ecology*):

Reproducibility of flower scent emissions in two wild subspecies of the snapdragon, *Antirrhinum majus*

Suchet C., Simon V., Raynaud C., Chave J.

Article 2 (En préparation):

Variation of floral scent in F_1 and F_2 hybrids of two wild snapdragon subspecies (*Antirrhinum majus*)

Suchet C., Raynaud C., Chave J.

CHAPITRE 2 :

Article 3 (Accepté dans *Behavioral Ecology and Sociobiology*):

Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve bumblebees

Suchet C., Dormont L., Schatz B., Giurfa M., Simon V., Raynaud C., Chave J.

Article 4 (En préparation):

Wild pollinators learn the use of flower scents of two snapdragon subspecies (*Anttirrhinum majus*)

Suchet C., Raynaud C., Chave J.

CHAPITRE 3 :

Article 5 (En préparation):

Associative patterns between floral odor-color and nectar traits in two subspecies of snapdragon (*Antirrhinum majus*)

Suchet C., Mathieu C., Raynaud C., Chave J.

J'ai également participé au projet de biogéographie des populations d'*Antirrhinum* de l'Est des Pyrénées *via* la collaboration entre l'équipe *Antirrhinum* d'EDB (Toulouse, France) que Christophe Thébaud dirige et l'équipe *Antirrhinum* du Jardin Botanique de Madrid dirigé par Pablo Vargas (Madrid, Espagne). Suite à la collecte des données qui est encore en cours, la rédaction d'un article est aussi planifiée.

Résumé de thèse

Parmi les signaux floraux, les odeurs florales sont remarquables pour leur complexité en composés odorants et leur variation entre, et au sein des taxa. Elles interviennent dans de nombreuses interactions que les plantes entretiennent avec les organismes de leur environnement. Cette diversité chimique gouverne de multiples fonctions, telles que l'attraction de pollinisateurs, l'encouragement à la constance florale et la défense contre des antagonistes. Bien que les fonctions écologiques des odeurs florales soient relativement bien étudiées, les facteurs évolutifs qui gouvernent la composition et les variations de ce signal complexe sont très mal connus.

C'est dans ce contexte que ma thèse s'inscrit. J'ai étudié les variations de ce trait floral particulier : les odeurs florales. Ma thèse se focalise sur une espèce de plante, la gueule-de-loup, *Antirrhinum majus*, utilisée comme espèce modèle en biologie depuis des décennies. Cette espèce, native des Pyrénées, elle présente deux sous-espèces, l'une à fleurs magenta, *A. m. pseudomajus*, et l'autre à fleurs jaunes, *A. m. striatum*. Alors que ces deux sous-espèces peuvent s'inter-féconder, elles ne coexistent jamais dans la nature et leurs hybrides, reconnaissables par une grande diversité de colorations florales, sont peu fréquents. Le mécanisme de cet isolement reproducteur n'est pas connu, mais le comportement des pollinisateurs a été envisagé dans de précédentes études.

Les principaux résultats de ma thèse montrent que les deux sous-espèces d'*A. majus* se distinguent par leurs odeurs florales. Certains composés volatils, en particulier trois benzénoïdes, ne sont émis que par *A. m. pseudomajus*, et ceci de manière constante entre les populations et pour différents environnements. Quant aux hybrides, les ratios de composés volatils floraux sont très variables par rapport aux signaux reproductibles parentaux, avec un patron de ségrégation chez les hybrides F_2.

En utilisant des bourdons commercialisés (*Bombus terrestris*), donc naïfs de toutes odeurs florales, j'ai montré que ces bourdons sont capables de détecter les principaux composés d'odeurs d'*A. majus* et qu'ils préfèrent de manière innée un mélange de composés volatils d'*A. m. striatum*. Finalement, en conditions naturelles, c'est-à-dire avec des odeurs florales naturelles et des pollinisateurs sauvages, ces derniers sont attirés préférentiellement par les odeurs florales de leur sous-espèce d'origine.

J'ai finalement montré que le patron associatif odeur-nectar qu'apprennent les pollinisateurs fait intervenir uniquement les composés odorants floraux et la quantité de nectar, puisque les différences d'odeurs florales entre les deux sous-espèces sont associées à une plus grande quantité de nectar par fleur chez *A. m. pseudomajus* mais à une plus faible concentration en sucres. En d'autres termes, les plantes contiennent autant de sucre total dans leurs fleurs dans une sous-espèce ou dans une autre.

Ces résultats, pris dans leur ensemble, semblent montrer que les composés volatils floraux sont bien impliqués dans l'isolement reproducteur de ces deux sous-espèces. Même si les odeurs florales ne peuvent pas expliquer à elles seules la distribution spatiale des deux sous-espèces d'*A. majus*, elles peuvent jouer un rôle supplémentaire de barrière aux flux de gènes. En effet, les pollinisateurs sont susceptibles de montrer un phénomène de constance envers l'un des phénotypes floraux, limitant ainsi les flux de gènes entre les deux sous-espèces. Dans cette thèse, je propose différentes perspectives possibles à mes résultats de thèse.

INTRODUCTION GENERALE

L'interaction plantes-pollinisateurs

L'étude des caractéristiques des fleurs telles que, la taille, la forme, la couleur et l'odeur, impliquées dans le système de reproduction des plantes, est un domaine qui a intéressé les biologistes depuis les débuts de la botanique, et reste encore aujourd'hui un champ de recherche actif. Les plantes à fleurs, ou angiospermes recèlent une multitude de stratégies de reproduction, dont celles qui mènent à la fécondation, appelée la **pollinisation**. Les grains de pollen (les gamétophytes mâles portés par les étamines) sont transportés jusqu'aux ovules de la même espèce (les gamètes femelles situés dans le pistil) soit par le vent (anémogamie), soit par l'eau (hydrogamie) soit par des animaux vecteurs de pollen (zoogamie). Les trois quarts des espèces d'angiospermes sont zoogames (Kearns et al. 1998, Kremen et al. 2007). Les deux partenaires tirent un bénéfice du service écologique rendu : la plante assure sa reproduction et l'animal se nourrit. L'**interaction** plantes-pollinisateurs est donc mutualiste lorsque le succès reproducteur individuel des deux ou plusieurs partenaires augmente directement ou indirectement lors de l'interaction. Plus les traits floraux sont adaptés à la **perception** de l'animal, plus la pollinisation est efficace. Ce sont les fleurs et les pollinisateurs les plus compétents à l'interaction qui maximiseront leurs chances de se reproduire. Leurs descendances, plus nombreuses que ceux dont les parents présentaient des traits moins optimaux, présenteront alors leurs habilités transmises par leurs parents. Ainsi, par **sélection naturelle**, chacun des partenaires peut façonner l'apparence, ou encore le **phénotype**. En dépit de ses aspects bénéfiques, l'interaction plantes-pollinisateurs engendre des **conflits d'intérêts** qui peuvent exercer des pressions de sélection considérables, telles que la compétition pour les récompenses entre pollinisateurs, la différence d'efficacité des espèces pollinisatrices ou la dispersion du pollen à trop faible distance. La relation mutualiste entre les partenaires est alors instable et tend vers une interaction de parasitisme, c'est-à-dire une interaction où le bilan des coûts et des bénéfices est positif pour l'un des partenaires et négatif pour l'autre. C'est par exemple très répandu des pollinisations par tricheries. Le palmier nain femelle *Chamaerops humilis,* par exemple, trompe son pollinisateur spécifique, *Derelomus chamaeropsis,* qui trouve en ces fleurs un lieu de reproduction et de ponte, en détruisant les œufs à l'aide d'une résine (Dufaÿ 2003). Même à l'intérieur d'une relation mutualiste les partenaires peuvent s'imposer des coûts. Il s'agit là d'exploitation réciproque, où les agents en

interaction minimisent au mieux leurs coûts au détriment de leur(s) partenaire(s) (Maynard Smith et Szathmary 1995).

Trois thématiques peuvent être définies lorsque l'on s'intéresse aux interactions plantes-pollinisateurs. Elles sont à l'interface entre **l'écologie**, la science qui étudie les relations entre organismes vivants et leur environnement, et **l'évolution**, la science qui étudie la modification des êtres vivants au cours du temps (Figure 1). Comprendre les conséquences évolutives des variations de traits floraux impliqués dans un système de pollinisation d'une espèce de plante, est une tâche complexe car nécessitant une approche multidisciplinaire, allant de la biologie végétale jusqu'aux processus d'adaptation. Le sujet de ma thèse s'inscrit dans cet enjeu. Il fait partie intégrante de la thématique : « **Ecologie fonctionnelle et adaptative des traits floraux** » (Figure 1). Ici, l'enjeu est de comprendre dans quelle mesure les traits phénotypiques des fleurs remplissent des fonctions spécifiques au sein du réseau d'interactions (de pollinisation mais aussi d'herbivorie et/ou de parasitisme) et parallèlement, de déterminer si chacun de ces traits relève d'une histoire de vie façonnée par la sélection naturelle ou par des moteurs évolutifs neutres ou sous contraintes.

L'étude « des dynamiques de transfert de pollen » est basée sur une approche principalement génétique qui vise à déterminer l'histoire évolutive des croisements de plantes *via* la pollinisation qui façonnent les espèces (Figure 1). C'est par exemple le cas des études actuelles menées sur le rhododendron (*Rhododendron ferrugineum*) dont le système de pollinisation peut varier de l'allogamie (c'est-à-dire d'une fécondation croisée entre individus), à l'autogamie (où les gamètes femelles sont fécondés par les gamètes mâles d'un même individu) en fonction de l'altitude et de la fragmentation de l'habitat qui influencent l'abondance des espèces pollinisatrices.

Une dernière thématique intitulée sur la Figure 1 « Niche et compétition dans les communautés de pollinisateurs », davantage du point de vue animal, étudie comment la compétition entre pollinisateurs variant dans le temps et l'espace influe sur les valeurs sélectives des fleurs qui peuvent notamment rentrer en conflit dans le cas de fleurs unisexuées (soit mâles, soit femelles).

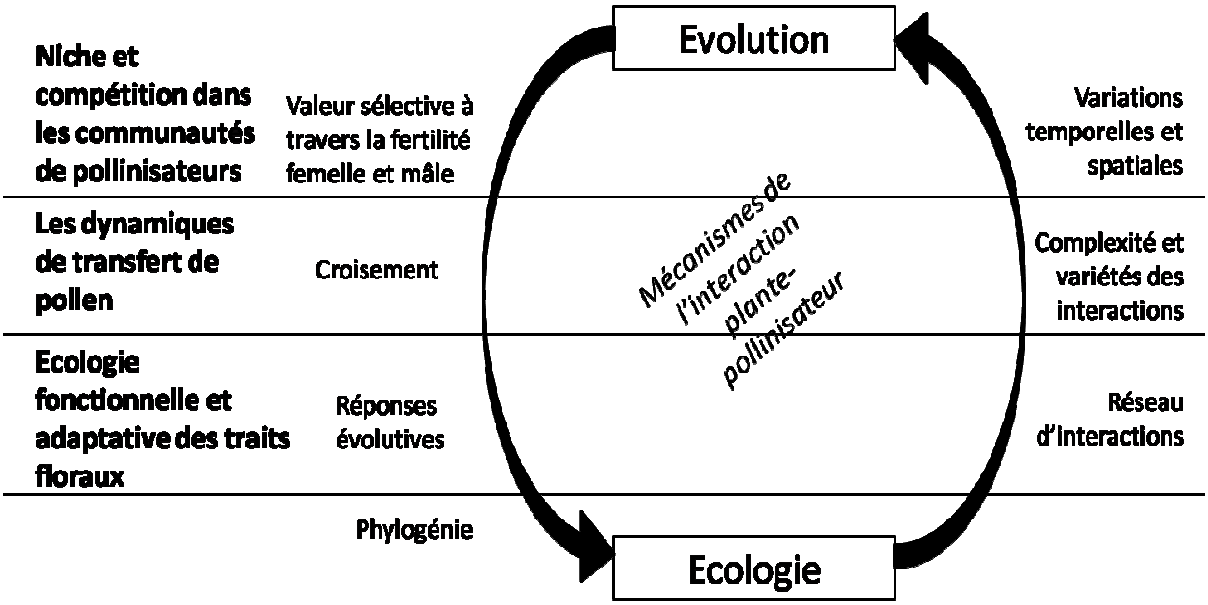

Figure 1 : Représentation conceptuelle de l'interface entre l'écologie et l'évolution dans l'étude de l'interaction plante-pollinisateur (tiré et adapté de Mitchell et al. 2009)

Alors que l'on sait depuis longtemps que la forme, la taille et la couleur des fleurs sont autant de signaux visuels utilisés par les pollinisateurs dans la détection des fleurs, le rôle des **odeurs florales** n'a été que récemment exploré. L'odorat si faiblement développé chez les humains en est peut-être la cause – peu de personnes, dites « nez », sont pourvues de capacités olfactives exceptionnelles. Les moyens techniques nécessaires à l'exploration de ces impalpables signaux chimiques floraux ont été mis au point relativement récemment (la découverte de la chromatographie ne date que de 1952). Pourtant, les parfumeurs comme les phytothérapeutes le savent bien, les fleurs recèlent une diversité considérable de **composés odorants**. Alors que 2010 est l'année de la biodiversité et que la diversité des formes, des espèces et des gènes a été exposée et mise en avant, la diversité chimique du monde vivant n'a été que peu évoquée. Cette présente thèse présente mes travaux qui ont exploré la fonction écologique des odeurs florales d'une plante modèle, bien connue des biologistes, la gueule-de-loup (« snapdragon », en anglais) ou *Antirrhinum majus*, et les conséquences potentielles des variations d'odeurs florales sur son évolution.

A. Qu'est-ce qu'une odeur florale ?

1. Les composés organiques volatils et leur diversité

Une odeur florale consiste en un **mélange** de molécules organiques légères, dites volatiles parce qu'elles s'évaporent facilement à température ambiante des surfaces de la fleur. Ce sont les **Composés Organiques Volatils,** ou COV (VOCs en anglais). Parce que ces composés interagissent avec les récepteurs olfactifs des êtres vivants qui y sont sensibles, ils sont dits **composés odorants,** et sont, depuis 50 ans, le sujet d'études aussi bien mécanistes, fonctionnelles, qu'évolutives (Hartmann 2007). Leur intérêt est croissant comme l'illustre la Figure 2 ci-dessous. Les COV font partie de ce qu'a nommé, en 1891, Albrecht Kossel, les « métabolites secondaires » qui désignaient tout composé produit par les plantes dont le rôle n'était pas impliqué directement dans les fonctions primaires (Hartmann 2007). On sait aujourd'hui que ces composés sont essentiels aux plantes, notamment pour la reproduction ou la survie, tout autant que les « métabolites primaires » (tels que les glucides, les lipides, les acides aminés, les protéines et les acides nucléiques). **Plus de 1700 COV** on été identifiés chez un total de 991 espèces de plantes à fleurs (Knudsen et al. 2006).

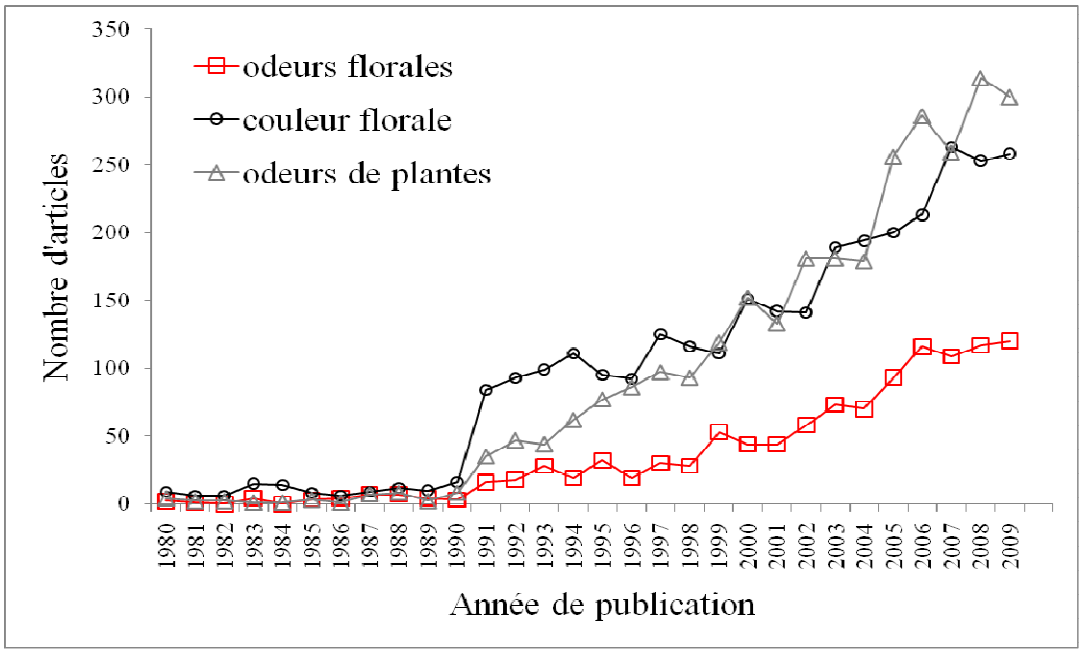

Figure 2 : Comparatif du nombre non-cumulatif d'articles publiés durant les 30 dernières années (en utilisant le moteur de recherche ISI Thomson 'Web of knowledge') qui portent sur les odeurs florales (en rouge, mots-clés : « flower scent », « floral scent », « flower odo*r », « floral odo*r » et « floral fragrance »), sur la couleur florale (en noir ; mots-clés : « flower colo*r », « floral colo*r ») et les composés organiques volatils chez les plantes en général (en gris ; mots-clés : « plant volatiles », « plant VOC »).

La caractérisation d'un bouquet de COV floraux d'une plante s'effectue généralement sur la base de quatre critères : i) **le nombre de molécules émises**, ii) **les structures et les fonctions chimiques des molécules**, iii) **l'intensité totale de l'odeur** et enfin iv) **les quantités relatives de chacun des composés**. Un mélange de COV floraux peut être plus ou moins complexe en fonction du nombre de molécules qui le constituent et de ses variations d'émissions dans le temps. Il peut varier grandement entre les espèces. Certaines variétés de roses, les plus riches en COV, peuvent émettre plus de 200 composés (ex. 275 composés volatils ont été décrit chez *Rosa x damascena*, Ohloff et Demole, 1987). En général, les fleurs d'une espèce de plante émettent entre 20 et 60 COV (Dudareva et al. 2006). Quant à savoir s'il existe des fleurs non-odorantes, la question est encore en suspens, étant donné que de faibles quantités de composés émises peuvent ne pas dépasser le seuil de détection des moyens analytiques utilisés et que les émissions sont variables dans le temps.

Les COV floraux sont souvent groupés en **5 classes de composés** : les dérivés d'acides gras, les terpénoïdes, les composés aromatiques, les composés azotés (composés d'atome(s) d'azote) et les composés soufrés (composés d'atome(s) de soufre). Leurs critères de différentiation est **leur structure** (Figure 3) et leur mode de production, c'est-à-dire **leur voie de biosynthèse**.

- **Les dérivés d'acides gras** sont rarement caractéristiques de la note de l'odeur florale. On ne sait pas, si leur production est active et remplit une fonction précise, ou alors s'ils sont des déchets de certaines réactions. Certains d'entre eux (les composés de 6 à 9 carbones Figure 3) sont synthétisés abondamment par les parties végétatives de la fleur, telles que les sépales, les tépales, le réceptacle, le pédoncule et les folioles et semblent avoir un rôle de défense. C'est pourquoi ils sont aussi appelés « Green Leaf Volatiles » GLV, ou « composés volatils de feuilles vertes ».
- **Les terpènes** sont en revanche très caractéristiques des odeurs florales. Par définition, ils ne sont constitués que d'atomes de carbone et d'hydrogène, pas d'oxygène. On les distingue par leur nombre de carbone (Cn) : les **monoterpènes** (C10) (Figure 3), les sesquiterpènes (C15), homoterpènes (C11 et C16) et les diterpènes (C20) ou des dérivés terpéniques irréguliers (ex. le 6-methyl-5-hepten-2-one qui est très répandu). Parmi les plus rencontrés, on distingue les monoterpènes suivants : le limonène, le *cis*-β-ocimène, le myrcene, le linalool et le α- et β-pinène (Knudsen et al. 2006).

- **Les composés aromatiques** sont aussi typiques des odeurs florales. Dans cette classe, où tous les composés ont en commun une structure moléculaire constituée d'un cycle de benzène (cycle à 6 carbones Figure 3), on différencie les **benzénoïdes** des phénylpropanoïdes. La note florale propre à la violette par exemple est due à la présence du *p*-dimethoxy benzène. Les benzénoïdes les plus fréquents chez les COV floraux sont le benzaldéhyde, le salicylate de méthyle, l'alcool de benzyle et l'éthanol 2-phényle, caractéristiques des odeurs florales de roses de Chine (Scalliet et al. 2008).

2. Introduction à la biosynthèse des composés volatils floraux

L'ingénierie des composés volatils floraux est une discipline qui a connu de rapides progrès ces dernières années. Connaître les modes de production des composés volatils permet à un biologiste de regrouper les composés en fonction de leur origine biosynthétique et de les considérer comme potentiellement liés à des évènements évolutifs communs.

Les plantes aromatiques ont souvent une valeur commerciale très importante. Elles sont la matière brute des parfumeurs, la source d'arômes pour l'industrie agro-industrielle et de propriétés convoitées par les industries pharmaceutiques. L'étude des **mécanismes de production** des composés volatils et **l'identification des gènes** impliqués dans leur biosynthèse offrent aussi des outils puissants en vue d'une meilleure compréhension des relations plantes-pollinisateurs. La modification génétique du bouquet floral en est un exemple probant bien qu'encore rare. Chez le tabac (*Nicotiana attenuata*), les fleurs, pour lesquelles l'émission de 4-phenyl butane-2-one a été génétiquement bloquée, sont moins visitées par les pollinisateurs que les fleurs sauvages chez qui ce composé est majoritaire (Kessler et al. 2008). Bon nombre de découvertes restent à faire puisque seule une petite partie des gènes a été identifiée, et que leur régulation reste encore mal connue (Hartmann 2007). Néanmoins, le réseau de voies de biosynthèse a été caractérisé (Figure 3). Comparé à la diversité considérable des composés volatils, les voies de biosynthèses sont peu nombreuses, et par conséquent, c'est la richesse en capacités enzymatiques qui est à l'origine de la grande diversité des composés volatils (Pichersky et al. 2006).

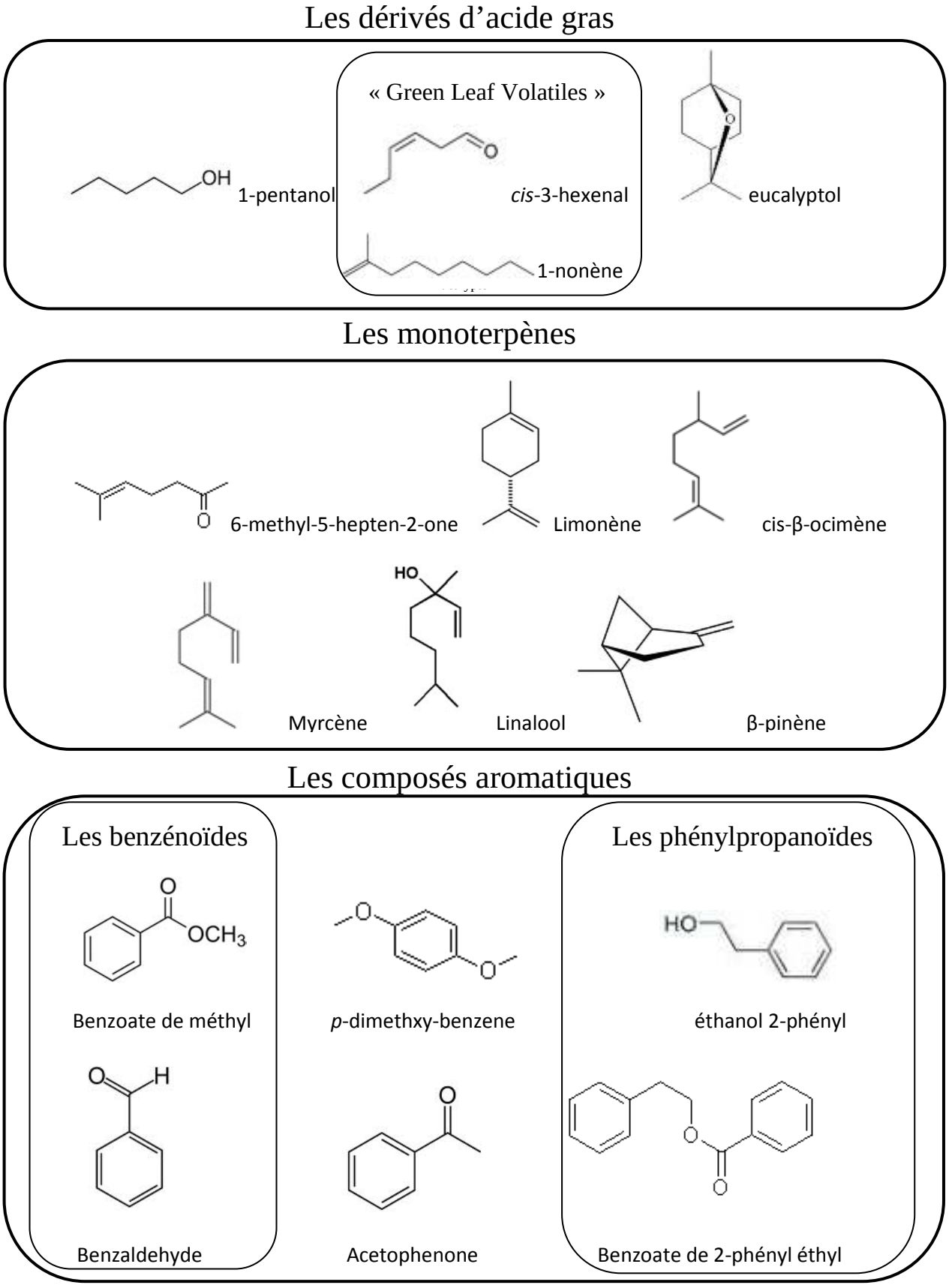

Figure 3 : Quelques exemples de structures chimiques de composés organiques volatiles

Ici, je résume les principales voies de biosynthèse chez les plantes (Figure 4). Les dérivés d'acide gras proviennent de la coupure des acides gras par des lipoxygénases (« *Lipoxygenase pathway* », Figure 3, en haut à droite). Les terpènes sont des composés qui dérivent tous d'un même précurseur, l'isoprène, mais leur production est compartimentée dans les plastides pour les monoterpènes et dans le cytosol des cellules pour les autres terpènes (Figure 4, au milieu et en bas à droite). Similairement, les composés aromatiques dérivent tous de la phénylalanine (Phe, Figure 4) mais leur voie de biosynthèse, la voie de l'acide shikimique (« Shikimic acid pathway », Figure 4, à gauche), est divisée en deux branches : celle des benzénoïdes et celle des phénylpropanoïdes.

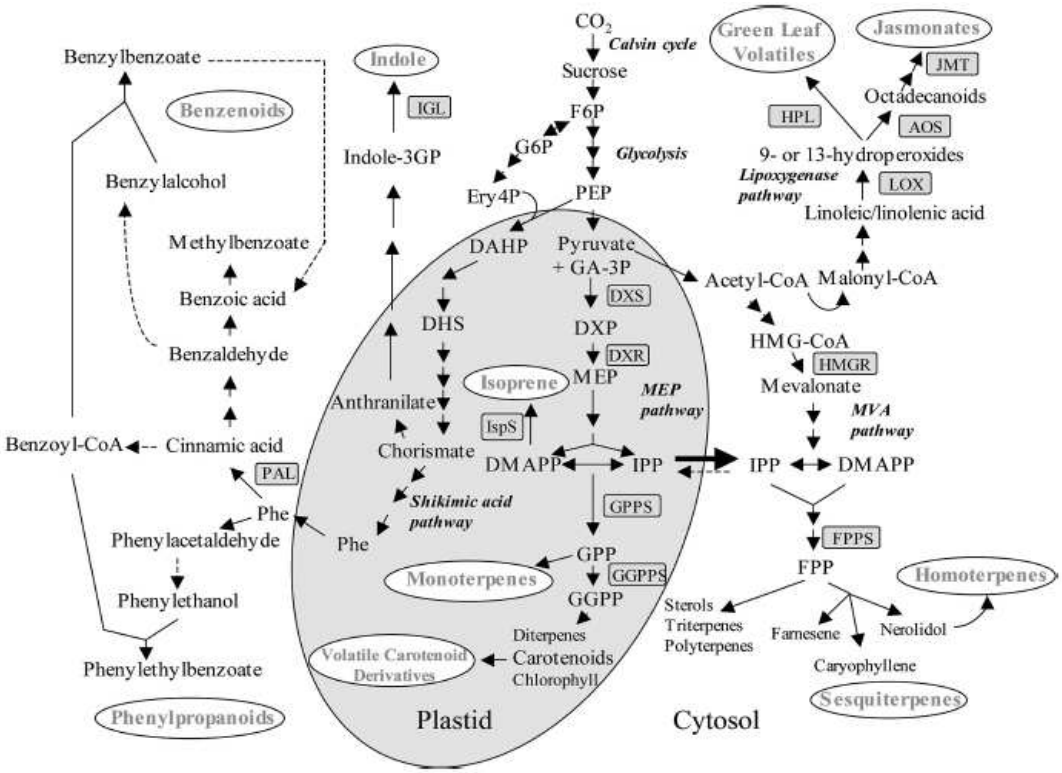

FIG. 2. Metabolic pathways leading to the biosynthesis of various volatile compounds in plants. Pathway names are italicized, volatile compounds are in bold and enzymes are boxed. Abbreviations: Acetyl-CoA, acetyl coenzyme-A; AOS, allene oxide synthase; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DHS, 3-dehydroshikimic acid; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; Ery4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FPP, farnesyl diphosphate; FPPS, FPP synthase; GA-3P, glyceraldehyde-3-phosphate; G6P, glucose 6-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; GPP, geranyl diphosphate; GPPS, GPP synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HPL, fatty acid hydroperoxide lyase; IGL, indole-3-glycerol phosphate lyase; Indole-3GP, indole 3-glycerol phosphate; IPP, isopentenyl diphosphate; JMT, jasmonic acid carboxyl methyl transferase; LOX, lipoxygenase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MVA, mevalonate; PAL, phenylalanine ammonia lyase; PEP, phosphoenolpyruvate; Phe, phenylalanine.

Figure 4 : Réseau de voies de biosynthèse des COV (tiré de Dudareva et al. 2006)

B. Fonctions écologiques des odeurs florales chez les plantes

1. Assurer sa reproduction

1.1. Signal d'attraction

L'odeur florale représente un **signal de reproduction** émis par les fleurs matures, c'est-à-dire les fleurs dont le stigmate est réceptif à l'apport de pollen et/ou pour lesquelles le pollen est prêt à être récolté. Elles sont **un attractant important** qui dirige les pollinisateurs dans leur quête de nourriture (Figure 5). Ce signal d'attraction peut être **de longue distance** pour les fleurs dont le taux d'émission est intense (Cunningham et al. 2004) et/ou **de proximité** pour guider l'animal lors de l'approche ou l'atterrissage sur la fleur (Dobson et al. 1999).

Figure 5 : Illustration de l'attraction d'un bourdon (*Bombus terrestris*) envers une fleur d'*Antirrhinum majus*.

Chez les fleurs qui récompensent leur pollinisateur par une solution sucrée, les pollinisateurs sont attirés de manière spontanée par certains COV (Andersson 2003). Ils sont aussi capables apprendre à associer le signal olfactif aux récompenses de la fleur (Wright et al. 2005). Par exemple Riffell et al. (2008) ont montré que le papillon de nuit *Manduca sexta*, en Arizona, change de préférence d'odeurs florales de *Agave palmeri* vers *Datura wrightii*, deux plantes dont les floraisons se succèdent au cours d'une saison, et que parallèlement, ce changement est régi par la combinaison d'un **apprentissage olfactif** (envers *A. palmeri*, davantage adapté à des visites de chauves-souris) et d'une **préférence olfactive innée** de *Manduca sexta* (envers *D. wrightii*, la plante hôte de ses larves). En cas d'apprentissage, les odeurs florales peuvent remplir plusieurs autres fonctions indirectes qui sont **(i)** d'identifier la fleur, **(ii)** de signaler le moment où un maximum de récompenses est disponible grâce à une forte intensité et une certaine diversité de COV et **(iii)** de rendre attractives les vieilles fleurs qui n'ont plus de récompenses mais qui sont encore réceptives (Dobson et al. 1999, Dobson et Bergström 2000, Raguso 2001). Ainsi, l'odeur florale est un **signal précis** qui guide et dirige le pollinisateur outrepassant la forme et la couleur florale essentiellement constante entre fleurs

(Dudareva et al. 2006). Idéalement, une odeur florale spécifique à l'espèce devrait **encourager la fidélité des pollinisateurs** et augmenter la probabilité d'un transfert de pollen entre fleurs de la même espèce, assurant ainsi la reproduction.

La production de COV floraux comme un signal de récompense doit donc offrir **un bénéfice net pour la plante**. Pour le démontrer, il faut examiner **comment ces odeurs augmentent l'attractivité des fleurs.** L'attractivité en-soi des odeurs florales a été démontrée par exemple chez le genre *Ficus* au sein duquel **les COV floraux suffisent à déclencher l'interaction** entre les espèces de figuiers et leur espèce de guêpes pollinisatrices spécifiques à chacune d'entre elles (Gibernau et al. 1998). Les COV floraux peuvent aussi indirectement être bénéfiques dans l'attractivité des fleurs lorsqu'ils fonctionnent **en synergie avec les signaux visuels,** puisqu'en leur absence, la discrimination des fleurs par les insectes est significativement réduite (Kunze et Gumbert 2001, Raguso et Willis 2002). Enfin, Majetic et al. (2009) ont démontré dernièrement chez *Hesperis matronalis* que les plantes aux **odeurs florales les plus intenses** pouvaient avoir une **meilleure valeur sélective** : en effet, elles produisaient plus de graines.

Sans offrir de récompense, les plantes peuvent aussi tirer un bénéfice de l'émission de composés odorants par d'étonnantes stratégies d'attraction du pollinisateur. L'odeur florale de l'orchidée *Ophrys sphegodes* déclenche par exemple un comportement de « pseudo-copulation » chez les abeilles solitaires mâles *Andrena nigroaenea* parce qu'elle contient certains composés odorants qui reproduisent les phéromones des femelles réceptives de *A. nigroaenea* et ceci dans les mêmes proportions (Schiestl et al. 1999). Après la pollinisation, l'orchidée maximise son succès reproducteur en émettant de l'hexanoate de farnésyle qui est le composé repoussant que dégagent les femelles non-réceptives *A. nigroaenea* (Schiestl et Ayasse 2001).

1.2. Le nectar, une récompense associée

L'attraction des pollinisateurs est maximisée quand le nectar abonde. Le nectar est la principale **source de nutrition** des visiteurs bien que les fleurs hermaphrodites offrent simultanément du pollen, et plus rarement, des huiles. De par leurs apports en sucres localisés dans les fleurs, les ressources en nectar **conditionnent la probabilité de transfert de pollen** parce que le nectar floral est directement récolté et consommé dans la fleur par les

pollinisateurs (Elias 1983). Cependant, un conflit d'intérêt peut apparaître lorsque les pollinisateurs deviennent des voleurs de nectar puiqu'ils n'entrent plus dans la fleur pour accéder à cette ressource et n'offrent donc pas de service de pollinisation (Richardson 2004).

Le nectar floral est sécrété par les glandes florales distinguables des glandes extra-florales par leur position et leur fonction (Fahn 1979). C'est une solution aqueuse, principalement sucrée, sécrétée par des organes spécialisés très répandus chez les angiospermes : les glandes nectarifères (Fahn 1979). **Les sucres** sont les principaux solutés totaux du nectar et représentent la source d'énergie majeure des visiteurs (Petanidou 2005). Le nectar pourrait être considéré comme la sève élaborée modifiée pendant la phase d'excrétion d'où résulte un mélange majoritaire de sucrose, fructose, et glucose en proportions diverses (Wykes 1952, Fahn 1979). Beaucoup d'autres substances, telles que les **acides aminés**, les lipides, et les phénols sont retrouvées dans le nectar, mais principalement sous forme de traces (Baker et Baker 1973). Les composants du nectar sont caractérisés par **un goût** et/ou **une odeur** qui peuvent jouer un rôle dans l'attraction des visiteurs (Raguso 2008, Kessler et al. 2008). Il existe des cas où le nectar contient des métabolites secondaires comme les alcaloïdes, les phénols, les saponins ou les amino-acides non protéiques, qui sont toxiques ou repoussants pour des visiteurs particuliers (Adler 2000, Kessler et Baldwin 2006 *Nicotiana attenuata*, Gegear et al. 2007 et Manson et al. 2010 *Gelsemium sempervirens*,).

La concentration en sucres du nectar a longtemps été le seul indice nectarifère utilisé pour comprendre les relations évolutives entre les plantes et les animaux parce qu'elle a un effet majeur sur plusieurs aspects du comportement et de l'écologie des nectarivores (Dafni et al. 2005). Par exemple, un nectar dilué est facile à ingérer alors qu'un nectar fortement concentré est difficile à extraire et peut augmenter le temps passé à la collecte. Baker et Baker (1973) suggéraient que les différences de concentration des solutés nectarifères sont **coadaptées aux principaux visiteurs**. La composition du nectar pourrait alors jouer un rôle de filtre de visiteurs adapté à leurs perceptions gustatives et olfactives et être soumis à sélection. Cependant, au sein d'une même espèce **la concentration du nectar peut varier** en fonction de l'âge de la fleur, de l'heure de la journée, et des conditions environnementales rendant difficile la question de l'héritabilité des traits du nectar floral (Mitchell 2004). Plus récemment, l'argument de Baker and Baker (1973), qui **associe la composition du nectar aux guildes de visiteurs** a été réactualisé sur la base de la composition en acides-aminés du nectar et du sucre à l'échelle de la communauté. Petanidou

et al. (2006) ont montré une corrélation positive entre les fleurs méditerranéennes d'Athènes pollinisées par des Apidae (abeilles, bourdons…) à longues langues et la concentration de phénylalanine dans le nectar, qui a un pouvoir phago-stimulant. Les acides aminés du nectar pourraient constituer un facteur de discrimination que les différents types de pollinisateurs de cette communauté de plantes à fleurs utiliseraient pour les sélectionner. La composition en solutés du nectar serait alors impliquée dans la **coévolution** entre les deux partenaires.

2. Survivre

L'origine des odeurs florales a été liée à des **fonctions de défense** (Theis et Lerdau 2003) en examinant les compositions de COV chez les plantes à fleurs parmi les premières à être apparues et encore présentes actuellement (Magnolidés Pellmyr et Thien 1986, Cycas Pellmyr et al. 1991, Annonaceae Goodrich et Raguso 2009). Les premières structures apparentées aux fleurs actuelles composées de pollen et d'ovules qui émettaient des composés dissuasifs contre pestes et pathogènes auraient complété le régime alimentaire de certains animaux. Ces herbivores, qui y trouvaient aussi un lieu de reproduction, auraient agit par inadvertance comme des vecteurs de pollen et auraient ainsi commencé à exercer des **pressions de sélection sur les COV** des fleurs (Pellmyr et Thien 1986). Par des visites récurrentes et constantes, ce passage de l'antagonisme au mutualisme serait à l'origine de l'interaction plantes-pollinisateurs.

Aujourd'hui, on pense que les odeurs florales maintiennent encore cette fonction primaire de défense par **une dualité de fonctions attractive et défensive** de certains composés volatils. La synthèse bibliographique de Junker et Blüthgen (2010) montre que **les odeurs florales peuvent agir comme un filtre** qui attire les visiteurs obligatoires, dépendants des ressources qu'offrent les fleurs, et qui repoussent les visiteurs facultatifs commensaux ou antagonistes. Le plus étonnant dans l'article de Junker et Blüthgen (2010) est l'identification de certaines classes biosynthétiques de COV floraux, comme les monoterpènes (notamment le linalool), ou certaines fonctions de molécules, comme les cétones, qui induiraient une réponse positive chez les visiteurs obligatoires et une réponse négative chez les visiteurs facultatifs (Figure 6).

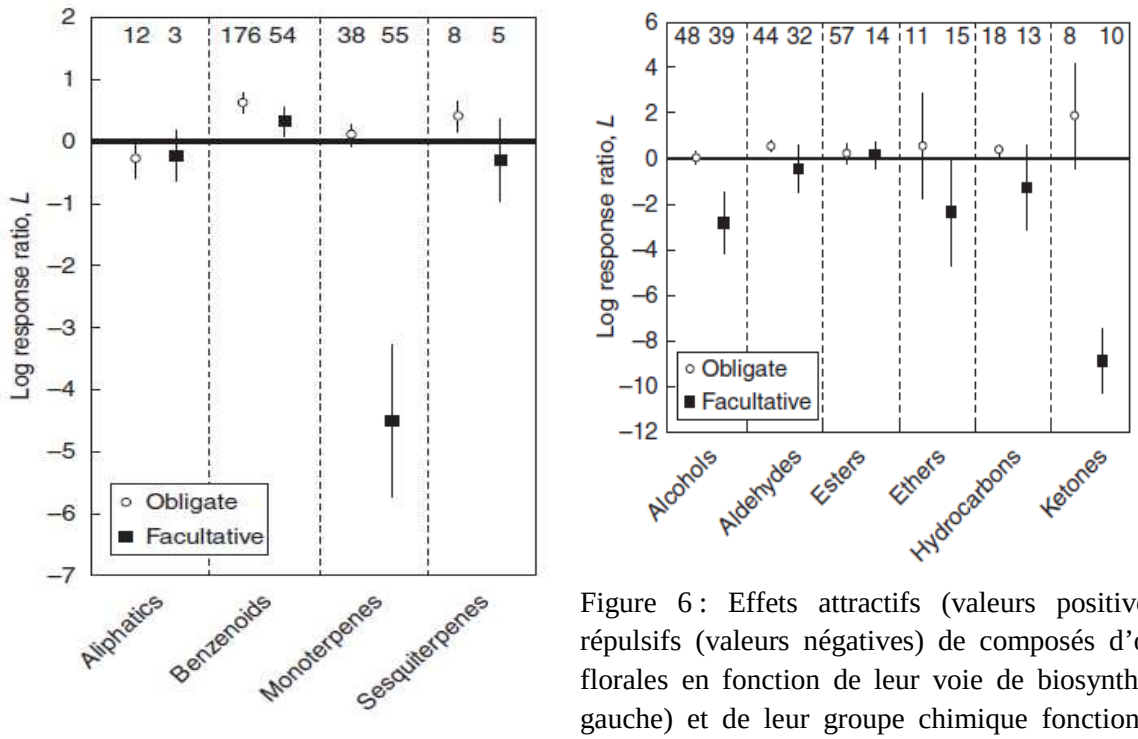

Figure 6 : Effets attractifs (valeurs positives) et répulsifs (valeurs négatives) de composés d'odeurs florales en fonction de leur voie de biosynthèse (à gauche) et de leur groupe chimique fonctionnel (à droite). Les moyennes pondérées et les intervalles de confiance de 95% de l'intensité de l'effet de la réponse d'un animal à une odeur (exprimée en log ratio de réponse, appelé L, qui est le ratio entre l'effet de l'odeur et l'effet du control) sont représentés. Un effet est significatif si l'intervalle de confiance n'inclus pas 0 (Tiré de Jünker et Blüthgen 2010).

Les émissions florales sont dans certains cas un **moyen de défense direct** contre des visiteurs non appropriés (Ghazoul 2001). Chez l'olivier odorant (*Osmanthus fragrans*, dont le nom provient du fait que les fleurs émettent une agréable odeur de pêches mûres), l'odeur des fleurs **repousse** le papillon *Pieris rapae*, la piéride de la rave, principalement à cause de la présence de γ-decalactone (Omura et al. 2000). Les pathogènes peuvent aussi être repoussés par les COV floraux (ex. propriétés antifongiques, Steinerbrunner et al. 2008) et les stress abiotiques peuvent être physiologiquement diminués par les composés volatils floraux (Dudareva et al. 2006, Knudsen et al. 2006).

Le signal informatif qu'est une odeur florale n'est donc pas seulement associé à la présence de nectar. Plusieurs études ont mis en évidence la **double fonction d'attraction et de défense** de certaines odeurs florales (chez le cycas *Macrozamia lucida* Terry et al. 2007, et le tabac *Nicotania attenuata* Kessler et al. 2008). La diversité des messages que transmet les odeurs florales à leurs différents partenaires, nommés « canaux privés de communication » par Robert Raguso (2008), est encore peu explorée, mais ces études indiquent que le message « Venez ici » pourrait être aussi important que « Circulez, il n'y rien à voir » (Raguso 2009).

3. Les inconvénients

Produire des odeurs florales est **coûteux** d'un point de vue métabolique et **risqué** puisqu'elles peuvent attirer des visiteurs inattendus (Balwin et al. 1997). Les exemples de ses inconvénients ne sont pas rares. Le chardon *Cirsium arvense* attire par ses COV floraux à la fois ses pollinisateurs et ses ennemis naturels (Theis et al. 2007). L'une des araignées crabes, *Thomisus spectabilis*, qui chasse ses proies en faisant le guet à la surface des fleurs dont elle mime sa couleur, utilise les COV floraux de *Chrysanthemum frutescens* pour localiser son lieu de prédation (Heiling et al. 2004).

Par ailleurs le rôle des odeurs florales peut **être relié à des perturbations de la physiologie de l'organisme**. Une infection microbienne dans le nectar chez *Agave palmeri* induit un changement des odeurs de nectar qui pourrait altérer les messages encodés par les odeurs florales globales de cette plante (Raguso 2004). Les plants de tabac (*Nicotiana attenuata*) modifiés génétiquement pour ne pas produire de nicotine présent dans le nectar (la nicotine a un goût repoussant pour les pollinisateurs nocturnes) sont significativement plus visitées (au moins 68% de nectar extrait en plus par nuit par rapport aux plants sauvages) (Kessler et Baldwin 2007). Enfin, certaines contraintes biochimiques pourraient induire des associations de traits floraux utilisés par les pollinisateurs telles des co-variations odeur-couleur (Majetic et al. 2007).

Pour comprendre la véritable fonction des émissions de composés odorants par les fleurs, il ne faut donc pas se restreindre à l'étude du signal chimique floral de reproduction et à l'interaction plante-pollinisateur ; une approche plus globale s'impose (Raguso 2009).

C. Conséquences évolutives des variations d'odeurs florales

1. L'isolement reproducteur

On estime aujourd'hui sur la Terre plus de 350 000 espèces de plantes à fleur (Paton et al. 2008). Cette immense diversité a été façonnée par des processus évolutifs. La **sélection naturelle** en est un, et celle-ci agit notamment *via* les interactions plantes-animaux, comme par exemple la pollinisation animale (Grant 1994, Johnson 2006). Les fleurs sont en effet plus ou moins attractives pour les pollinisateurs et ces derniers exercent aussi un choix parmi les formes florales rencontrées. Selon une vision darwinienne, les **phénotypes** des deux agents mutualistes sont ainsi **sélectionnés** au cours des temps évolutifs de sorte à ce qu'ils soient **adaptés** à interagir efficacement (Darwin 1859). Mais d'autres processus évolutifs peuvent être à l'origine de la diversité des espèces. La **dérive génétique** est l'évolution des populations / des espèces par des phénomènes dûs au hasard. Dans ce cas, des fluctuations aléatoires de fréquences d'allèles peuvent aboutir au remplacement de vieux allèles par de nouveaux, résultant à l'évolution non-adaptative (Wright 1931). La modification d'un gène, une mutation, peut avoir des conséquences sur plusieurs traits phénotypiques, et ces conséquences dites **pléitropiques** peuvent également être une source de diversité. La sélection naturelle et la dérive génétique sont reconnues comme étant les deux processus évolutifs les plus importants mais leur part d'action respective fait encore débat (exemple du dimorphisme de pigmentation florale chez *Linanthus parryae* Schemske et Bierzychudek 2007). Aussi, l'étude des **variations des traits floraux** apparaît comme centrale dans la compréhension de ces processus. Mais quels sont les mécanismes à l'origine de cette diversité ? Et, comment les limites entre les espèces sont-elles maintenues ?

Si deux populations n'échangent peu ou pas de gènes (ne se reproduisent pas entre elles) on dit qu'elles présentent un **isolement reproducteur** (Mayr 1942, Coyne et Orr 2004). Deux types de processus sont à l'origine de l'isolement reproducteur chez les plantes à fleur. Le premier a lieu une fois que le pollen a été transféré sur la pièce réceptrice femelle, le stigmate. Il s'agit là de **barrières post-pollinisation**. Parmi ces barrières, on compte l'incompatibilité entre le pollen et les ovules, duquel découle le concept de la limitation en pollen (Ashman et al. 2004). Les ovules d'une fleur ne peuvent pas être fécondés par n'importe quel pollen. Les grains de pollen de la même espèce peuvent être en compétition

pour la surface stigmatique avec les grains de pollen de différentes espèces déposés par les pollinisateurs généralistes. Il est également possible que, même si la fécondation ait lieu, les hybrides ne soient pas viables et/ou qu'ils soient stériles induisant ainsi une valeur sélective plus faible (Rieseberg et al. 1999).

Le deuxième type de processus d'isolement reproducteur est celui qui empêche que le pollen ne soit déposé sur le stigmate (**barrières pré-pollinisation**). C'est ce qui arrive, par exemple, lorsque des plantes ne vivent pas dans le même habitat (isolement géographique), ou lorsque leur floraison n'est pas synchrone (isolement temporel).

Des barrières pré-pollinisation peuvent également se mettre en place alors que des plantes vivent au même endroit, c'est-à-dire en sympatrie, et fleurissent en même temps. Leurs phénotypes peuvent s'être modifiés par sélection divergente et empêcher ainsi que les visiteurs ne transfèrent efficacement le pollen (isolement mécanique). Par exemple, il existe une barrière mécanique entre les fleurs de *Ipomopsis arizonica* et *I. aggregata* parce qu'elles diffèrent par la longueur de la corolle, des étamines et du style et que, seules les fleurs de morphologie similaire permettent le dépôt du pollen sur le corps du pollinisateur qui convient à atteindre le stigmate d'une fleur conspécifique (Wolf et al. 2001).

Il se peut aussi que des plantes attirent différentes espèces de pollinisateurs parce qu'elles diffèrent par leurs traits floraux. Fréquemment, une divergence florale présentant une co-variation de traits floraux (forme, couleur, odeurs etc…) est liée à une barrière pré-pollinisation où **les pollinisateurs se spécialisent en un type de traits floraux** (Stebbins 1970, Armbruster et al. 1993, Fenster et al. 2004). L'exemple le plus élégant est celui de *Mimulus lewisii* (Figure 7a) et *M. cardinalis* (Figure 7c) chez qui, une seule et unique mutation, contrôlant la présence de la pigmentation florale jaune, augmente dramatiquement les visites de colibris chez les fleurs orange par rapport aux visites de bourdons chez les fleurs roses, et induit donc un changement adaptatif drastique (Bradshaw et Schemske 2003).

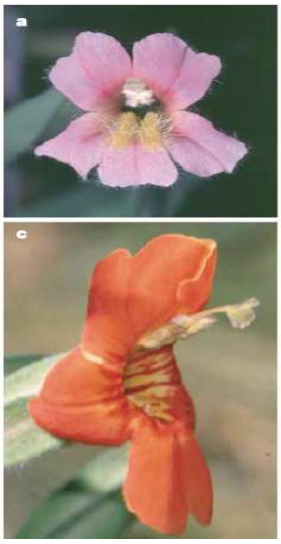

Figure 7 : Phénotypes des deux espèces du genre *Mimulus, M. lewisii* et *M. cardinalis* qui sont adaptées à différents pollinisateurs (tiré de Bradshaw et Schemske 2003)

Enfin, lorsque les pollinisateurs sont partagés, la barrière pré-pollinisation dépend exclusivement de la fidélité des pollinisateurs

envers un morphe ou l'autre par **phénomène de constance de visite** (Jones 2001). En d'autres termes, si le comportement des pollinisateurs est influencé par le phénotype des fleurs de sorte à ce que le choix des visites entre les morphes ne soit pas aléatoire, alors il existe des transferts de pollen qui sont restreints entre les morphes et accentués au sein des morphes.

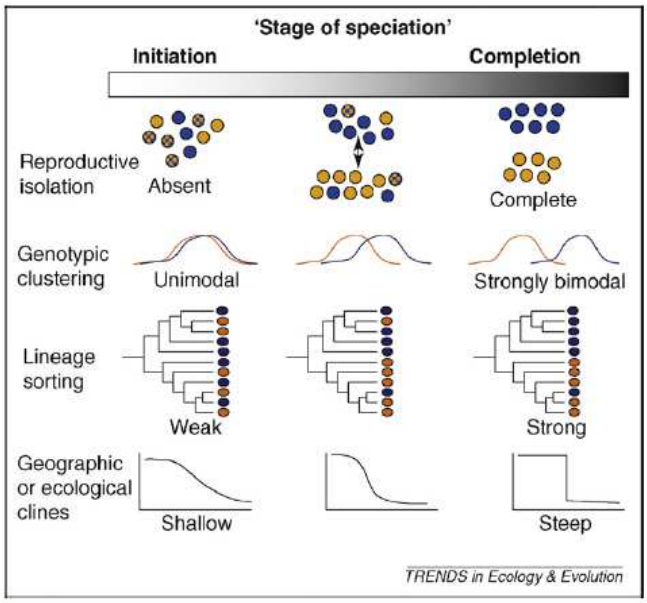

Figure 1. The continuous nature of divergence during speciation. Divergence during speciation can vary quantitatively, for numerous types of differentiation. Thus, different means of quantifying divergence can be used to measure arbitrary 'stages' of speciation, representing stages from the initiation through to the completion of the speciation process (when two populations are depicted, one is shown in blue and the other in orange). For example, reproductive isolation can vary from absent through to complete. Likewise, the distribution of gene frequencies in individuals sampled from two populations, depicted here as genotypic clustering, can vary from unimodal through to strongly bimodal. The extent of lineage sorting can vary from weak to strong. Finally, the steepness of geographic or ecological clines in gene frequency can vary, with the latter stages of speciation being characterized by steep or stepped clines.

Figure 8 : Les processus graduels de l'isolement reproducteur qui tendent à la spéciation (tiré de Nosil et al. 2009).

L'ensemble de ces processus évolutifs **limite les flux de gènes** entre populations ayant divergé et participe à la cohésion des espèces (Mayr 1942, Dobzhansky 1951). Tant que l'isolement reproducteur est partiel, les plantes isolées peuvent échanger des gènes (Figure 8). Mais celui-ci peut être renforcé et devenir complet au point que les plantes isolées ou divergentes ne puissent plus se croiser (Figure 8). Ce phénomène de **spéciation**, où deux espèces découlent d'un ancêtre commun, explique l'origine des espèces (Darwin 1859, Coyne and Orr 2004).

Le cas spécifique où les pollinisateurs induisent des barrières d'isolement en sympatrie est bien documenté (Fulton et Hodges 1999, Ramsey et al. 2003, Ippolito et al. 2004). L'un des mécanismes de ces isolements dirigés par le système de pollinisation est indépendant d'un isolement mécanique lié à la forme florale. Il advient lorsqu'il y a présence de **barrières post-zygotiques**, c'est-à-dire lorsque le développement, suite à la fécondation, avorte plus ou moins tôt, et ne repose que sur le comportement des pollinisateurs lorsqu'ils choisissent les fleurs à visiter. Une controverse existe sur le fait que de tels **isolements éthologiques** (Grant 1964, Stebbins 1970) puissent induire, à eux seuls, une dissolution complète d'espèces comme lors de la spéciation (Johnson 2006). Les systèmes de pollinisation sont rarement suffisamment spécialisés pour une telle conséquence évolutive (Waser 2001).

2. Les odeurs florales et le phénomène d'isolement reproducteur

Quand les odeurs florales jouent un rôle clé dans l'attraction des pollinisateurs, elles sont susceptibles, tout comme les autres traits floraux, d'être **soumises à une sélection dirigée par les pollinisateurs**. Peu de fleurs sauvages sont considérées comme inodores ; ce qui peut être considéré comme une preuve en-soi de l'importance fonctionnelle des odeurs florales. Aussi, l'étude des **variations des émissions de COV floraux** promet d'en savoir davantage sur l'évolution des plantes à fleur. Malgré leur importance, les COV floraux ont été bien moins étudiés que les autres traits floraux, et leurs processus de diversification restent encore à découvrir (Raguso 2008, Whitehead et Peakall 2010).

Il est un cas où la spéciation due à l'isolement éthologique ne fait plus controverse, et ce cas exceptionnel fait intervenir des odeurs florales. C'est celui des **orchidées sexuellement trompeuses** dont le système de pollinisation est hautement spécialisé (Schiestl et Ayasse 2002, Schiestl et al. 2003). Chez ces orchidées, qui attirent des mâles hyménoptères en émettant les phéromones des femelles, un mutant ou un hybride avec une nouvelle odeur peut attirer un nouvel assemblage de pollinisateurs non-commun à ses conspécifiques (Schiestl et Ayasse 2002, Vereecken et al. 2010). Quant à savoir si ces spéciations ont eu lieu en sympatrie ou non, des analyses génétiques montrant que des flux de gènes entre les espèces d'orchidées sont communs, laissent croire qu'il est plus probable qu'elles aient eu lieu en allopatrie (Mant et al. 2005).

Trois espèces sympatriques d'**Araceae** en Guyane française (*Anthurium sagittatum*, *A. thrinax* et *Spathiphylum humboldtii*) émettent des odeurs florales qui leurs sont spécifiques et qui attirent différents spectres d'espèces d'abeilles euglossines des genres *Euglossa*, *Aglae*, *Eulaema* et *Exaerete* (Hentrich et al. 2010). Leur spadice (inflorescence en épi entourée d'une grande bractée) oblige un dépôt de pollen uniforme sur le corps des abeilles qui, si elles visitent d'autres espèces d'Araceae, induit des interférences entre pollen hétérospécifiques affectant la valeur reproductive des plantes. Ces abeilles pollinisatrices collectent des composés odorants que l'on pense utiles lors de leur accouplement (Hentrich et al. 2010). Un isolement reproducteur, de type éthologique, sur la base de différences d'odeurs florales a été mis en avant pour expliquer l'origine de ces espèces et le maintien de leur coexistence actuelle (Hentrich et al. 2010).

Ces cas restent des exemples exceptionnels. La plupart du temps si les variations d'odeurs florales participent à des flux de gènes limités, elles fonctionnent en synergie avec d'autres traits floraux pouvant expliquer des co-variations (Gegear et Laverty 2005, Dobson 2006). L'étude des variations d'odeurs florales de deux genres d'**Annonaceae** (*Asimina* et *Deeringothamus*) montre des co-variations entre la couleur florale, les odeurs florales et la présence ou non de récompenses. Les espèces d'Annonaceae étudiées à fleurs marron tendent à émettre des odeurs florales de fermentation et n'offrent pas de récompenses, alors que les fleurs blanches, qui émettent une agréable odeur de composés aromatiques, offrent quant à elles des récompenses (Goodrich et Raguso 2009). Cependant, il est délicat de comprendre les mécanismes qui sous-tendent les co-variations odeur-couleur parce qu'il n'est pas exclu que des contraintes biochimiques en soient la cause (Majetic et al. 2008). On sait par exemple, qu'il y a un lien biosynthétique entre les benzénoïdes et les anthocyanines (pigmentations sur la base de la couleur rouge) puisque lorsque l'expression d'une enzyme centrale dans les voies de production des anthocyanines est bloquée chez l'œillet, une surproduction de benzoate de méthyle est provoquée (Zuker et al. 2002).

3. Evolution des odeurs florales

Bien que les fonctions écologiques des odeurs florales soient relativement bien étudiées, **les mécanismes évolutifs** qui gouvernent la composition et les variations quantitatives de ce signal complexe restent peu étudiés.

Les variations d'odeurs florales sont globalement plus marquées entre les espèces qu'au sein des espèces (Dobson 2006, Raguso et al. 2006, Raguso 2008). La comparaison des cas d'études des variations d'odeurs florales au sein de l'espèce tend à montrer que l'intensité des variations pourrait être corrélée avec les stratégies de pollinisation. La composition en COV floraux peut être, par exemple, très variable chez les espèces visitées par un grand nombre de pollinisateurs généralistes, comme chez *Magniola kobus* (Azuma et al. 2001) ou présenter un conservatisme marqué chez les espèces hautement spécialisées, telles les Yuccas (Svensson et al. 2005, Svensson et al. 2006). Cependant, l'étude des variations des émissions de COV floraux est délicate dans la mesure où les quantités émises de COV floraux d'une espèce sont souvent dynamiques. Leurs patrons de variation dans le temps et l'espace représentent des indices pour mieux comprendre les facteurs qui gouvernent l'évolution des

odeurs florales. Les canaux privés de communication entre les fleurs et certains de leurs partenaires pourraient induire des pressions de sélection qui agissent sur un sous-ensemble seulement de COV émis (Raguso 2008). Par exemple, les quantités de COV floraux qui varient les moins entre les populations d'*Ophrys exaltata* correspondent aux composés qui reproduisent les phéromones du pollinisateur *Colletes cuniculiarius* (Mant et al. 2005).

L'étude de la distribution des composés volatils floraux entre les espèces pose la question de savoir si la neutralité ou la sélection expliquent préférentiellement l'évolution des odeurs florales. Si les COV floraux sont distribués de manière aléatoire entre les espèces alors la théorie neutre, qui propose des facteurs stochastiques sont à l'origine de la répartition des traits au sein des communités (Hubbell 2001), peut être avancée. En revanche, des patrons phylogénétiques significatifs, c'est-à-dire lorsque certains COV ou classes de COV sont sur-dispersés ou sous-dispersés, sont expliqués soit par des contraintes phylogénétiques, soit par la sélection (Webb et al. 2002, Cavenders-Bares et al. 2004).

D'un point de vue plus fonctionnel, les composés émis par des groupes de plantes non reliés phylogénétiquement sont susceptibles d'être des molécules ancestrales intervenant dans diverses fonctions (ex benzaldehyde Schiestl 2010) alors que les composés plus rares pourraient avoir évolué plus récemment et correspondre à des fonctions plus spécifiques. Mais de telles études à grande échelle pourraient-elles montrer que la répartition des composés volatils floraux est aléatoire, contrainte ou adaptative ? L'une des seules études utilisant cette approche, celle de Andreas Jürgens (2009) sur les Annonaceae, montre que des **contraintes phylogénétiques** existent dans la distribution des composés volatils floraux au sein de cette famille, mais également que **certaines corrélations apparaissent entre l'occurrence des COV floraux et les stratégies de pollinisation.** Ceci suggère ainsi que les odeurs florales jouent un rôle dans l'évolution des angiospermes. Des études récentes tentent de prendre en compte **les potentiels conflits de sélection dus à la pollinisation et à l'herbivorie** dans l'étude de l'évolution des odeurs florales bien que très peu de données appropriées existent encore pour tester ces prédictions (Kessler et Halitschke 2009, Raguso 2009).

On sait que les insectes sont très sensibles aux composés volatils floraux et qu'ils ont la faculté d'en détecter un très grand nombre et d'y répondre activement (Raguso 2001). Chez l'abeille, le système olfactif exige des processus cognitifs complexes des centres nerveux, et il induit que l'occurrence d'un composé volatil a un impact sur la perception d'un autre (Guerrieri et al. 2005, Wright et al. 2005, Chittka et Raine 2006, Wright et Schiestl 2009, Reinhard et al. 2010). Ce n'est que récemment qu'une méta-analyse a permis de valider

l'hypothèse que **l'évolution de certains COV floraux a été façonnée par la communication chimique avec les insectes** (Figure 9, Schiestl 2010) alors que la neutralité était mise en avant auparavant (Knudsen et Gershenzon 2006).

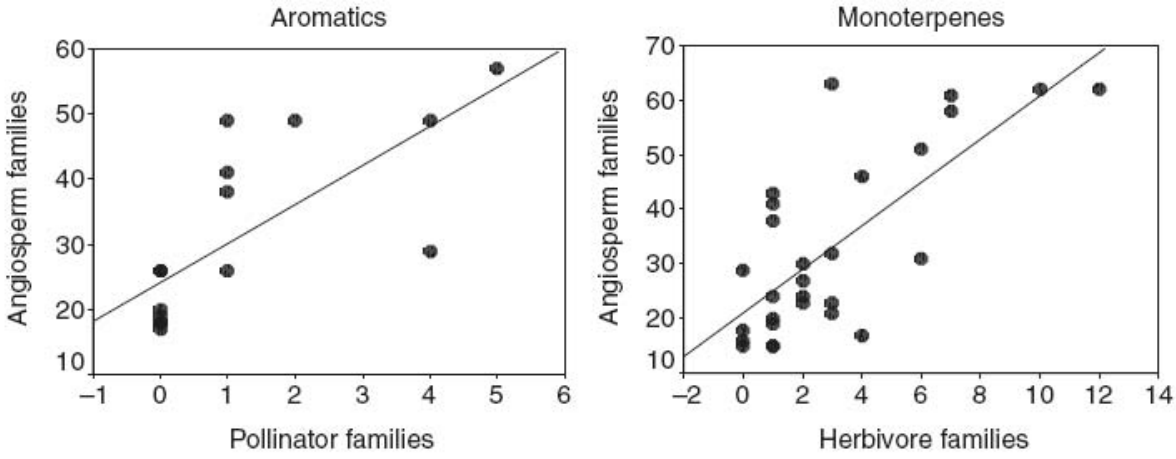

Figure 9 : Relation entre le nombre de familles de pollinisateurs et d'angiospermes qui produisent des composés aromatiques (à gauche) et des monoterpènes (à droite) (Tiré de Schiestl 2010).

D. Le modèle biologique d'étude : *Antirrhinum majus*

Antirrhinum majus (Scrophulariaceae, parfois inclus dans les Plantaginaceae selon le marqueur moléculaire utilisé dans les reconstructions phylogénétiques, Albach et al. 2005) est une herbacée pérenne qui pousse, en Europe, dans l'Est des Pyrénées françaises et espagnoles. A la floraison, elle présente des racèmes de fleurs zygomorphes à cinq pétales partiellement fusionnés qui forment un tube fermé par deux lobes. Dans notre région d'étude, deux sous-espèces sont clairement distinguées par leurs phénotypes : *A. m. pseudomajus* a des fleurs magenta, et *A. m. striatum*, des fleurs jaunes (Figure 10). Ces deux phénotypes floraux ne diffèrent pas morphologiquement et fleurissent de manière synchrone. Elles sont auto-incompatibles, et entièrement dépendantes des pollinisateurs pour leur reproduction (Andalo et al. 2010).

C'est une espèce généraliste pour la pollinisation, qui impose à ses pollinisateurs d'être capables d'ouvrir les deux lobes de la corolle pour avoir accès aux récompenses (le pollen et le nectar). Les pollinisateurs semblent principalement être des Apidés parmi lesquels les bourdons (*Bombus sp.*) sont prédominants. A l'heure actuelle, cependant, aucune étude n'a encore clairement caractérisé les cohortes de pollinisateurs associées aux deux sous-espèces

d'*A. majus*. On sait par ailleurs qu'*A. majus* subit deux interactions antagonistes qui lui sont spécifiques. La chenille du papillon *Mellicta deione* peut causer des dommages très importants en se nourrissant exclusivement des feuilles d'*A. majus*. L'explication la plus probable au fait qu'*A. majus* ne soit attaqué uniquement par cet herbivore est qu'*A. majus* produirait des composés non volatils de défenses (des iridoïdes) identifiés chez les variétés commerciales qui sont toxiques pour des herbivores généralistes et liés à la stratégie de reproduction de la plante (Beninger et al. 2007, 2008 et 2009). Une autre force de sélection négative est exercée sur *A. majus* par les jeunes larves du charançon *Rhinusa vestita* qui se développent dans ses fruits en consommant les graines.

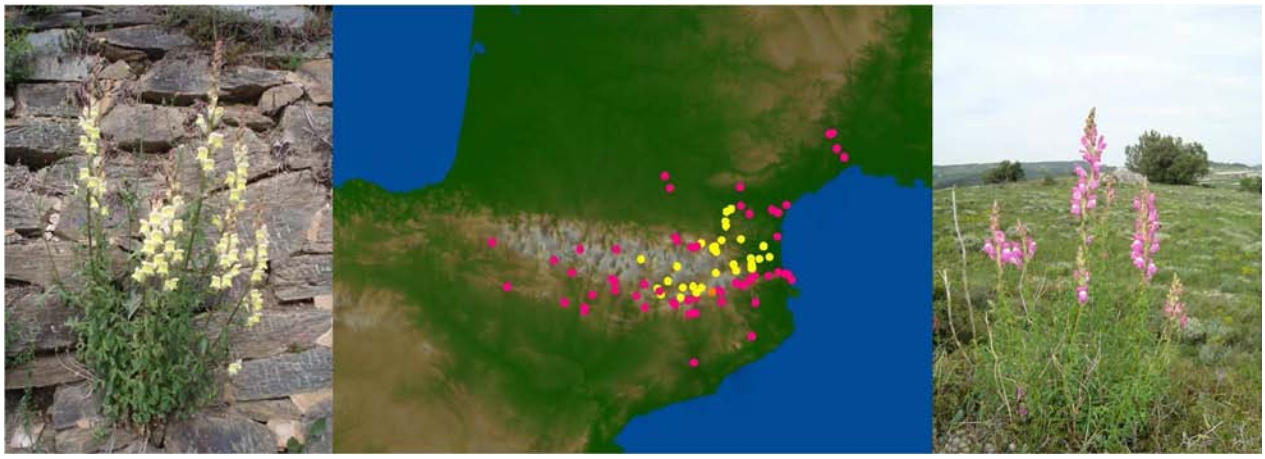

Figure 10 : Aire de distribution dans les Pyrénées d'*A. m. striatum* (en jaune sur la carte, à gauche en photo) et d'*A. m. pseudomajus* (en magenta sur la carte, à droite en photo).

Les deux sous-espèces d'*Antirrhinum majus* ne coexistent pas. L'aire de distribution d'*A. m. striatum* est enclavée au sein de l'aire d'*A. m. pseudomajus* (Figure 10) où les populations des deux sous-espèces sont soit allopatriques, soit parapatriques, mais jamais sympatriques. Cependant, elles ne sont pas considérées comme des espèces car leur isolement est partiel du fait qu'elles peuvent se croiser sans barrières post-zygotiques. Le nombre de fruits et de graines par fruit, le poids des graines, la taille des fruits, le taux de germination, la taille des parties aériennes, le ratio des parties végétatives et des racines ainsi que la probabilité de survie ne diffèrent pas significativement entre des individus issus de croisements intra et inter sous-espèces (Andalo et al. 2010). Des zones hybrides sont d'ailleurs présentes dans l'aire de distribution d'*A. majus* lorsque les deux sous-espèces rentrent en contact. L'une d'entre elle est particulièrement étudiée de part son ampleur. Elle se

situe dans la vallée de Toses, à Planoles, dans les Pyrénées catalanes (en orange sur la Figure 10) à 1150 mètres d'altitude en moyenne, au milieu d'un versant de montagne et de deux populations parentales : l'une d'*A. m. striatum*, près du village de Toses, à environ 9 kilomètres à l'ouest et l'autre d'*A. m. pseudomajus*, près du village de Pardines, à environ 13 kilomètres à l'est. La couleur des fleurs ségrége alors en une grande diversité de colorations le long de ce transect de la zone hybride (Figure 11 A) qui décrit deux clines étroits des deux couleurs florales parentales magenta et jaunes (Figure 11 B, Whibley et al. 2006). L'isolement reproducteur des deux sous-espèces est aussi caractérisé par un autre cline étroit, superposable à ces derniers, d'un locus qui gouverne la couleur magenta (Figure 11C, Whibley et al. 2006).

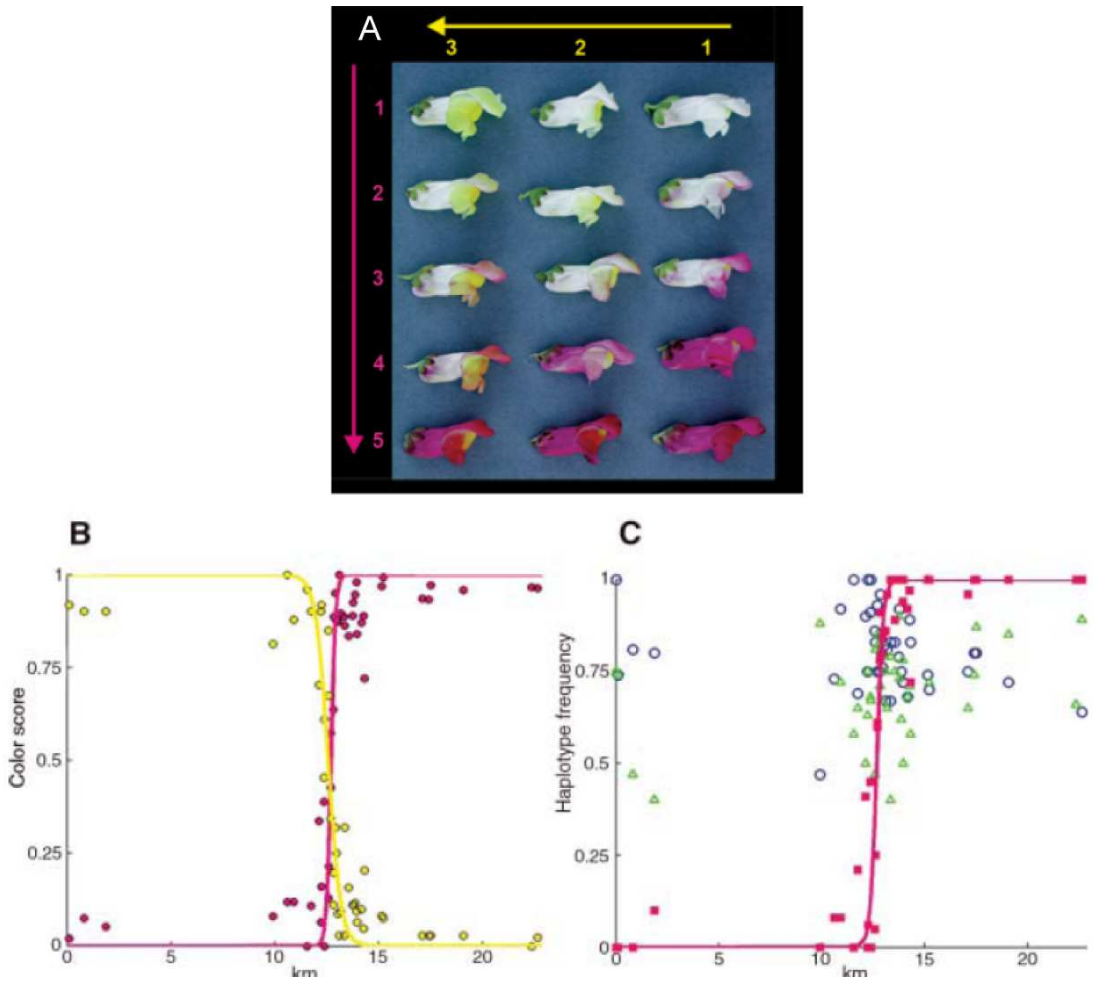

Figure 11 : (A) Spectre de la diversité de la couleur florale des hybrides rangés en fonction de leur intensité de jaune (sur une échelle de 1 à 3, en haute du spectre) et de magenta (sur une échelle de 1 à 5, à gauche du spectre), (B) clines des scores des deux couleurs florales parentales, jaune et magenta, le long d'un transect de la zone hybride, (C) fréquences des allèles *ROS1* (du locus ROSEA qui code pour l'intensité du pigment d'anthocyanine magenta, carrés magenta), *PAL* (cercles bleus) et *DICH* (triangles verts) (des loci PALLIDA et DICHOTOMA liés à ROSEA) (tiré et adapté de Whibley et al. 2006).

Les travaux de thèse d'Emmanuelle Tastard (2009), eux aussi concentrés sur cette même zone hybride, ont démontré que certains phénotypes hybrides sont contre-sélectionnés, notamment les hybrides transgressifs à fleurs oranges et blanches (de coordonnées respectives 3;5 et 1;1 sur le spectre de couleur, Figure 11 A). Ces hybrides ont en effet un taux de fructification et une charge pollinique sur le stigmate significativement plus faibles que les phénotypes parentaux ainsi qu'un pourcentage de fruits charançonnés et un volume de nectar à un instant t (mesure indirecte du taux de visite de pollinisateur) significativement plus important (Tastard 2009).

Tous ces travaux réunis laissent croire que le choix des pollinisateurs pourrait avoir un rôle prédominant dans la limitation en flux de gènes qui maintiennent les deux sous-espèces isolées. Ainsi, Emmanuelle Tastard a également étudié l'influence de la couleur florale sur le comportement des bourdons (*Bombus terrestris*). Elle a tout d'abord mis en évidence que *B. terrestris* était capable de discriminer les différentes couleurs rencontrées chez *A. majus* (Tastard et al. 2008). En plus de différencier les phénotypes floraux, les bourdons se comportent différemment en présence de ces derniers. De manière expérimentale, ils préfèrent significativement visiter les fleurs artificielles jaunes et magenta quand ils font face à une gamme diversifiée de couleurs rencontrées chez les hybrides, mais ils ne montrent pas forcément de préférence entre les deux phénotypes parentaux (Tastard et al. soumis). En somme, la couleur florale semble jouer un rôle dans la contre sélection des hybrides mais n'intervient pas dans le maintien des deux sous-espèces isolées.

E. Objectifs de thèse et approches expérimentales

Afin de participer à une meilleure compréhension de la diversité des fonctions écologiques et de l'évolution des odeurs florales, il est important de multiplier les cas d'études parce que la déduction de conclusions globales et objectives dépend de telles connaissances approfondies et homogènes. *Antirrhinum majus* apparait comme un modèle de choix de par les nombreuses connaissances multidisciplinaires accumulées à son sujet depuis les croisements réalisés par Charles Darwin en 1876. Sa situation particulière en milieu naturel permet une approche évolutive à l'étude de ses émissions d'odeurs florales. Pour le nez humain, *Antirrhinum majus* émet des odeurs florales très intenses qui ont attiré l'attention de

plusieurs chercheurs dans le passé sans aboutissements (Thébaud C., Coen E., Giurfa M. et Raguso R.). Son système de pollinisation généraliste en fait un modèle intéressant parce qu'aujourd'hui nos connaissances sur l'écologie évolutive des odeurs florales sont biaisées par des systèmes hautement spécialisés, notamment dans la famille des Orchidaceae. Ainsi, il s'agissait concernant ma thèse de mener à bien des travaux qui cherchent à déterminer si les odeurs florales pouvaient être une dimension supplémentaire au mécanisme d'isolement reproducteur d'*A. m. striatum* et d'*A. m. pseudomajus*.

La première problématique à traiter était celle des variations du signal chimique floral émis par *A. m. pseudomajus* et *A. m. striatum* (article 1). Cette approche est primordiale parce que les variations de COV floraux en intra-spécifique, mises en évidence ces dernières années (Raguso 2009), décrivent des patrons qui diffèrent au cas par cas et qui peuvent être hautement informatifs quant aux causes de ces variations (qu'elles soient environnementales, génétiques ou dues à de la plasticité phénotypique). C'est une approche qui peut, par conséquent, fournir des renseignements précieux sur l'évolution des odeurs florales du modèle, sous réserve d'une méthode scientifique rigoureuse. Pour ce faire, j'ai échantillonné les émissions de COV floraux à l'aide de la même méthode, « l'headspace dynamique » (Tholl et al. 2006), à la fois en conditions contrôlées (en serre) et naturelles (Figure 12). L'approche comparative étant respectée, six populations ont été étudiées, dont trois pour chaque sous-espèce, afin de caractériser les variations intra et inter populationnelles, et sous-spécifiques. Des hybrides de première génération, F_1, (provenant d'un stock de graines issu de l'étude de Andalo et al. 2010) et de deuxième génération, F_2, (que j'ai produit par pollinisation artificielle des F_1) ont également été échantillonnés en conditions contrôlées. Tous ces échantillons ont ensuite été analysés par les mêmes méthodes de chromatographie en phase gazeuse et de spectrométrie de masse. Ces recherches relevaient

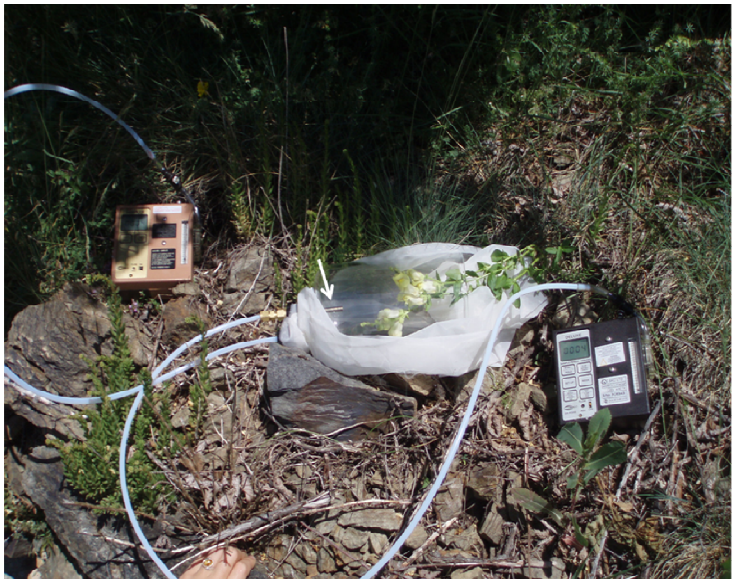

Figure 12 : Photographie du design expérimental mis en place durant ma thèse pour échantillonner les odeurs florales d'*A. majus* (ici *A. m. striatum*, au col de Toses, en Espagne, détails en Annexe 1).

donc d'un travail de coordination d'échantillonnage dans différentes conditions dont certaines, notamment de terrain, ont demandé une organisation parfois fastidieuse pour respecter une rigueur de travail justifiée. Une formation en chimie analytique poussée a surtout été nécessaire parce qu'elle n'était pas acquise en début de thèse, alors qu'elle demandait, avant d'être appliquée, un effort de travail considérable de mise au point et de calibration d'analyses.

La deuxième problématique qui à été développée a été celle de la perception olfactive des pollinisateurs et de l'influence des variations d'odeurs florales observées sur leur comportement. Il s'agissait en d'autres termes, de tester indirectement une des hypothèses sur les causes des variations d'odeurs florales, à savoir si celles-ci pouvaient représenter un patron adaptatif dans le contexte de pollinisation. Il a d'ores et déjà fallu connaître quels étaient les pollinisateurs en adaptant les méthodes de caractérisation de la cohorte des pollinisateurs au modèle et en s'ouvrant à leur mode d'identification. J'ai ainsi pu observer que les bourdons représentaient le pollinisateur principal, tout particulièrement *Bombus terrestris*. Une étude d'électro-antennographie (EAG) (SYNTECH®, Kirchzarten, Allemagne) (article 3) a alors été mise en place pour savoir si les bourdons étaient capables de détecter les composés majoritaires de l'odeur florale d'*A. majus*, en utilisant des bourdons commercialisés (*B. terrestris*, Koppert®, Berkel en Rodenrijs, Pays-Bas). Cette méthode consiste à enregistrer l'activité électrique induite par la réception de composés volatils d'une antenne montée entre deux électrodes. Parmi de multiples méthodes possibles, dont certaines testées sans succès, c'est l'olfactrométrie à l'aide d'un labyrinthe en « Y » (Figure 13) qui a été appliquée afin de tester l'influence des COV floraux sur le comportement des pollinisateurs. Dans un premier temps, des bourdons commercialisés, autrement dit vierges de toutes odeurs, ont été testé dans ce dispositif, dans lequel des odeurs artificielles recomposées à partir de molécules synthétisées leur étaient présentées (article 3). Cette approche permet de mettre en exergue les composés pour lesquels les bourdons sont le plus sensibles. Dans un deuxième temps, j'ai répété cette expérience en utilisant des bourdons

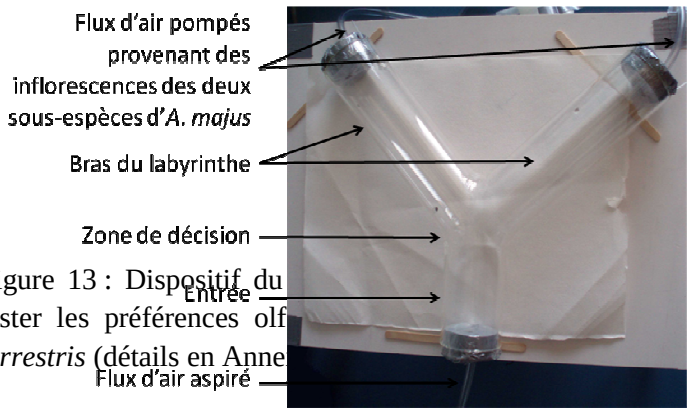

Figure 13 : Dispositif du
tester les préférences olf
terrestris (détails en Anne

naïfs mais avec, cette fois-ci, des odeurs florales naturelles d'*A. m. pseudomajus* et d'*A. m. striatum* afin de valider les patrons observés qui peuvent être des artéfacts de l'utilisation d'odeurs artificielles. En dernier lieu, j'ai appliqué ce test d'olfactométrie avec des bourdons et des halictides sauvages expérimentés en utilisant des odeurs florales naturelles afin de tester leur réponse suite à un apprentissage (article 4). Pour faire ce travail, une découverte et une mise en application de méthode de cognition et de comportement animal auprès d'experts ont été nécessaires.

Enfin, il s'agissait de chercher à savoir si les pollinisateurs pourraient présenter des préférences entre les deux sous-espèces en examinant s'il existait des patrons associatifs entre les odeurs florales et la composition en nectar (article 5). Le nectar des fleurs cultivées en conditions contrôlées a été prélevé par capillarité en évitant toutes contaminations extérieures. Le volume a été directement mesuré. Ensuite les sucres contenus dans le nectar ont été dosés par chromatographie en phase liquide à haute performance, ce qui permet de séparer les composés en fonction de leur hydrophobicité. Ceci permet l'identification et la quantification des sucres. Avec le même outil analytique, les acides-aminés contenus dans le nectar ont également été identifiés.

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CHAPITRE 1

Variabilité des composés volatils floraux chez *Antirrhinum majus*

Résumé

Dans ce chapitre mes travaux cherchent à déterminer les facteurs de variation des émissions en composés volatils floraux chez les deux sous-espèces d'*Antirrhinum majus* (Article1) ainsi que chez les hybrides de première génération (F_1) et de deuxième génération (F_2) (Article 2). Mon approche consiste à mettre en place la même méthode d'échantillonnage dit d'headspace dynamique des COV floraux d'individus provenant de différents environnements tels que le milieu sauvage et en serre, où les conditions sont contrôlées. La comparaison des patrons d'émission entre les environnements permet de dissocier les variations dues à un déterminisme génétique de celles engendrées par des facteurs environnementaux. Tous les échantillons ont été analysés en utilisant les mêmes paramètres de CPG-SM-DIF.

L'étude focalisée sur les parentaux (Article 1) montre que le facteur sous-espèce est le facteur le plus explicatif des variations d'odeurs florales. En effet, *A. m. pseudomajus* et *A. m. striatum* se distinguent systématiquement par leurs composés volatils floraux. Certains composés volatils, en particulier trois benzénoïdes, ne sont émis que par *A. m. pseudomajus* et ceci de manière constante entre les populations et entre les environnements. Cette différence qualitative engendre une différence d'intensité d'odeur florale puisque l'un des benzénoïdes, l'acétophénone, est émis en grande quantité. L'étude a également révélée que les émissions de COV floraux sont légèrement influencées par l'environnement puisque celles-ci étaient significativement plus variables en milieu naturel qu'en serre. Les sous-espèces d'*A. majus* étaient davantage différenciées en milieu naturel.

Quant aux hybrides (Article 2), leur composition en COV floraux est très variable par rapport aux parentaux en terme de ratio relatifs des composés. En effet, de nouveaux composés volatils ont été détectés mais ne participent pas significativement à leur différenciation. Les hybrides F_1 émettent des COV en concentrations intermédiaires par rapport aux parents alors que certains composés, principalement les dérivés d'acides gras, ségrégent (sont émis en surabondance) chez les hybrides F_2. Les benzénoïdes spécifiques à *A. m. pseudomajus* sont omniprésents dès la première génération d'hybrides et présentent des différenciations quantitatives en fonction des lignées.

Les résultats de ses deux études convergent pour conclure qu'une base génétique est à l'origine des différences d'odeurs florales observées entre les deux sous-espèces de la gueule-de-loup. Les processus évolutifs aboutissant à de tels patrons peuvent être adaptatifs ou non-adaptatifs ; soit les deux phénotypes ont été sélectionnés de sorte à être adaptés de manière différente à leur environnement, soit il existe une contrainte biochimique liée à la différence de coloration florale existant également entre *A. m. pseudomajus* et *A. m. striatum*. Par conséquence, les odeurs florales sont susceptibles d'être impliquées dans l'isolement reproducteur des deux sous-espèces d'*A. majus*.

Article 1

REPRODUCIBILITY OF FLOWER SCENT EMISSIONS IN TWO WILD SUBSPECIES OF THE SNAPDRAGON, *Antirrhinum majus*

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Abstract-The reproducibility of flower scent is critical for a pollinator to learn that a reward is available. We studied the inter-populational variation in floral volatile organic compounds (VOCs) emitted by the flowers of *Antirrhinum majus*, to test whether flower scent significantly varies across populations. We selected six natural populations of *A. majus*, three from the magenta-flowering subspecies, *A. m. pseudomajus*, and three from the yellow-flowering subspecies, *A. m. striatum*. A seed stock collected in the same six populations was also grown in a greenhouse to control for possible environmental variability. Floral VOC emissions of both the wild and greenhouse-grown plants were sampled using the dynamic headspace sampling technique. VOCs were identified and quantified using a GC-MS-FID. The variability in the flower scent of *A. majus* was mostly explained by systematic differences between the subspecies, both in the wild-grown and in the greenhouse-grown plants. The

qualitative and quantitative differences in flower scent between the two subspecies were reproducible among the populations. The floral emissions were more variable in the wild plants than in the greenhouse-grown plants, but we failed to explain this variability by available micrometeorological variables. We conclude that the flower scent is reproducible within each *A. majus* subspecies, and that this signal may be used as a cue by pollinators for detection and recognition of flowers.

Key Words-Flower scent, *Antirrhinum majus*, wild snapdragon, genetic determinism, environmental variability, VOC.

INTRODUCTION

Many insect-pollinated flowers attract their pollinators through chemical cues (Raguso, 2008), but a variety of other functions of floral emissions have also been evidenced. For instance, pollen odors in *Acacia* repel their mutualistic ant partners to prevent a conflict with the pollinators when they access the flowers (Ghazoul, 2001). In the sweet olive tree *Osmanthus fragrans*, the presence of γ-decalactone in flower scent deters the cabbage butterfly *Pieris rapae*, a potential flower visitor and plays a role of filter for pollination (Omura et al., 2000). Volatile organic compounds (VOCs) may also be used as a protection against the attacks of opportunistic enemies that may compromise the plant's reproduction (Gershenzon and Dudareva, 2007). Thus the floral VOCs convey bits of information to a wide array of ecological partners. To be useful as a message-passing signal (Shannon and Weaver, 1949), a floral scent should be (a) adapted to an ecological function (i.e. meaningful), (b) perceptibly efficient for the receiver (i.e. based on a simple 'syntax'), and (c) reproducible (i.e. minimizing message error).

The first of these issues, the adaptive nature of the signal (point (a) above) has motivated a great deal of interest in the scope of plant-pollinator interactions (Raguso, 2008). A major result emerging from these studies is that floral VOCs are finely tuned to optimize pollinators' visit, and are therefore an important component of the plant's fitness. Indeed, the flower scent of *Antirrhinum majus* is emitted in maximal amounts precisely when the flower is mature (dehiscent anthers), and comparatively less either before (Dudareva et al., 2000), or after pollination (Negre et al., 2003). Likewise, the ability to perceive the stimulus by the pollinators (point (b) above) has generated a great deal of research (Giurfa, 2007; Wright and Schiestl, 2009). The question of whether the flower scent is reproducible within a species

(point (c) above) is also critical. If the scent is reproducible across the individuals of the same species, the fidelity of pollinators is encouraged because pollinators then learn to associate a VOC profile with its rewards (Wright and Schiestl, 2009). In the special case of non-rewarding flowers, this scenario does not apply; in food-deceptive orchids for instance, a highly variable floral VOC composition prevent the pollinators from learning the dupery (Salzmann et al., 2007, Salzmann and Schiestl, 2007). Therefore, to study whether pollinators may use floral scent as a cue in the wild, it is important to document whether the floral scent varies across individuals and populations. In the literature, several examples are *Magnolia kobus* in Japon (Azuma et al., 2001), *Geonoma macrostachys* in Western Amazon (Knudsen, 2002), Orchidaceae in Italy and France (Salzmann et al., 2007, Salzmann and Schiestl, 2007, Dormont et al., 2009), *Yucca* sp. in US (Svensson et al., 2005, 2006), *Linanthus dichotomus* in US (Chess et al., 2008), and *Echinopsis ancistrophora* in Bolivia and Argentina (Schlumpberger and Raguso, 2008). In the two sympatric purple and white flower color morphs of *Hesperis matronalis*, another example, a consistent difference in flower scent composition was detected among purple flowered plants but not among white flowered plants (Majetic et al., 2007). With a larger sample size (n=5 populations) of the same plant species, it was shown that flower scent variability is mostly explained by genetic drift, founder effect, population membership and environmental conditions (Majetic et al., 2008, 2009). The study of intra-specific variability of the floral VOC composition of a species is therefore important, as it may be used to detect the chemicals that are the most consistently emitted. Such chemicals may act as private channels of communication with ecological partners (Raguso, 2008).

Here we study the variability in the floral VOC emissions of the wild species *Antirrhinum majus* by comparing the chemical variability of the same populations in homogeneous growth

conditions (in greenhouse), which control for potential genetic differences, to the variability in heterogeneous growth conditions (in the wild). A great deal of knowledge has been assembled on the biosynthesis and the regulation of some major VOCs emitted by cultivars of *Antirrhinum majus* (Dudareva et al., 2000, 2003; Goodwin et al., 2003; Negre et al., 2003; Dudareva et al., 2006), because pure lines have been cultivated for a long time (Schwarz-Sommer et al., 2003). *Antirrhinum majus* grows naturally in the Eastern Pyrenees, a mountainous region bordering Spain and France (Whibley et al., 2006; Andalo et al., 2010) and the phenotypic variability of this species may then be studied *in natura*. An experimental quantification of the intraspecific variability of flower scent of wild plant species would consist in manipulating the plant's environment, by varying for example temperature or light intensity, and study the response in VOC emissions (e.g. Jakobsen and Olsen, 1994). However, these studies only quantify the amount of short-term plastic response to an environmental stressor (acclimation). Rather, we chose compare the emissions of plants grown in the wild with plants of the same populations but cultivated in greenhouse conditions.

The variability of VOCs may be further studied in this species by contrasting two subspecies of *Antirrhinum majus* that both occur in the area of study (Whibley et al., 2006). One of the subspecies has magenta flowers (subspecies *A. m. pseudomajus*) and the other has yellow flowers (subspecies *A. m. striatum*). To assess the degree of inter-population variation of flower scent, we sampled the VOCs emitted by the inflorescence of *A. majus* in three natural populations of each of these two subspecies (for a total of 6 populations) across the natural range of the species. We then compared the VOCs emitted by the inflorescences in the wild to thoses emitted by the inflorescences of plants from the same populations, but cultivated in greenhouse under controlled conditions. Four factors causing variation may be tested. If the differences between the two subspecies are constant among populations and growing

environment, heritable variation, that causes a subspecies differentiation, is then evidenced. If it is differences between the two growing environment that explained the more the floral scent variation, otherwise a growing environment effect, it means that the floral scent is environmentally conditioned. Two cases are possible in case of a population effect: either similar differences of floral scent from particular populations are found in the two growing environments, or differences relative to populations are observed only in the wild. In the first case, it is also an evidenced of heritable variation due to a lineage effect. In contrast, in the second case, when floral scents vary relative to populations only in the wild, it is more likely a variation due to local adaptations to abiotic and/or biotic conditions, which are not heritable. In the last case, there could have any variation in function of subspecies, growing environment and population, because variations are only shaped by inter-individual differences. We asked the following questions: (i) Which factors explain the variability of flower scent in this *Antirrhinum majus* sampling: the growing environment, the subspecies, the population or the inter-individual effect? (ii) To what extent do the floral VOCs emissions differ between the greenhouse and the wild conditions (henceforth "environmental condition"), and do micrometeorological variables (temperature, light intensity and humidity) explain the observed variation in the wild? Finally (iii) Do the two *A. majus* subspecies consistently differ in their floral scent across populations and environmental conditions, and if so which VOCs may contribute to this difference, both in the greenhouse and in the field?

MATERIALS AND METHODS

Study Species. The genus *Antirrhinum* (Scrophulariaceae) has semi-perennial plants with zygomorphic flowers. It is pollinated by insects (mostly Apidae), flowers are self-incompatible and bear closed corollas, which are mostly visited by large-bodied insect visitors

able to force their way through the tubular corollas (Andalo et al., 2010; Vargas et al., 2010). *Antirrhinum majus* grows in Southern Europe at an elevation of 0-1600 m asl in the Eastern Pyrenees mountains. One widespread subspecies, called *A. m. pseudomajus* has magenta flowers. A more restricted subspecies, *A. m. striatum*, has yellow flowers. Both subspecies display a metapopulation spatial structure, and the two phenotypes are not found in sympatry but only in allopatry or in parapatry when the two subspecies come into contact. Near the village of Planoles in Spain, a remarkable hybrid zone between the two subspecies populations is observed. There, hybrids display a striking diversity of floral color. Two abrupt and inverse clines of the parental floral colors, magenta and yellow, have been evidenced and these match ithw a cline for the ROSEA gene, directly implicated in the production of anthocyans present in petals of *A. majus* (Whibley et al., 2006). Based on these results, Whibley et al. (2006) speculated that the hybrid flowers are counter-selected suggesting that the hybrid zone is under stabilizing selection.

Sampling Sites. We selected six wild populations of *A. majus*, three for each subspecies. These populations were chosen in order to maximize the elevation range for each subspecies and their relative isolation status in term of distances from the Planoles hybrid zone and other conspecific populations (Figure 1). The Pardines (PAR) and Collada de Toses (TOS) populations of *A. m. pseudomajus* and *A. m. striatum* respectively are separated by only 30 km, and the hybrid zone is found approximately midway between them, over a distance of 5 km (Whibley et al., 2006). We also selected two populations in an area where secondary contacts between the subspecies are possible but which do not display the floral color range of hybrid zone: Lagrasse (LAG) and Camurac (CAM). In Camurac, hybrids of the subspecies were occasionally observed (C Suchet pers. observation). Finally, we selected two

populations well within the range of each subspecies: Martinet (MRT) for *A. m. pseudomajus*, and Lles (LLE) for *A. m. striatum*.

In each of these populations, seeds were collected between 2000 and 2006 at the end of the growing season from mature fruits. Seeds from ten fruits from ten individual plants for each of the six populations were grown in greenhouse conditions between November 2008 and May 2009 (16 hr/day of light, at 25°C average temperature, in individual pots with universal compost and with no extra nutrients). Ten adult plants were selected by population at the flowering time for their similar phenotypes and their diversified genetic sources. We minimized the number of sampled plants originated from the same fruit. In total, between 5 to 7 maternal lineages are represented among the ten selected plants by population.

Sampling of Floral VOCs. In May and June 2009, we sampled the VOC emissions of ten adult plants for each of the six populations directly in the field (Figure 1). These plants were chosen within each population in order to maximize the pool of genetic diversity by sampling geographically distant plants within each population. Indeed, since the snapdragon seed dispersion occurs only by gravity, geographically closed plants more probably originate from the same maternal lineage. We could not analyze five of the 60 sampled individuals because of a technical issues, and our results then report data on a total of 55 individuals grown in the wild (Figure 1). The 60 plants grown in the greenhouse from the same six populations were also sampled for floral VOCs between February and May 2009.

Preliminary analyses showed that daily variations in VOC emissions were comparable with those described by Dudareva et al. (2000, 2003). Sampling was therefore conducted during the peak of emission intensity, between 11:00 am and 4:00 pm both in the field and in

greenhouse conditions. To minimize biases due to flower developmental stages (Dudareva et al., 2000, 2003; Goodwin et al., 2003), the VOCs were sampled when the inflorescence had at least four open flowers with dehiscent anthers. The total number of flowers varied between 4 to 8 comprising all stages of development of open flowers.

The sampling of floral VOC emissions was based on the dynamic headspace method (Tholl et al., 2006). Each inflorescence was enclosed *in vivo* into a 2 l glass chamber, and the VOCs were adsorbed on TenaxTA 60/80 (100 mg) trap connected to a battery-operated vacuum pump operated at 200 ml min^{-1}. This design optimizes the signal/threshold ratio without exceeding the breakthrough volumes of each compound (Kesselmeier et al., 1996; Simon et al., 2005a, b). The flow rate of the enclosure purge air was maintained at 600 ml min^{-1}. Sample duration was fixed at 10 min. To control for possible environmental contamination, ambient air was also trapped during each sampling session. Sample tubes were stored in the dark at constant temperature (0-4°C) until analysis. Special care was paid to cool the samples after sampling either in the field or in the greenhouse. Directly after the sampling session, all samples were cooled by using a motor-cool-box connected to a car battery for a maximum of five hours or in a refrigerator. All samples were analyzed in the lab at most four days after the sampling sessions.

Floral VOC emissions are regulated by light intensity and temperature (Guenther et al., 1995; Dudareva et al., 2000, 2003, 2006). For each sampling of VOCs, both variables were recorded from the headspace of the sampled inflorescence. Temperature and humidity of the floral headspace were measured with a EL-WIN-USB datalogger (Lascar Electronics LTD., United Kingdom), and photon flux with a LI250A light meter connected to a LI190SA Quantum Sensor (LI-COR Biosciences, Lincoln, USA). To normalize the emission rates among plants,

274 we cut the inflorescences after VOC sampling, and we measured their oven-dry weight
275 (flowers, sepals and buds of inflorescences were dried at 100°C during 48hr).
276
277 *Chemical Analyses.* The VOC samples were thermodesorbed using a Turbomatrix TD
278 desorber (Perkin Elmer) and they were analyzed by a gas chromatograph coupled with a mass
279 spectrometer and a flame ionization detector (FID) (Clarus 500, Perkin Elmer). The
280 separation of VOCs was performed using DB-5 non-polar capillary column (30m x 0.25 mm
281 ID x 0.25 µm film thickness). Oven temperature was held at 35°C for 5 min, heated to 160°C
282 at 5°C min^{-1} and then up to 220°C at 15°C min^{-1}. The carrier gas was helium. Mass spectra
283 were recorded in the electron impact mode at an ionization voltage of 70eV and scanned from
284 m/z = 33 to 450.
285
286 The identification of VOCs was based on their Kovats index relative to C_5 – C_{18} n-alkanes
287 and their mass spectra, which was matched with those from the NIST library (2005) and those
288 reported in the literature (Adams, 2001).
289
290 The quantification of VOC emissions was made on the basis of their FID peak area. Ocimene
291 (TCI Chemical®, Stockholm, Sweden, 90.0%) and nonanal (Extrasynthese SAS, Genay,
292 France, pure) were used as external standards. The calibration was carried out in laboratory
293 conditions by injecting a liquid volume of standard solutions directly into the sample tube. A
294 linearity range from 2 x 10^{-5} to 9.2 x 10^{-4} µg was observed for the two analyzed VOCs
295 (R^2=0.99 for both compounds). The theoretical response factor of the studied VOCs was
296 computed using the theory of the effective carbon number (Jorgensen et al., 1990). Applying
297 corrections to the mean response factors allowed us to quantify VOCs that were not calibrated
298 individually (Komenda et al., 2001). The emission rate of each VOC was obtained from the

difference between the quantity of VOCs recorded inside and outside the glass chamber. The emission rates E (µg. g (dry flowers weight)$^{-1}$. hr^{-1}) were computed using the following equation:

$$E = \left(\frac{m_2}{q_2} - \frac{m_1}{q_1} \right) \frac{Q}{Mt}$$

where m_2 and m_1 are the mass of the VOC in the outlet and inlet flow rates (µg), q_2 and q_1 are the outlet and inlet flow rates (ml min^{-1}), Q is the flow rate of the enclosure purge air (ml min^{-1}), M is the dry weight of the enclosed flowers (g) and t the sampling time (hr) (Sabillon and Cremades, 2001). This quantification method makes it possible to compare among plants because it normalizes the amount of VOCs to the dry flower weight, regardless of the difference in flower number per inflorescence. A uniform sampling and analytical uncertainty of ca. 30% is associated with the chamber design (Moukhtar et al., 2005).

Statistical Analyses.

Exploration of the variability of floral scent. A discriminant function analysis (DFA) based on VOC emission rate was performed to determine which VOCs most discriminate the two subspecies plus the two growing environment, and to test the probability that a plant was assigned with other plant of the same subspecies and of the same origin (field or greenhouse) based on its floral scent.

Causes of the variability of floral scent. We globally explored the causes of variability in floral VOCs emission in *A. majus* by testing for (i) a growth condition effect (greenhouse vs. wild conditions, henceforth "sampling condition") (ii) a subspecies effect, (*A. m. pseudomajus* vs. *A. m. striatum*) and (iii) a population effect (among the six populations, Figure 1). We applied a non-parametric multivariate analysis of variance (Anderson, 2001), as implemented

in the "adonis" routine of the R package "vegan" (Oksanen et al., 2010). This method is suited to compare metric or semi metric distance matrices. The Euclidean distance in the VOC emission rates between pairs of samples was used to build a distance matrix. Such a distance function weighs the VOCs proportionally to their abundance. The MANOVA performs an additive partitioning of the variance for complex models, while maintaining the flexibility and lack of formal assumptions of other non-parametric methods. The statistical test is a multivariate analogue to Fisher's *F*-ratio and is calculated directly from any symmetrical distance or dissimilarity matrix; significance levels are then obtained using permutations over the rows or columns of the matrices (Anderson, 2001).

Differences between the greenhouse and wild growth conditions. We compared the diversity of VOCs between both environmental conditions. We also tested separately the population effect between both environmental conditions. Finally, to disentangle the part of the variability of floral VOC emissions due to environmental growth conditions and natural heritable variation fixed among populations and subspecies, we analyzed only the dataset of the wild-grown individuals using a non-parametric MANOVA and incorporating the micrometeorological variables, such as temperature, photosynthetic active radiation (PAR), and relative humidity, as independent variables.

Differences between the two subspecies. Finally, we tested whether the two subspecies differed in their absolute emission rate using analyses of variance (ANOVA) based on log-transformed VOC emission rates of the greenhouse and the wild datasets separately. We also used an ANOVA to determine which VOCs contributed to the differences between the two subspecies by testing whether the inter-subspecies variability of each VOC emission is higher

than the intra-subspecies variability (as measured by the variability among the three populations of the same subspecies), based on log-transformed VOC emission rates.

All statistical analyses were carried out with the R statistical software version 2.9.2 (http://cran.r-project.org/).

RESULTS

Growing environment effect. The non-parametric MANOVA on the pooled greenhouse and wild datasets (Table 1) showed that the growing environment has a significant effect on floral VOC emissions because 1.7% of the flower scent variation were explained by the differences between greenhouse and wild conditions (P=0.045). However, it is not the primary cause of flower scent variation detected in the present study. This is characterized in the result of the discriminate function analysis, figure 2, because the second discriminant function (DF2) discriminates the plants from greenhouse than from the wild. The flower scent of *A. majus* was composed of 37 VOCs in the greenhouse and 41 VOCs in the wild (Table 2). Most of the VOCs emitted by the flowers of the greenhouse-grown plants were also detected in the wild, except the following five VOCs: one nitrogen-containing compound (syn-3-methyl-butyl-aldoxime), ethyl acetate, hemimelitene, and two highly volatile fatty acid derivatives (2-methyl-propanal and 3-methyl-propanal). Linalool was detected in the wild populations but not in the greenhouse populations. In addition, nine fatty acid derivatives were detected in the field, and were absent from the scent of the plants grown in the greenhouse. These VOCs poorly contributed to the discrimination between plants from greenhouse and plants from the wild. In contrast, the most correlated VOCs with DF2 were common to the two growing

environments except octane (Appendix 1). This means that the environmental effect on floral scent influences more the relative ratio of compounds than their occurrences.

None of the micrometeorological variables had an effect on the variation in VOCs across the wild populations (Table 3) despite large ranges of variation measured inside the headspace of VOC sampling for each inflorescence (n=55): temperature varied from 19°C to 47°C (mean 31 ± 1.2°C), light intensity varied from 161 to 1501 PAR (mean 687 ± 31 PAR) and relative humidity varied from 15 to 65% (mean 35 ± 1.8%). This indicates that other environmental factors, not measured here, are responsible of the additive variability observed in the wild.

Subspecies effect. The non-parametric MANOVA on the pooled greenhouse and wild datasets (Table 1) showed that the subspecies effect explained the larger proportion of variance in the floral VOC emissions (32.5%, P=0.001). In fact, the subspecies clusters were not overlapped in the DFA (Figure 2) and they were distributed along the first discriminant function. When considering other DFA projections including DF3 the subspecies clusters of plants from greenhouse also do not overlapped (not shown). High assignation levels were found when the plants were predicted as belonging to a cluster based on their flower scent: 100% for plants of *A. m. striatum* from the wild, 96% of *A. m. pseudomajus* from the wild, 93% of *A. m. striatum* from greenhouse and 87% of *A. m. pseudomajus* from greenhouse. The absolute VOC emission rate was far higher in *A. m. pseudomajus* than in *A. m. striatum* in both natural (591.95 ± 109.72 vs. 154.84 ± 49.45 $\mu g\ g^{-1}_{DW}\ hr^{-1}$, F=27.41, P<0.001) and greenhouse conditions (539.65 ± 81.04 vs. 149.9 ± 59.64 $\mu g\ g^{-1}_{DW}\ hr^{-1}$, F=22.23, P<0.001). We found that the two subspecies differed in their scent for five VOCs (Table 2). Moreover this difference was consistent in both environmental conditions. Three of these VOCs, acetophenone, benzaldehyde and methyl benzoate (benzenoids VOCs), were emitted only by *A. m.*

pseudomajus with acetophenone as the most abundant floral VOC of this subspecies. In addition 5-methyl-6-hepten-2-one which was emitted significantly more abundantly in *A. m. pseudomajus*, and limonene was emitted significantly more abundantly in *A. m. striatum*. Six additional VOCs showed significant differences of abundance between the two subspecies in the wild, but not in the greenhouse (Table 2). Importantly, all the differences of floral VOCs composition observed for the greenhouse plants between the two subspecies persisted in the wild populations. Focusing on the wild-grown populations only, the subspecies effect indeed persisted (Table 3). The subspecies effect was also significant in the greenhouse conditions, but to a lesser extent (percent of variance explained by subspecies: 50.8% and 26% in wild and greenhouse conditions, respectively).

Six plants of *A. m. striatum* sampled in the Camurac population (CAM) displayed an unusual pattern. Unlike all the other *A. m. striatum* plants, they did emit low abundances of acetophenone and four of them emitted traces of benzaldehyde (Table 2).

Population effect. We detected a significant population effect in the wild (Table 3, P=0.039) but neither in the pooled greenhouse and wild datasets (Table 1, P=0.572) nor in the greenhouse (P=0.757, not shown). This illustrating in figure 2 because the plants of the two clusters from the wild are more dispersed than those from the greenhouse (Figure 2). This suggests that local environmental conditions that differ among populations in the wild influence the floral VOC emissions of *A. majus*.

DISCUSSION

In this study, we explored the natural variation of the VOCs mixtures emitted by the flowers of two subspecies of *Antirrhinum majus*. We showed that floral VOCs mixtures were characteristics of the subspecies and that they were partially under environmental control because VOC emissions from populations of the same provenance were more variable in wild conditions than in greenhouse conditions despite their similar diversity of genetic sources.

Multiple causes may be responsible for flower scent variability in the wild. It is well known that floral VOC emissions depend on the daily variation in abiotic conditions (e.g. Jakobsen and Olsen, 1994; Dudareva et al., 2000, 2003). We found no clear relationship between VOC emissions and instantaneous micrometeorological measurements such as temperature, light intensity and humidity, hence micrometeorological variables were not important in explaining the variability of *A. majus* flower scent. However, we were unable to directly test the effect of these particular abiotic factors. To perform such a test, it would have been necessary to grow the plants under variable and controlled environmental conditions. Other abiotic factors such as soil composition or hydric stress could also explain the variability in floral VOC emissions in wild populations.

At the individual level, the history of pollinator visitation may in part explain the higher variability observed in the wild: the emissions of the main VOCs in the Maryland Pink True snapdragon cultivar were shown to decrease in intensity after pollination (Negre et al., 2003). Moreover visited flowers usually hold less nectar and this nectar may have olfactory properties (Raguso, 2004; Kessler and Baldwin, 2007). Biotic interactions may in fact play a major role in the variability of floral VOC emissions in wild populations. One remarkable example of a possibly biotically-mediated manipulation of the floral phenotype was offered

by Wolfe et al. (2005). They showed that the presence of mutualist arbuscular mycorrhizal fungi in the roots of *Chamerion angustifolium* (Onagraceae) increased pollinator visitation. Several mechanistic explanations of such a pattern have been offered, some of which involve differential VOC emission between the fungus-infected, and uninfected plants (Gange and Smith 2005, Bruinsma and Dicke 2008, Gehring and Bennett 2009). Similarly above ground aggressions like leaf herbivory can also led to changes in floral blends which may in turn influence pollinator attraction (Strauss, 1997). Finally, it has been recently shown that environmental variation explains at least in part the floral scent variation in *Hesperis matronalis* (Majetic et al., 2009).

We also showed that the two *A. majus* subspecies consistently emitted a different VOC mixture, irrespective of the population origin or environmental conditions. This difference was both qualitative and quantitative: three benzenoids were only emitted by *A. m. pseudomajus* (especially acetophenone, the most abundant VOC of this subspecies), and some other VOCs were emitted in both subspecies but in different quantities. Experimental studies on bees have convincingly demonstrated that variation in floral scent among flowers affects the pollinator learning ability (Wright and Smith, 2004; Wright et al., 2009). Here we showed remarkably constant differences of floral scent between subspecies, even though we attempted to maximize the range of environmental variation by sampling three populations by subspecies. Such systematic differences between the two subspecies may indicate that the floral scent of wild *A. majus* plants could be a useful learning cue for pollinators. Indeed, because the flower scents of the two *A. majus* subspecies were reproducible across populations, they may convey informative messages to pollinators.

Conservatism in floral scent emissions can be an important mechanism of stabilizing selection. For instance, the strong conservatism of flower scent in *Yucca filamentosa* has been related to an important stabilizing selection by obligate and highly specialized pollinators (Svensson et al., 2005). An even more extreme situation was offered by Mant et al. (2005), who showed that the more stable floral VOCs emissions in *Ophrys* species were the VOCs that were shown to be mimicking the sexual pheromones of the pollinator, *Colletes cunicularius*. Even though *A. majus* does not display such a highly specialized pollination system, if the flower scent is consistently different between the two subspecies then this signal may be used by pollinators to discriminate between the subspecies. Our analysis does confirm that the floral scent signal differs consistently between the two subspecies both in the wild and under greenhouse conditions. This is a necessary and important test of an often made assumption that plants grown under a greenhouse display the same floral scent than wild-grown plants. However here, we did not show that pollinators do indeed use this signal nor did we show that they had an advantage to discriminate among the subspecies. Our result thus raises new questions on the behavior of *A. majus* pollinators, their ability to discriminate between the floral scents of the two subspecies, and the evolutionary significance of this behavior for the plant system. Future experiments would be crucial to explore the role played by these differences on the pollinator behavior.

In the wild, the floral scents of the two subspecies were even more differentiated than under greenhouse conditions. Since we observed no significant population effect for *A. majus* plants grown in greenhouse conditions, we concluded that the two subspecies may differentially respond to this effect of environment in the wild. It would be important to understand the genetic basis for the observed differences between the two subspecies. Crosses may offer a useful insight on this genetic basis (Falconer and MacKay 1996). Even though we have still

no information to discuss the genetic basis of VOC emissions, we showed that six plants of *A. m. striatum* from the same population of Camurac, emitted low quantities of acetophenone and sometimes of benzaldehyde and one explanation would be that they result from introgression with *A. m. pseudomajus* plants. This phenotype was found only in the Camurac population, where a few hybrid phenotypes were also observed over the course of our sampling. It is possible that these plants of *A. m. striatum* may be hybrids, displaying a maternal lineage of *A. m. pseudomajus*. Introgression should thus have noticeable consequences on flower scent variability.

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Table 1: Explanatory factors of the variability of *A. majus* floral VOC emission rates (µg g^{-1}_{DW} hr^{-1}) using a non-parametric MANOVA ("adonis"). The proportion of the explained variance is given by the R^2 and test significance (*P* value) is performed by *F*-tests based on sequential sums of squares from 1000 permutations of the raw data. The dependent factors were environmental conditions (greenhouse or wild), subspecies (*A. m. pseudomajus* or *A. m. striatum*), and populations and their interactions.

	DF	SumsOfSqs	MeanSqs	F.Model	R^2	*P*
Environmental condition	1	1.86E+05	1.86E+05	3.43E+00	0.017	0.045*
Subspecies	1	3.55E+06	3.55E+06	6.54E+01	0.325	0.001 ***
Population	6	2.73E+05	4.55E+04	8.39E-01	0.025	0.572
Environmental condition*subspecies	1	4.01E+04	4.01E+04	7.41E-01	0.004	0.402
Environmental condition*population	4	2.54E+05	6.34E+04	1.17E+00	0.023	0.302
Residuals	122	6.61E+06	5.42E+04		0.606	
Total	135	1.09E+07			1.000	

Table 2: Mean emission rate ($\mu g\ g^{-1}_{DW}\ hr^{-1}$) and standard error (SE) of floral VOC emissions between the two subspecies of *A. majus* sampled in the greenhouse and in the wild. The significance of the differences in VOC emissions between the two subspecies were tested by ANOVAs, NS: not significant and ND: not detected because lower than the threshold of detection estimated at $2 \times 10^{-5}\ \mu g\ g^{-1}_{DW}\ hr^{-1}$.

	In greenhouse		Differences of VOC emissions between subspecies	**In the wild**		Differences of VOC emissions between subspecies
	***A. m. pseudomajus* (n=30)**	***A. m. striatum* (n=30)**		***A. m. pseudomajus* (n=27)**	***A. m. striatum* (n=28)**	
	Mean Emission rate	Mean Emission rate		Mean Emission rate	Mean Emission rate	
Monoterpenes	**106.64 ± 16.49**	**108.98 ± 34.26**		**133.24 ± 22.34**	**41.23 ± 12.45**	
cyclic						
α-pinene	0.71 ± 0.14	0.3 ± 0.07	NS	2.23 ± 0.47	2.79 ± 0.68	NS
β-pinene	0.09 ± 0.03	0.09 ± 0.05	NS	2.46 ± 1.53	0.99 ± 0.32	NS
p-cymene	0.37 ± 0.24	0.37 ± 0.2	NS	2.18 ± 1.48	1.01 ± 0.3	NS
Limonene	1.64 ± 0.69	2.63 ± 0.73	$P < 0.001$ **	3.46 ± 1.53	5.89 ± 0.84	$P < 0.001$ ***
γ-terpinene	0.26 ± 0.15	0.45 ± 0.23	$P < 0.001$ ***	0.65 ± 0.19	0.41 ± 0.18	NS
non-cyclic						
β-myrcene	10.87 ± 1.47	14.9 ± 4.12	NS	30.54 ± 4.74	7.33 ± 1.96	$P < 0.001$ ***
(Z)-β-ocimene	1.87 ± 0.72	1.21 ± 0.3	NS	1.99 ± 0.9	0.22 ± 0.09	NS
(E)-β-ocimene	84.82 ± 11.58	84.68 ± 26.56	NS	85.61 ± 10.83	18.27 ± 6.5	$P < 0.001$ ***
Linalool	ND	ND	NS	ND	2.11 ± 0.82	NS
3,4-dimethyl-2,4,6-octatriene	3.15 ± 0.72	2.17 ± 0.94	NS	1.2 ± 0.22	0.38 ± 0.18	$P < 0.001$ **
(E,E)-2,6-dimethyl-1,3,5,7-octatrene	1.88 ± 0.64	1.71 ± 0.95	NS	1.82 ± 0.28	0.46 ± 0.09	$P < 0.001$ **
irregular						
6-methyl-5-hepten-2-one	0.98 ± 0.11	0.47 ± 0.11	$P < 0.001$ ***	1.1 ± 0.17	1.37 ± 0.49	$P < 0.001$ ***
Benzenoids	**439.47 ± 78.17**	**0.19 ± 0.09**		**321.19 ± 33.3**	**2.3 ± 1.01**	
Benzaldehyde	101 ± 0.1	ND	$P < 0.001$ ***	5.87 ± 3.02	0.05 ± 0.03	$P < 0.001$ ***
Methyl benzoate	2.18 ± 0.94	ND	$P < 0.001$ ***	0.44 ± 0.13	ND	$P < 0.001$ ***
Acetophenone	336.14 ± 77.04	ND	$P < 0.001$ ***	314.45 ± 30	1.52 ± 0.81	$P < 0.001$ ***
Hemimetilene	0.05 ± 0.03	0.05 ± 0.04	NS	ND	ND	NS
Mesitylene	0.10 ± 0.06	0.14 ± 0.05	NS	0.43 ± 0.15	0.73 ± 0.17	NS
Total	**546.11 ± 94.66**	**109.17 ± 34.35**		**454.43 ± 55.64**	**43.53 ± 13.46**	

711 **Table 2**: Continued

	In greenhouse		Differences of VOC emissions between subspecies	In the wild		Differences of VOC emissions between subspecies
	***A. m. pseudomajus* (n=30)**	***A. m. striatum* (n=30)**		***A. m. pseudomajus* (n=27)**	***A. m. striatum* (n=28)**	
	Mean Emission rate	Mean Emission rate		Mean Emission rate	Mean Emission rate	
Fatty acid derivatives	**43.99 ± 14.56**	**44.41 ± 14.4**		**85.22 ± 25.4**	**106.46 ± 46.18**	
Aldehhydes						
2-methyl-propanal	6.95 ± 3.18	5.84 ± 2.35	NS	ND	ND	NS
3-methyl-butanal	5.67 ± 3.99	0.75 ± 0.44	NS	ND	ND	NS
Pentanal	4.35 ± 0.54	5.49 ± 0.78	NS	8.61 ± 1.89	12.59 ± 1.59	NS
(Z)-3-hexenal	0.29 ± 0.09	0.35 ± 0.12	NS	1.64 ± 0.38	3.74 ± 1.97	NS
Hexanal	1.51 ± 0.28	1.68 ± 0.33	NS	8.50 ± 3.62	10.21 ± 3.39	NS
Heptanal	0.93 ± 0.18	1.21 ± 0.31	NS	3.35 ± 0.99	2.06 ± 0.39	NS
Octanal	1.57 ± 0.23	2.03 ± 0.61	NS	3.61 ± 1.07	2.49 ± 0.54	NS
Nonanal	8.52 ± 1.7	8.00 ± 3.29	NS	21.13 ± 7.56	8.34 ± 1.47	NS
Decanal	1.02 ± 0.32	2.42 ± 1.3	NS	7.63 ± 1.34	2.28 ± 0.72	NS
Alcenes						
1,3,5-cycloheptatriene	1.51 ± 0.41	1.58 ± 0.34	NS	3.91 ± 1.14	4.03 ± 1.65	NS
1-octene	0.42 ± 0.16	0.09 ± 0.04	NS	0.82 ± 0.31	1.28 ± 0.35	NS
2,4-dimethyl-1-heptene	ND	ND	NS	ND	0.29 ± 0.15	NS
1-nonene	ND	ND	NS	2.76 ± 0.93	2.06 ± 0.41	NS
Alkanes						
1,1-diethoxy-ethane	0.17 ± 0.07	0.88 ± 0.46	NS	ND	2.69 ± 1.86	NS
Octane	ND	ND	NS	1.71 ± 0.43	1.32 ± 0.23	NS
2,4-dimethyl-heptane	ND	ND	NS	ND	0.06 ± 0.06	NS
Nonane	0.52 ± 0.16	0.86 ± 0.29	NS	4.30 ± 1.32	1.96 ± 0.34	NS
Decane	0.55 ± 0.22	0.22 ± 0.08	NS	2.22 ± 0.62	0.63 ± 0.10	NS
Undecane	ND	ND	NS	3.71 ± 0.71	0.97 ± 0.20	NS
Dodecane	1.13 ± 0.42	1.18 ± 0.56	NS	10.88 ± 2.86	0.38 ± 0.08	$P < 0.001$ ***

712

713 **Table 2**: Continued

	In greenhouse		Differences of VOC emissions between subspecies	**In the wild**		Differences of VOC emissions between subspecies
	***A. m. pseudomajus* (n=30)**	***A. m. striatum* (n=30)**		***A. m. pseudomajus* (n=27)**	***A. m. striatum* (n=28)**	
	Mean Emission rate	Mean Emission rate		Mean Emission rate	Mean Emission rate	
Alcohols						
1-pentanol	0.4 0± 0.09	0.35 ± 0.11	NS	ND	1.46 ± 0.62	NS
Esters						
Ethyl acetate	0.04 ± 0.03	0.18 ± 0.11	NS	ND	ND	NS
Methyl ester, 2-methyl, butanoic acid	ND	ND	NS	0.44 ± 0.23	0.35 ± 0.17	NS
Hexyl acetate	0.09 ± 0.07	0.49 ± 0.36	NS	ND	2.97 ± 1.73	NS
Ketones						
2-butanone	8.25 ± 2.33	10.79 ± 2.50	NS	11.12 ± 3.60	43.88 ± 27.98	NS
Ether cyclic						
Eucalyptol	0.10 ± 0.09	0.02 ± 0.02	NS	ND	0.42 ± 0.18	NS
Nitrogen-containing compounds	**1.85 ± 0.50**	**1.26 ± 0.70**		**ND**	**ND**	
syn-3-methyl-butyl-aldoxime	1.85 ± 0.50	1.26 ± 0.70	NS	ND	ND	NS
Total	**45.84 ± 15.06**	**45.67 ± 15.01**		**85.22 ± 25.4**	**106.46 ± 46.18**	

Table 3: Explanatory factors of floral scent variability in the wild-grown *Antirrhinum majus* populations Subspecies and population effects were included in a non-parametric MANOVA, together with three meteorological variables (temperature, PAR, and relative humidity (%)). The proportion of the explained variance is given by the R^2 and test significance (*P* value) was performed using *F*-tests based on sequential sums of squares from 1000 permutations of the raw data. The dependent factors were subspecies (*A. m. pseudomajus* or *A. m. striatum*), population, temperature, PAR, and relative humidity.

	DF	SumsOfSqs	MeanSqs	F.Model	R^2	*P*
Subspecies	1	1.45E+06	1.45E+06	54.827	0.491	0.001 ***
Population	5	2.43E+05	6.08E+04	2.300	0.082	0.039 *
Temperature	1	3.05E+03	3.05E+03	0.116	0.001	0.928
PAR	1	3.14E+04	3.14E+04	1.187	0.011	0.300
Relative humidity	1	1.04E+04	1.04E+04	0.394	0.004	0.692
Residuals	46	1.22E+06	2.64E+04		0.412	
Total	54	2.95E+06			1.000	

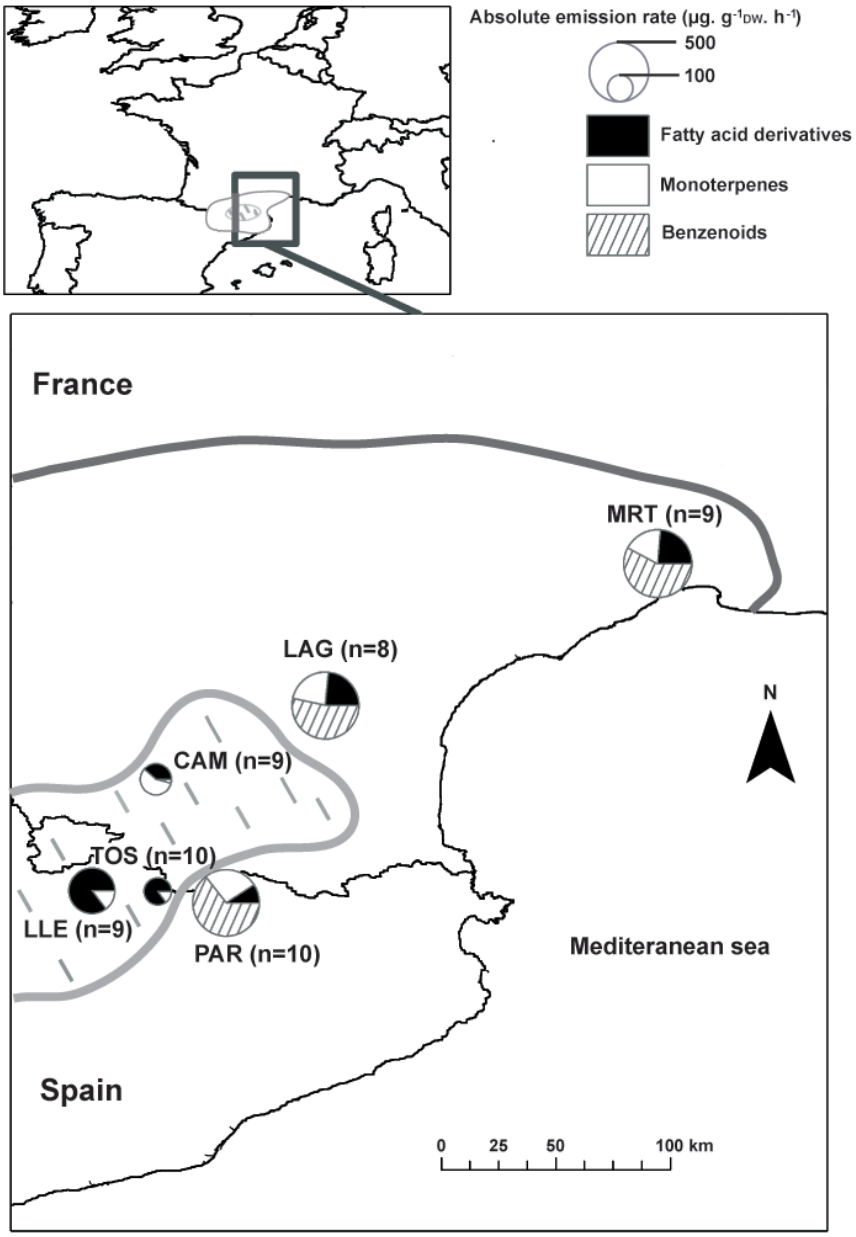

Figure 1: Map of the six sampled populations of the two subspecies of *Antirrhinum majus* in the Eastern Pyrenees. Circle diameter is proportional to absolute emission rates (in µg g^{-1}_{DW} hr^{-1}), and color pies show three main groups of VOCs (black: fatty acid derivatives, white: monoterpenes, shaded: benzenoids). The distribution area of *A. m. striatum* (hatched zone) is enclosed within the area of *A. m. pseudomajus*. The abbreviations of populations correspond to population locations: MRT, Le Martinet (France, 43.64N, 3.89E, 40m asl), LAG, Lagrasse (France, 43.08N, 2.58E, 149m asl), PAR, Pardines (Spain, 42.31N, 2.19E, 1118m asl), CAM, Camurac (France, 42.28N, 1.55E, 1241m asl), LLE, Lles (Spain, 42.36N, 1.66E, 960m asl), and TOS, Toses (Spain, 42.36N, 1.92E, 1492m asl).

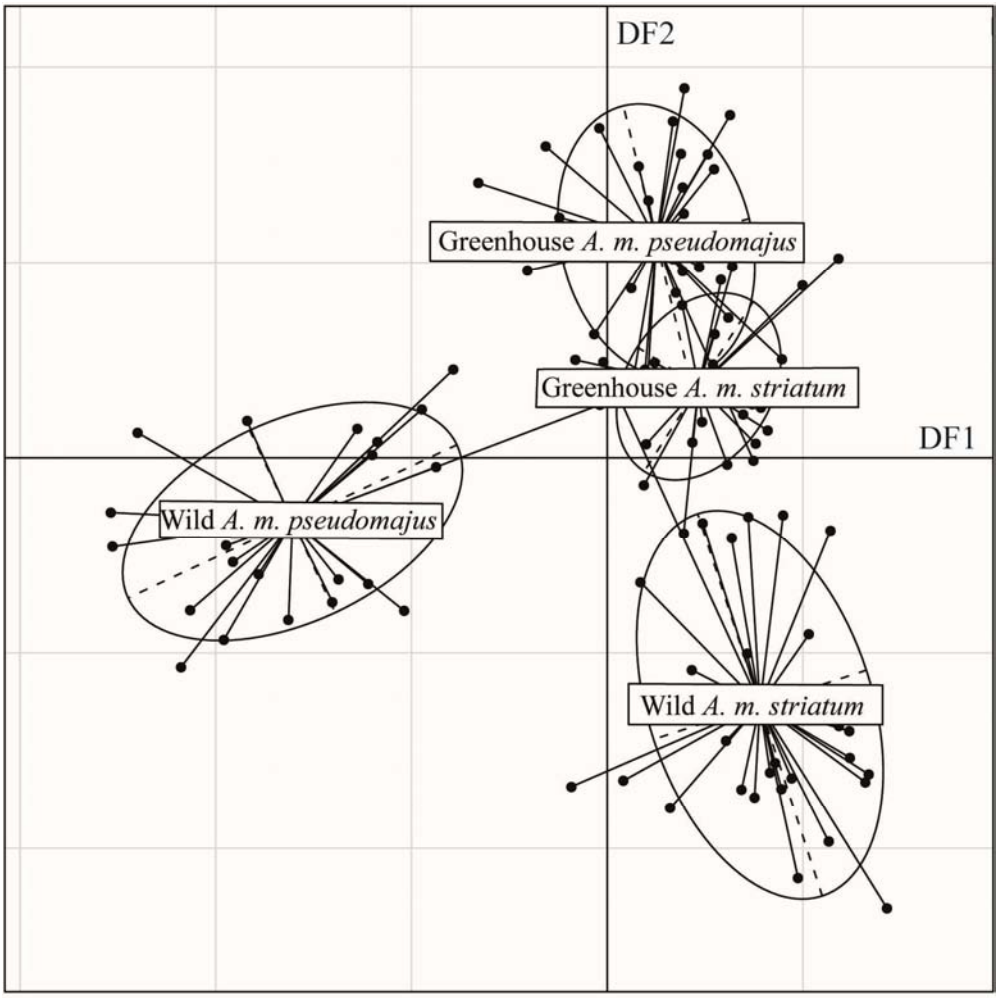

Figure 2: Discriminant function analysis (DFA) based on the floral scent with the subspecies defined as discriminant factor. The first discriminant function (DF1) represents 44% of the the total variability and the secund discriminant function (DF2) 36%. DF1 discriminates the two groups of plants from the wild that belong to the two *A. majus* subspecies. DF2 discriminates the floral VOCs emission of plants from greenhouse and from the wild.

Appendix 1: Coefficients of correlation of the 44 VOCs with the two first discriminant functions (DF1 and DF2, 0.83 and 0.77 their respective eigen values) based on the floral VOC emissions of *A. majus.* Here, they are listed by a decreasing order of the absolute value of the DF2 coefficients.

	DF1	DF2
α-pinene	-0.13	-0.49
Octane	-0.33	-0.49
Pentanal	0.02	-0.47
Mesitylene	0.00	-0.42
3,4-dimethyl-2,4,6-octatriene	0.04	0.41
1-nonene	-0.28	-0.39
syn-3-methyl-butyl-aldoxime	0.14	0.38
Acetophenone	-0.45	0.35
Limonene	0.06	-0.34
Undecane	-0.62	-0.32
Hexanal	-0.09	-0.32
(E)-β-ocime	-0.19	0.32
1-octene	-0.02	-0.31
2-methyl-propanal	0.12	0.29
2,4-dimethyl-1-heptene	0.16	-0.28
(Z)-3-hexenal	0.03	-0.28
Methyl benzoate	-0.02	0.28
Methyl ester, 2-methyl, butanoic acid	-0.16	-0.25
Nonane	-0.36	-0.24
Eucalyptol	0.19	-0.24
Hexyl acetate	0.16	-0.24
Heptanal	-0.28	-0.23
1-pentanol	0.24	-0.23
3-methyl-butanal	0.07	0.23
1,3,5-cycloheptatriene	-0.09	-0.22
(E,E)-2,6-dimethyl-1,3,5,7-octatrene	-0.11	0.20
(Z)-β-ocime	-0.16	0.19
Decanal	-0.45	-0.18
Hemimetilene	0.08	0.17
2,4-dimethyl-heptane	0.09	-0.15
β-pinene	-0.22	-0.15
Octanal	-0.18	-0.15
Decane	-0.43	-0.14
Ethyl acetate	0.11	0.13
p-cymene	-0.17	-0.11
γ-terpinene	-0.13	-0.09
Dodecane	-0.55	-0.07
Nonanal	-0.27	-0.06
Benzaldehyde	-0.33	-0.03
β-myrcene	-0.49	-0.01

Article 2

Variation of floral scent in F_1 and F_2 hybrids of two wild snapdragon subspecies (*Antirrhinum majus*)

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In preparation

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Abstract

The study of floral trait variation in hybrids provides clues on the genetic basis of these floral traits. In this study, we explored patterns of floral volatile organic compound (VOC) emissions in the hybrids of two wild subspecies of *Antirrhinum majus* with a clearly distinct phenotype for both their floral color and scent. Previous research has shown that the magenta flowers of *A. m. pseudomajus* emit consistently different blends of VOCs than the yellow flowers of *A. m. striatum*, especially due to three benzenoids (acetophenone, benzaldehyde and methyl benzoate) present in *A. m. pseudomajus* flowers and absent in *A. m. striatum* flowers. We crossed the subspecies to produce F_1 and F_2 hybrids. Floral VOCs of hybrid lineages were sampled by dynamic headspace method and they were then identified and quantified by GC-MS-FID. We found that F_1 hybrids emitted blends of VOCs that were intermediate between the emissions of the parental lineages. In addition, we found that

benzenoid emissions were influenced by the identity of the maternal lineage. In the F_2 hybrids, some floral VOCs (e.g. methyl benzoate) were over-emitted, leading to transgressive chemical phenotypes. These findings suggest that hybridization induces a break-up of the genetic architecture of *A. majus* parental floral VOC emissions. The potential consequences from pollination efficiency of these changes in flower scent in snapdragon hybrids as compared to parent lines are also discussed.

Key words: hybridization, floral scent, *Antirrhinum majus*, wild snapdragon, pollination

Introduction

In flowering plants, one of the main roles of the flower is the attraction of pollinating insects. Among floral traits such as form and color, flower scent is an important mode of pollinator attraction (Raguso 2008). The intra-specific variation of flower scent emissions has been well documented floral emissions vary with floral sex, circadian rhythm or phenology (Wright and Schiestl 2009). For instance, the daily variation of the intensity of floral emissions is tuned to the time activity of pollinators: the diurnally-pollinated-flowers emit higher abundances of VOCs at daylight (Kolosova et al., 2001, Chess et al., 2008). Also, the composition of the VOCs blends changes in pre and post-mating flowers of the same inflorescence, so as to guide the insects towards unpollinated flowers (Schiestl and Ayasse 2001, Theis and Raguso 2005). Insect pollinators are sensitive to differences of the composition of VOCs but also to changes in the relative ratio of the mixture of VOCs (Wright et al., 2005, Raguso 2008, Stökl et al., 2009). In some species with a large geographical distribution, it was found that the geographical variation in floral scent was low throughout the study area, which may be interpreted as an evidence of a strong selection for floral scent (*Yucca filamentosa*, Svensson et al., 2005, *Y. filamentosa* and *Y. elata* Svensson et al., 2006). In other species, floral scent variation was high across the distribution but did not display a spatial structure (*Magnolia kobus* DC, Azuma et al., 2001, *Geonoma macrostachys* Knudsen et al., 2002). In such cases, it is likely that this variation is related to another mechanism than pollination per se.

The components of the floral scents that do play a role in pollination should have a genetic basis. Hence, much of the above problem amounts to quantifying the degree of genetic versus environmental determinism for a complex suite of quantitative traits. Statistical methods in quantitative genetics help disentangle these effects on a quantitative character (Roff 1992,

Lynch and Walsh 1998). Quantitative genetic suggests that when two populations are crossed, the hybrids lineages show either intermediate traits, more likely in F_1, or segregated traits (also called transgressive, the opposite of regressive), more expected in F_2 (Rieseberg et al., 1999). The latter transgressive phenotypes are best understood as extreme phenotypes relative to the parental lines (Rieseberg et al., 1999). Such studies of the nature of hybrid traits rely on crossing experiments, and these have seldom been conducted on complex traits such as flower scent. The scent of F_1 crosses in three inbred horticultural cultivars of *Anthurium armeniense* was quantitatively intermediate between parental profiles and it presented both parental compounds (Kuanprasert et al., 1998). However if transgressive segregation occurs for some VOCs in hybrids, then hybridization may yield an altogether new flower scent fostering niche divergence (Rieseberg et al., 1999). Another example is offered by *Clarkia breweri*, that emits high abundance of floral linalool, while *Clarkia concinna* emits only traces of floral linalool. In interspecific hybrids of the two species, the *LIS* allele from *C. breweri* is dominant over the *LIS* allele from *C. concinna* in F_1 hybrids but transgressive emissions of linalool are observed in F_2 hybrids (Raguso and Pichersky 1999).

In the present work, we endeavor to study the profiles of flower scent in the hybrids formed from two subspecies of wild snapdragon populations (*Antirrhinum majus*). These subspecies display two clearly distinct phenotypes in the wild, characterized by a floral scent-color association. One magenta-flowered subspecies, *A. m. pseudomajus*, emits three floral benzenoids not detected in the yellow-flowered subspecies, *A. m. striatum* (Suchet et al., in press). These subspecies, upon hybridization, display a wide array of floral color. In a hybrid zone between the two subspecies, Whibley et al. (2006) have evidenced abrupt clines for the parental floral colors and for one locus coding for the magenta pigment, strongly suggesting that hybrids are counter-selected. Tastard et al. (2008) showed that the variation in flower

color is visually perceived by pollinators and that artificial parental colored flowers are more visited than hybrid phenotypes (Andalo et al., submitted). However, the mechanism for the counter-selection of the hybrids remains poorly understood. Flower scent might be a necessary dimension of the floral phenotype to understand the maintenance of subspecies in this wild snapdragon species.

Here, we characterize the floral scent of the F_1 and F_2 hybrids of the two subspecies of *A. majus* reared in greenhouse. Our questions are the following (1) Can flower scents of hybrids be discriminated of parental chemical profiles? (2) If so, can we evidence any pattern of segregation in floral scent profiles between parents and hybrids? (3) Can one detect a maternal effect in the floral emissions of hybrids?

Material and Method

Plant model

In the Pyrenees Mountains between France and Spain, the wild snapdragon (*Antirrhnium majus* L. Scrophulariaceae) displays two subspecies phenotypes. The flowers of this species are strictly self-incompatible and insect pollinated. Further, there is no post-zygotic barrier between these two subspecies (Andalo et al., 2010). Among the three benzenoid compounds only emitted by the magenta flowering subspecies, one of them, acetophenone, is the most abundant compound in *A. m. pseudomajus* and it is also innately aversive for the most frequent pollinator species in the wild, *Bombus terrestris* (Suchet et al., in press). Several contact zones among the non-overlapping distribution areas of the two subspecies are known, but only few sites produce hybrids. One particular hybrid zone has been studied because its hybrids display a striking diversity of floral colors reflecting a high level of crosses among

hybrids themselves (Whibley et al., 2006). The color of the flower is correlated with the level of crosses: plants with pink upper lobe and yellow lower lobe are often the first crosses (F_1) of the two parental lines. Orange and white corollas come from successive crosses between hybrids lineages, they are transgressive phenotypes. Finally, the corollas mainly pigmented of either yellow or magenta are the result of backcrosses between the F_1 hybrids and the respective phenotypes of parents (Whibley et al., 2006).

Plant material

Between 2000 and 2006, seeds were collected from seven wild parental locations (Table 1). From this field census, we selected 10 seeds from four populations of *A. m. pseudomajus* (Np=40), and from three populations of *A. m. striatum* (Ns=30, Table 1). All of these seeds were grown from wild stock, and three of them were manually crossed within populations so these populations can be more reliable considered as pure lineages (TOS, PAR, PRE, Table 1). One population of *A. m. striatum* from Pomas, France was initially included in our experimental design but germination showed a low rate of success, and it was subsequently discarded. The F_1 hybrids were produced either from pollen of *A. m. striatum* (F_1-a, n=8) or from pollen of *A. m. pseudomajus* (F_1-b, n=12). All possible combinations of crosses between F1-a and F1-b hybrids resulted in 4 lineages of F_2 hybrids (see figure 1). For F_2 hybrid of the category F_2-c the fructification and the germination rate were too low. All seeds were grown under similar greenhouse conditions (16 h/day of light, at 25°C in average temperature, in individual pots with universal compost and with no extra nutrients) between November 2008 and July 2009.

Sampling and analyses of floral VOC emissions

Scent sampling was carried out between February and July 2009. The floral emission measurements and the analyses of VOCs were performed based on the method detailed in Suchet et al. (in press) summarized as follows. A special care was paid to sample floral scents in standardized conditions. Sampling was conducted during the peak of emission intensity, that is, between 11 am and 4 pm. To minimize biases due to flower development (Dudareva et al., 2000, 2003, Goodwin et al., 2003), the VOCs were collected by dynamic headspace when the inflorescence had at least four open flowers with dehiscent anthers. The dry weight of inflorescences was measured to normalize the emission rates of floral VOCs because inflorescence weights were positively correlated to the absolute VOC quantities. To identify and quantify the sampled VOCs, we performed analyses using gas chromatography coupled with a mass spectrometer (GC-MS Clarus 500, Perkin Elmer) and a flame ionization detector (FID, Clarus 500, Perkin Elmer). Absolute emission intensity was based on external standard calibration. We finally computed the emission rates (µg. g (dry flowers weight)$^{-1}$. h^{-1}) using the method of Sabillon and Cremades (2001).

Statistical analyses

In each sample, compounds with relative amount less than at 0.01% of the total were excluded. All analyzes were carried out with the R statistical software (http://cran.r-project.org/).

ANOVA was applied to determine if the four taxa, two parents and the two first generations of hybrid differed in absolute emission rate.

A discriminant function analysis (DFA) based on VOC emission rate was performed to determine which VOCs discriminate among the four taxa (the two subspecies plus the two generations of hybrids) and to detect a signature of these taxa based on the flower scent.

Finally we tested whether the variances of the emission rates per VOC differed between $\mathbf{F_1}$ hybrids, $\mathbf{F_2}$ hybrids and pooled-parents (**P**). To this end, we used generalized linear models (GLM) based on log-transformed data. These analyses detect a break-up of VOCs emissions caused by hybridization. It is the case when one VOC is under or over-emitted in $\mathbf{F_1}$ or $\mathbf{F_2}$. We carried out these analyses only if the VOC was detected in at least a third of the plants sampled in each of the three genetic groups (**P**, $\mathbf{F_1}$ and $\mathbf{F_2}$). We tested the effect of two factors, the genetic group, and the lineage, the latter being a test of the maternal effect. The lineage factor was nested within the genetic group factor because it is differentially defined among genetic groups. In the parental lineages, it corresponds to the two subspecies (*A. m. pseudomajus* and *A. m. striatum*), whereas in hybrids it corresponds to the type of cross in $\mathbf{F_1}$ and in $\mathbf{F_2}$ (see Figure 1).

Results

Floral scent differentiation in the F_1 and F_2 hybrids and their parents

Parents and F_1 hybrids had the same chemical diversity but VOC compositions of F_1 were more variable within them than within parental lines. Quantitatively, the VOC emissions of F_1 hybrids did not differ from those the two subspecies (Table 2) because they were intermediate as the absolute emission rate (Figure 2). Among the VOCs that discriminated *A. m. pseudomajus* to *A. m. striatum*, acetophenone was always found in F_1 hybrids but it was emitted twice less than in *A. m. pseudomajus* on average. Benzaldehyde was also always

present in F_1 hybrids in similar amount than in its parent whereas methyl benzoate had an occurrence of 70% but with a more than twice emission rate than in *A. m. pseudomajus* (Table 2). A global analysis of the dataset was conducted using the discriminant function analysis (DFA). In the DFA, F_1 hybrids and *A. m. pseudomajus* overlapped (Figure 3). One F_1 hybrid was assigned in *A. m. pseudomajus* and another in *A. m. striatum*. In total, 90% of the F_1 hybrids were assigned to their group.

F_2 hybrids emitted five VOCs not detected in the parental lineages: four FADs and linalool, a monoterpene (compounds marked by an asterisk in Table 2). The floral scent of F_2 hybrids was quantitatively over-expressed for a number of VOCs compared to that of the parental lines (Table 2). Figure 2 reports the comparisons: the absolute emission rates of F_2 hybrids were significantly higher than in *A. m. striatum* and than in F_1 hybrids and they were marginally higher than in *A. m. pseudomajus*. The high emission rate of F_2 hybrids was mainly due to an over emission of fatty acid derivatives and of some monoterpenes (Table 2 and Figure 4). The five compounds detected only in the F_2 hybrids did not have a significant weight in the DFA (Figure 3, Appendix 1). Indeed, it may be that linalool was detected in F_2 emissions because it passed the detection threshold in F_2 hybrids but not in parents. They therefore do not represent discriminant compounds between F_2 to parents. The cluster of F_2 hybrids was well discriminated by DFA and 96.5% of them were assigned to their group.

Segregation in floral scent profiles between parents and hybrids

To test if segregation patterns occur in VOC emissions, we compared the variances among parental lines, F_1 hybrids and F_2 hybrids for n=27 VOCs, with the null expectation that all VOCs were emitted in same quantities in these three groups (Table 3). For only two VOCs the emission rate differed between the two parental lineages (6-methyl-5-hepten-2-one and limonene) and they show significant lineage effects in F_2 hybrids. This suggests that there is a

genetic determinism to the difference between parents on these two traits. In the remaining 25 VOCs, 10 were emitted in similar amounts among groups (i.e. no over-expression). Two VOCs, (Z)-3-hexenal and syn-methyl-butyl-aldoxime, had similar emission rate among F_1 hybrids and parents, but were absent in F_2 hybrids. Finally, the F_2 over-emitted thirteen VOCs (Table 3). There is a trend of heritability patterns according to the class of VOC. Most of the segregated VOCs emission were fatty-acid-derivatives (mainly alkanes and alcenes), except one benzenoid, methyl benzoate. The constant VOC emission rates among genetic groups are mainly represented by monoterpenes and aldehydes and correspond to the most abundant compounds in parental lines.

Maternal effect

The F_1 hybrids with a maternal lineage from *A. m. pseudomajus* (F_1-a) emitted on average twice more benzenoids than the F_1 hybrids with a maternal lineage from *A. m. striatum* (F_1-b; 227 ± 54 and 94 ± 12 $\mu g\ g^{-1}_{DW}\ h^{-1}$ respectively, Figure 4), suggesting that there is indeed a maternal effect in the expression of benzenoids. The three lineages of F_2 hybrids had different absolute emission rates (F_2-a 480 ± 129, F_2-b 604 ± 189, F_2-d 705 ± 147 $\mu g\ g^{-1}_{DW}\ h^{-1}$) that did not significantly differ among them because there is a high variability of chemical emission among and within each lineage (Figure 4).

Discussion

By studying the profiles of floral VOC emissions in F_1 and F_2 hybrids resulting from crosses of two subspecies of *Antirrhinum majus*, we showed that hybridization alters the snapdragon floral scent. The emissions of floral VOCs in hybrids were much more variable than the two floral scents of parental snapdragon subspecies shown to be reproducible across

sites and populations (Suchet et al., submitted). New floral VOCs were detected in F_2 hybrids but they did not discriminate with F_1 and parents. We observed qualitatively and quantitatively intermediate flower scent profiles in F_1 hybrids. However, there was a clear difference with the floral scent of F_2 hybrids, which segregated, compared to the two subspecies of *A. majus*. These findings point to interesting research avenues in the study of the genetic basis of the floral VOC biosynthesis. This holds especially for benzenoids for which the biosynthetic pathways remain poorly known. They also allow discussing of the adaptive value of the floral scent in the snapdragon.

Patterns of VOC emission under hybridization and biosynthesis implications

Variation in volatile compound emissions in plant crosses surveys under controlled conditions are mostly due to changes of biosynthetic activity due to genomic reorganization induced by hybridization. The wild snapdragon emitted monoterpenes including (E)-β-ocimene, β-myrcene and limonene, the three most abundant monoterpenes. They are synthesized by the methyl-erythritol-phosphate (MEP) pathway (Dudareva et al., 2006). Their emission rates were constant among hybrid lineages and subspecies, except for α-pinene. The regulation of monoterpenes production was therefore maintained even after hybridization. Extensive past hybridization is apparent from phylogenetic studies among species of the *Antirrhinum* genus (Vargas et al., 2009). In spite of this high level of hybridization, the production of monoterpenes is constant, then one may suspect strong selective pressures on monoterpene functions, hence a direct selective advantage. For example, Mant et al. (2005) evidenced that the more stable floral VOCs emissions in *Ophrys* species were those with an important ecological function since they mimic the sexual pheromones of the pollinator, *Colletes cunicularius.* In fact, the three previous cited monoterpenes have been shown to be

attractive compounds for *Bombus terrestris* one main flower visitor of *A. majus* (Suchet et al. in press).

In contrast, ten fatty acid derivatives, which are synthesized by the lipoxygenase pathway (Dudareva et al., 2006), were significantly over-emitted in F_2 hybrids compared with the parent lines. Hybridization has therefore an important effect on the emissions of FADs. Certain hybrid phenotypes such as those with white and orange flowers have been shown to be counter-selected in the hybrid zone (Whibley et al., 2006, Andalo et al., unpublished). To test if the flower scent is implicated on this phenomenon it would be interesting to focus on FADs and test if pollinators can detect them. New olfactometry tests with pollinators having the choice among hybrids and parental flower scent coupled with an EAD-GC study would also be a good test to know if the new chemical phenotypes of hybrids influence the attraction of pollinators.

Variation in benzenoids emissions is particularly interesting in our study because they represent the VOCs discriminating between the two wild subspecies snapdragon, and because the biosynthetic pathways have not been entirely discovered (Vogt 2010). Indeed, benzaldehyde, methyl benzoate and acetophenone were only detected in *A. m. pseudomajus* with acetophenone as the most abundant compound of the floral VOC blend (Suchet et al., in press). Benzaldehyde and methyl benzoate are known to be synthesized by the benzenoic acid pathway from phenylalanine (Dudareva et al., 2006, Long et al., 2009, Van Moerkercke et al., 2009). Methyl benzoate is the product of the last enzymatic steps of the benzenoic acid pathway, whereas benzaldehyde is synthesized by a basal enzymatic and it is the precursor of benzenoic acid, implicated in various plant functions (Long et al., 2009). Here hybridization has no effect on the emissions of benzaldehyde since they were constant among hybrids and

snapdragon subspecies, and all hybrids emitted it even in F_1 hybrids. In contrast, hybridization led to an over-emission of methyl benzoate in 29% of F_1 and 79% of F_2 hybrids. This is congruent with what was observed in some snapdragon cultivars such as the Marylan True Pink that emit methyl benzoate in high amounts (Dudareva et al., 2000, Wright et al., 2005), while it is detected as traces in the wild species (Suchet et al., in press). With FADs, methyl benzoate represents a good candidate in a possible role of VOCs in the counter-selection of hybrids. Methyl benzoate is known to be pheromone compounds in scarab beetles causing pests (Leal 1998) or floral scent compounds in the genus *Protea* that attract pollinating beetles (SL Steenhuisen, pers. comm.). In *A. majus*, it might be implicated in the specific relationship with the fruit parasite weevil, *Rhinusa vestita.*

Emissions of acetophenone in hybrids, the main compound in floral scent of *A. m. pseudomajus* were ubiquitous but never reached the same emission rate as in the parental line. We are altogether lacking information about the biosynthesis of acetophenone in plants. Variation of emission rate of this benzenoid differed than those of benzaldehyde and methyl benzoate, suggesting that the biosynthesis of acetophenone is likely not part of the same pathway. Nevertheless as the presence-absence of the three benzenoids co-occurs it would seem that their co-productions are constrained. Moreover, the fact that F_1 hybrids with a maternal lineage from *A. m. pseudomajus* emitted twice more benzenoids suggests that the cytosolic material is implicated in the benzenoid emission. Wild *A. majus* is the only known species to emit such high amount of acetophenone compared to the 22 species or horticultural cultivars known to also emit acetophenone (Knudsen et al., 2006; Jürgens et al., 2006). Snapdragon therefore represents the ideal model species to discover the biosynthetic pathway of acetophenone since various hybrid lineages, including backcrosses, are already available.

Hybridization, the way to smell different: a potential impact on pollinator choice?

Variation in flower scent under hybridization has been studied in the context of species boundaries to determine whether this trait can be implicated in a pre-zygotic barrier of reproductively isolated species. The bulk of knowledge on the topic is provided by deceptive orchids that mimic pheromones of solitary bees to ensure their reproduction. In these highly specialized pollination system, the floral scent is central and under strong selection. However different patterns of the effect of hybridization on flower scent profiles have been observed. For example, F_1 hybrids of *Ophrys arachnitiformis* and *O. lupercalis* produce a blend of VOCs in different ratios and with new compounds than parents that are less attractive for pollinators of their progenitors but more attractive for certain new pollinators (Vereecken et al., 2010). In contrast, F_1 hybrids of *Orchis masculata* and *O. pauciflora* emit intermediate and very variable flower scents despite a clear differentiation of parental flower scent (Salzmann et al., 2007). Such intra-specific polymorphism is advantageous in this deceptive system of pollination because pollinators cannot learn to associate the floral lure with a specific chemical signal.

In *Antirrhinum majus* a variable profile of hybrid VOC emission should not be advantageous because the flowers are providing a reward. Flower scent is therefore probably used to favor the fidelity of pollinator by emitting a consistent flower scent. The fact that hybrids cannot be chemically assigned contrary to the parental subspecies could be disturbing for pollination in a hybrid zone and contribute to the counter-selection of hybrid phenotypes. The profile of flower scent in F_2 hybrids found here describe the four known mechanisms by which flower odor learning contributes to the floral constancy during the pollinator foraging activity (Raguso 2008). First, the flowers of F_2 hybrids may be more strongly scented than parents. Even though we do not know if a higher concentration of the same scent is perceived

as a different stimulus, it has been evidenced that the same scent may lead to different spatial patterns of neural representation at different concentrations (Sachse and Galizia 2003). Second, the relative ratio of compounds may differ between parents and F_2 hybrids because some chemicals segregate. It is well known that ratio of VOCs make the informative message for insects (e.g. pheromones). For example, Wright et al. (2005) showed that honey bees can discriminate four snapdragon (*Antirrhinum majus*) cultivars that share the same chemical composition but differ in compound ratios. Third, F_2 hybrids emit additional compounds compared with the parental blends of volatile chemicals. Finally, F_2 hybrids may combine these three differences of flower scent with a difference in flower color. It is known that bumblebees (*Bombus impatiens*) are more constant and learn more quickly color or shape of flowers when these flowers are scented (Gegear and Laverty 2005; Kulahci et al., 2008). All these phenotypic features suggest that the differences of flower scent in hybrids compared to the two subspecies *A. m. pseudomajus* and *A. m. striatum* should influence the choice of the pollinators and may lead to a constancy of visitation between hybrid and parental phenotypes. However bio-assays and natural observations of snapdragon pollination in the hybrid zone are still needed to determine the impact of this multimodal floral signal on the pollinator behavior.

Conclusion

This study shows that hybridization leads to a wide array of hybrid floral scents that contrast with the constancy of the parental chemical phenotypes. It reinforces the idea that chemical floral trait coupled with floral color may play a role in a putative reproductive isolation of the two *A. majus* subspecies. Even though the two *A. majus* subspecies can exchange genes, pollinators might be influenced by the phenotypic differences in snapdragon

and the constancy of visits towards each parent is more likely to happens than towards hybrids. We hope to be able to test this hypothesis directly in the field in the future.

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Table 1: Origin and sample size of the parental seeds growing into the greenhouse. The origin of seeds states "pure wild lineages" result of greenhouse crosses between plants from the same wild populations.

Population code name	Subspecies	Origin of seeds	Locality	Elevation (m a.s.l.)	GPS coordinates	Isolation status	n
TOS	*A. m. striatum*	Pure wild lineages	Collada de Toses, Spain	1492	42.36 N, 1.92 E	Parapatric, hybrid zone	10
LLE	*A. m. striatum*	Wild lineages	Lles de Cerdanya, Spain	960	42.36 N, 1.66 E	Parapatric,	10
CAM	*A. m. striatum*	Wild lineages	Camurac, France	1241	42.48 N, 1.55 E	Parapatric, contact zone	10
PAR	*A. m. pseudomajus*	Pure wild lineages	Pardines, Spain	1118	42.31 N, 2.19 E	Parapatric, hybrid zone	10
LAG	*A. m. pseudomajus*	Wild lineages	Lagrasse, France	149	43.08N, 2.58 E	Parapatric	10
PRE	*A. m. pseudomajus*	Pure wild lineages	La Preste, France	1214	42.40 N, 2.39E	Parapatric	10
MRT	*A. m. pseudomajus*	Wild lineages	Le Martinet, France	40	43.64 N, 3.89 E	Allopatric	10

Table 2: Occurrence (O) and mean emission rate (µg. g^{-1}_{DW}. h^{-1}) of the volatiles in flower scent of *A. m. pseudomajus*, *A. m. striatum*, F_1 and F_2 hybrids in greenhouse conditions (* marked the VOCs only detected in F_2 hybrids).

	***A. m. pseudomajus* (n=40)**		***A. m. striatum* (n=30)**		**F_1 hybrid (n=20)**		**F_2 hybrid (n=29)**	
	O	Emission rate µg g^{-1}_{DW} h^{-1}	O	Emission rate µg g^{-1}_{DW} h^{-1}	O	Emission rate µg g^{-1}_{DW} h^{-1}	O	Emission rate µg g^{-1}_{DW} h^{-1}
Fatty acid derivatives		**47.16 ± 15.31**		**45.25 ± 14.68**		**29.56 ± 8.66**		**162.84 ± 44.35**
Aldehhydes								
2-methyl-propanal	25	11.31 ± 6.42	47	5.84 ± 2.35	15	1.21 ± 1.02	0	0
3-methyl-butanal	33	4.85 ± 2.78	20	2.22 ± 1.57	55	2.10 ± 1.02	0	0
Pentanal	100	4.60 ± 0.49	100	5.71 ± 0.75	100	9.23 ± 3.40	100	13.39 ± 3.52
(Z)-3-hexenal	65	0.21 ± 0.06	83	0.33 ± 0.11	60	0.06 ± 0.01	0	0
Hexanal	100	1.33 ± 0.20	100	1.88 ± 0.36	100	0.73 ± 0.07	100	3.20 ± 1.31
Heptanal	100	0.97 ± 0.17	97	1.19 ± 0.28	100	0.65 ± 0.17	100	1.89 ± 0.46
Octanal	100	1.45 ± 0.17	83	2.10 ± 0.57	100	1.21 ± 0.09	100	3.97 ± 1.42
Nonanal	100	9.68 ± 1.68	50	7.20 ± 2.99	100	5.88 ± 1.05	100	60.05 ± 13.26
Decanal	58	0.99 ± 0.25	37	2.24 ± 1.17	50	0.55 ± 0.25	100	13.81 ± 1.99
Alcenes								
1,3,5-cycloheptatriene	100	1.31 ± 0.30	100	1.65 ± 0.32	100	1.06 ± 0.20	97	3.20 ± 0.71
1-octene	68	0.34 ± 0.11	23	0.17 ± 0.09	50	0.12 ± 0.04	86	1.13 ± 0.43
1-nonene*	0	0	0	0	0	0	97	1.97 ± 0.99
2,4-dimethyl-heptene*	0	0	0	0	0	0	24	0.37 ± 0.22
Alkanes								
1,1-diethoxy-ethane	53	0.17 ± 0.05	33	0.84 ± 0.41	65	0.26 ± 0.07	52	7.76 ± 4.35
2,4-dimethyl-heptane*	0	0	0	0	0	0	24	1.13 ± 0.74
Octane*	0	0	0	0	0	0	100	1.12 ± 0.37
Nonane	100	0.48 ± 0.13	97	0.84 ± 0.26	95	0.15 ± 0.04	100	1.19 ± 0.25
Decane	45	0.38 ± 0.15	43	0.22 ± 0.07	50	0.06 ± 0.02	93	0.92 ± 0.18
Dodecane	38	1.04 ± 0.31	37	1.07 ± 0.51	35	0.45 ± 0.21	100	4.91 ± 0.68
Alcohol								
1-pentanol	83	0.58 ± 0.15	50	0.37 ± 0.11	85	0.32 ± 0.06	17	0.64 ± 0.41
Esters								
Ethyl acetate	8	0.03 ± 0.02	13	0.20 ± 0.10	0	0	0	0
Hexyl acetate	8	0.11 ± 0.07	20	0.44 ± 0.32	0	0	0	0
methylester-2-methyl-butanoic acid*	0	0	0	0	50	0.08 ± 0.02	59	0.59 ± 0.22
Ketones								
2-butanone	98	7.26 ± 1.74	100	10.72 ± 2.29	100	5.51 ± 1.12	100	46.51 ± 12.84
Ether cyclic								
Eucalyptol	5	0.07 ± 0.06	3	0.02 ± 0.1	15	0.02 ± 0.01	0	0
Nitrogen-containing compound		**1.90 ± 0.38**		**1.14 ± 0.63**		**2.74 ± 0.58**		**0.59 ± 0.31**
syn-3-methyl-butyl-aldoxime	68	1.90 ± 0.38	33	1.14 ± 0.63	80	2.74 ± 0.58	52	0.59 ± 0.31
Monoterpenes		**101.57 ± 13.39**		**103.67 ± 31.14**		**105.96 ± 15.70**		**165.53 ± 28.85**
Cyclic								

α-pinene	58	0.25 ± 0.10	70	0.33 ± 0.07	95	0.14 ± 0.02	100	1.03 ± 0.24
β-pinene	30	0.06 ± 0.02	27	0.08 ± 0.04	15	0.03 ± 0.02	7	0.08 ± 0.06
para-cymene	8	0.25 ± 0.16	30	0.40 ± 0.18	0	0	45	0.44 ± 0.25
Limonene	95	1.22 ± 0.47	97	2.69 ± 0.69	90	0.70 ± 0.17	100	0.96 ± 0.37
γ-terpene	33	0.20 ± 0.1	20	0.45 ± 0.21	20	0.10 ± 0.08	86	1.46 ± 0.54
Non-cyclic								
β-myrcene	100	12.82 ± 1.58	100	14.27 ± 3.72	100	8.08 ± 1.48	100	13.59 ±3.23
(Z)-β-ocimene	100	1.54 ± 0.49	77	1.47 ± 0.39	100	1.50 ± 0.23	93	0.94 ± 0.21
(E)-β-ocimene	100	78.91 ± 9.22	100	79.99 ± 24.02	100	91.62 ± 12.97	100	97.54 ± 16.27
Linalool*	0	0	0	0	0	0	21	8.95 ± 5.14
2,6-dimethyl-2,4,6-octatriene	75	3.27 ± 0.63	40	1.95 ± 0.85	70	0.88 ± 0.23	100	11.38 ± 1.71
(E,E)-2,6-dimethyl-1,3,5,7-octatriene	48	2.10 ± 0.53	20	1.54 ± 0.86	95	1.85 ± 0.34	100	28.35 ± 0.68
Irregulars								
6-methyl-5-hepten-2-one	100	0.95 ± 0.09	97	0.50 ± 0.11	100	1.06 ± 0.16	97	0.81 ± 0.15
Benzenoids		**339.86 ± 56.24**		**0.18 ± 0.08**		**147.11 ± 26.88**		**284.70 ± 45.97**
Acetophenone	100	337.39 ± 55.44	0	0	100	143.75 ± 25.88	97	271.94 ± 43.09
Benzaldehyde	100	0.88 ± 0.08	0	0	100	0.83 ± 0.09	100	0.75 ± 0.11
Methyl benzoate	20	1.47 ± 0.65	0	0	70	2.50 ± 0.90	62	11.32 ± 2.51
Hemimelitene	15	0.03 ± 0.02	10	0.05 ± 0.04	10	0.03 ± 0.01	0	0
Mesitylene	15	0.09 ± 0.05	27	0.13 ± 0.04	0	0	41	0.69 ± 0.26
Total		**490.49**		**150.24**		**285.37**		**613.66**

Table 3: Variances of VOC emission rates among the two subspecies (**P**) and the **F_1** and **F_2** hybrids (FAD, fatty-acid-derivatives, NS non-significant)

	Genetic group effect		Lineage effect		
	Pattern in **F_1** (**P** vs **F_1**)	Pattern in **F_2** (**P** vs **F_2**)	Difference among subspecies	Difference between **F_1** lineages	Difference among **F_2** lineages
Benzenoids					
Benzaldehyde	NS	NS	absent in *A. m. striatum*	NS	NS
acetophenone	NS	NS	absent in *A. m. striatum*	NS	NS
Methyl benzoate	NS	p<0.001	absent in *A. m. striatum*	NS	NS
FADs					
2-butanone	NS	p<0.001	NS	NS	NS
1,1-diethoxy-ethane	NS	p<0.001	NS	NS	NS
1,3,5-cycloheptatriène	NS	p<0.01	NS	NS	NS
Nonane	NS	p<0.001	NS	NS	NS
Decane	NS	p<0.001	NS	NS	NS
Nonanal	NS	p<0.001	NS	NS	NS
2,6-dimethyl-2,4,6-octatriene	NS	p<0.001	NS	NS	NS
E,E-2,6-dimethyl-1,3,5,7-octatriene	NS	p<0.001	NS	NS	NS
Dodecane	NS	p<0.01	NS	NS	NS
Decanal	NS	p<0.001	NS	NS	NS
1-octene	NS	NS	NS	NS	NS
1-penthanol	NS	NS	NS	NS	NS
cis-3-hexenal	NS	absent in F_2	NS	NS	-
Pentanal	NS	NS	NS	NS	NS
Hexanal	NS	NS	NS	NS	NS
Heptanal	NS	NS	NS	NS	NS
Octanal	NS	NS	NS	NS	NS
N-containing compound					
syn-methyl-butyl-aldoxime	NS	absent in F_2	NS	NS	NS
Monoterpenes					
β-myrcene	NS	NS	NS	NS	NS
cis-β-ocimene	NS	NS	NS	NS	NS
trans-β-ocimene	NS	NS	NS	NS	NS
α-pinene	NS	p<0.001	NS	NS	NS
Limonene	NS	NS	p<0.01	NS	p<0.01
6-methyl-5-hepten-2-one	NS	NS	p<0.001	NS	p<0.01

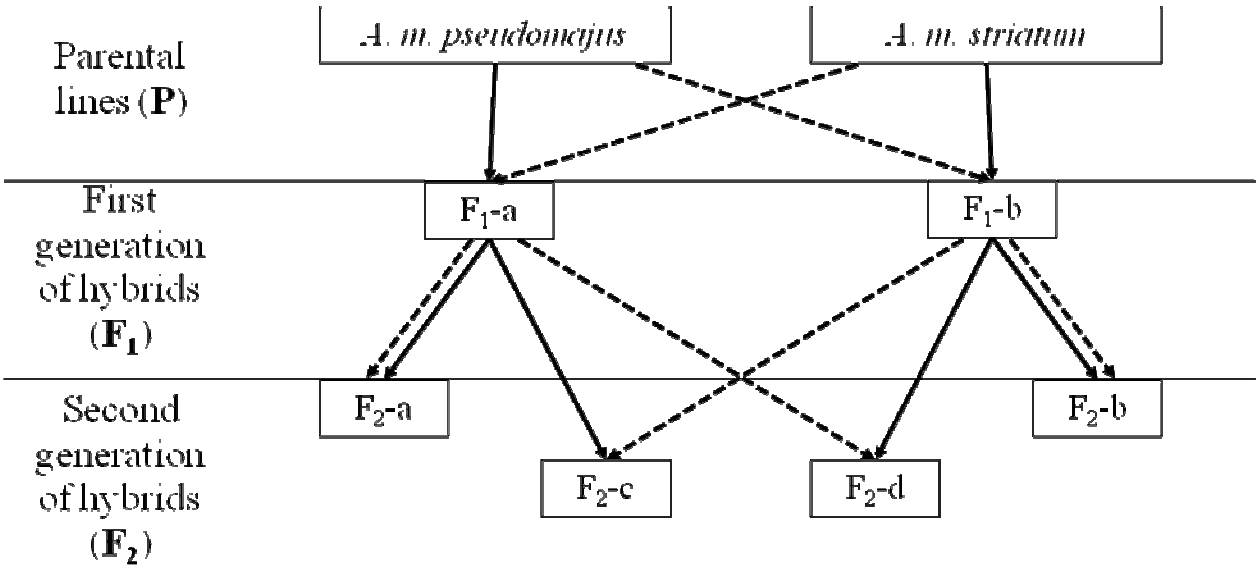

Figure 1: Plan of crosses of the three genetic groups (**P**, **F_1**, **F_2**) sampled for their floral scent. The full arrows represent the maternal lineage and the dotted arrows, the paternal lineage of the crosses. The first generation of hybrids was composed of two lineages: the F_1-a (n=8) with a maternal lineage from *A. m. pseudomajus* and the F_1-b (n=12) with a maternal lineage from *A. m. striatum*. The second generation was composed of four lineages, F_2-a (n=8) the crosses of F_1-a, F_2-b (n=7) the crosses of F_1-b, the F_2-c (n=0) and F_2-d (n=14) the two types of crosses between F_1-a and F_1-b.

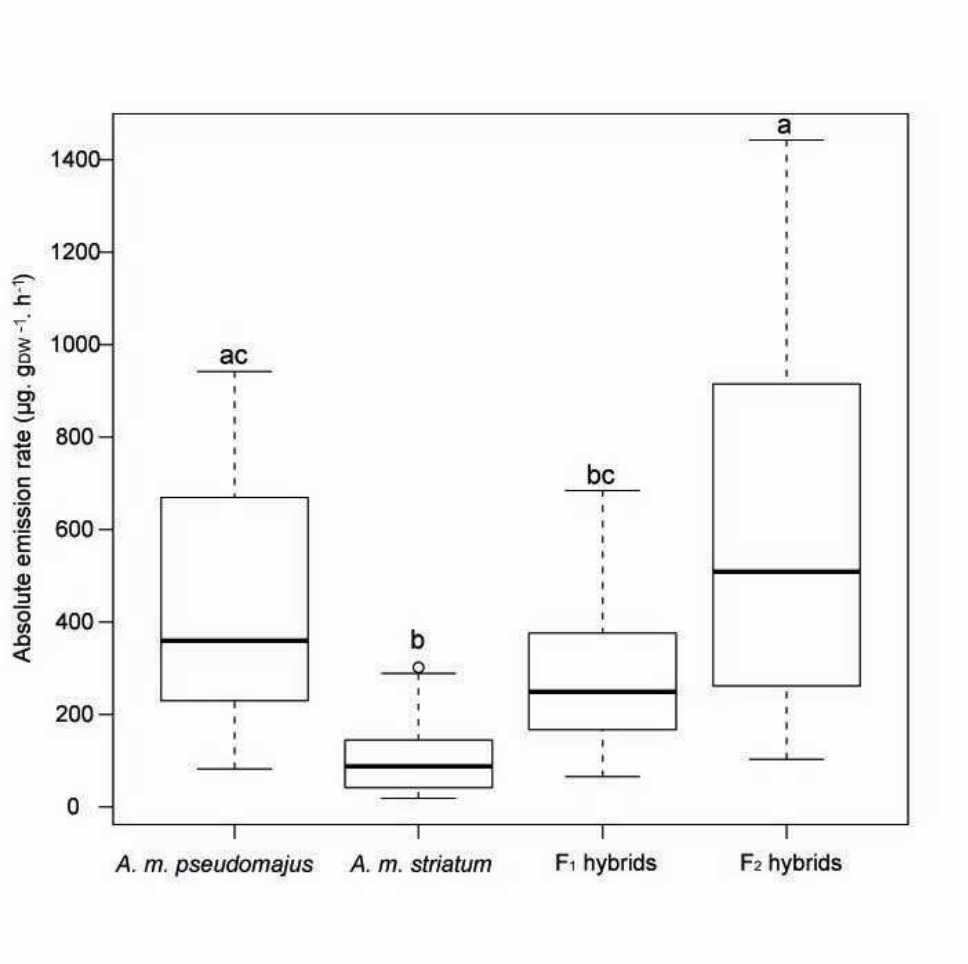

Figure 2: Absolute emission rates (µg. g^{-1}_{DW}. h^{-1}) of parental snapdragon subspecies, *A. m. pseudomajus* and *A. m. striatum*, and their F_1 and F_2 hybrids in greenhouse conditions.

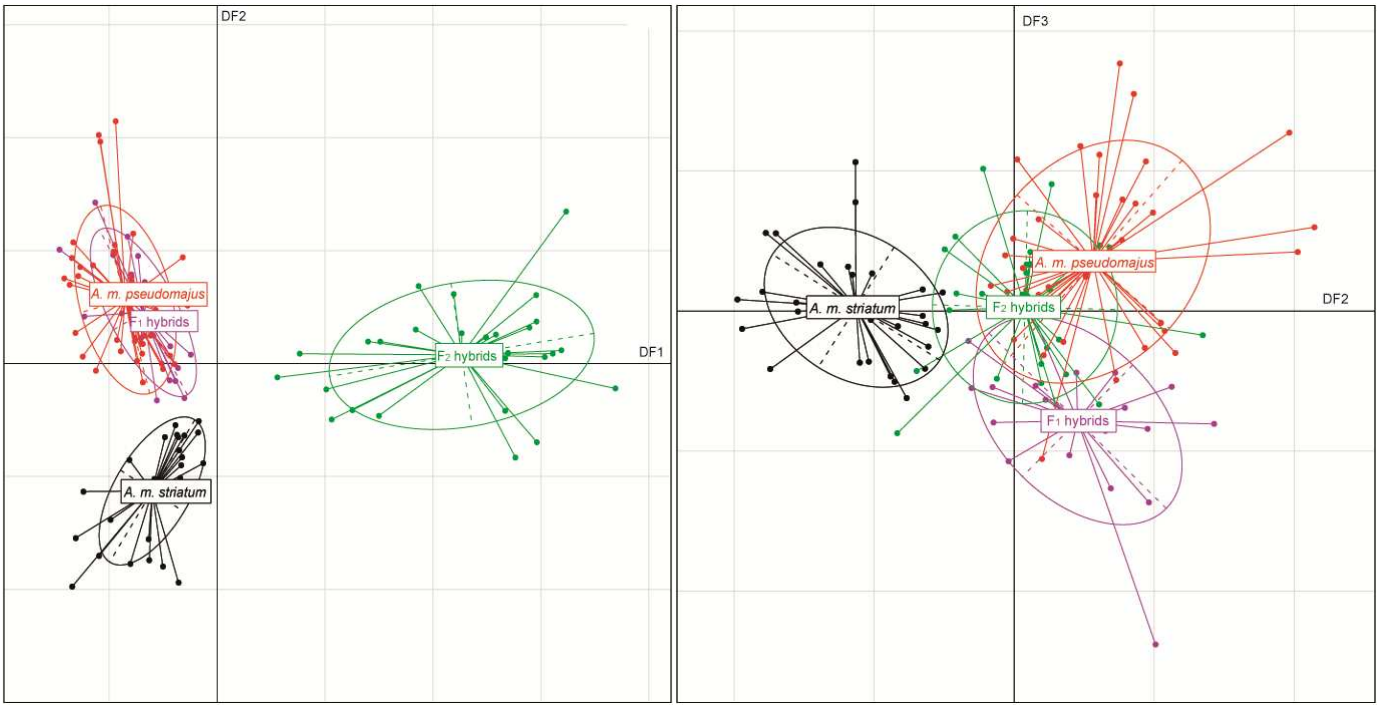

Figure 3: DFA analysis at the species level and hybrid generation. Cluster in red is *A. m. pseudomajus* plants, in black *A. m. striatum*, in purple F_1 hybrids and in green F_2 hybrids. The first discriminant function (DF1) represents 45.8% of the the total variability, the secund discriminant function (DF2) 34%, and the third discriminant function (DF3) 19.7%, for a total 99%. DF1 (at left) discriminates the F_2 hybrids cluster. DF2 (at left and right) discriminates the two parental lines. Note that DF3 (at right) dissociates *A. m. pseudomajus* and F_1-hybrids that are not entirely overlapped.

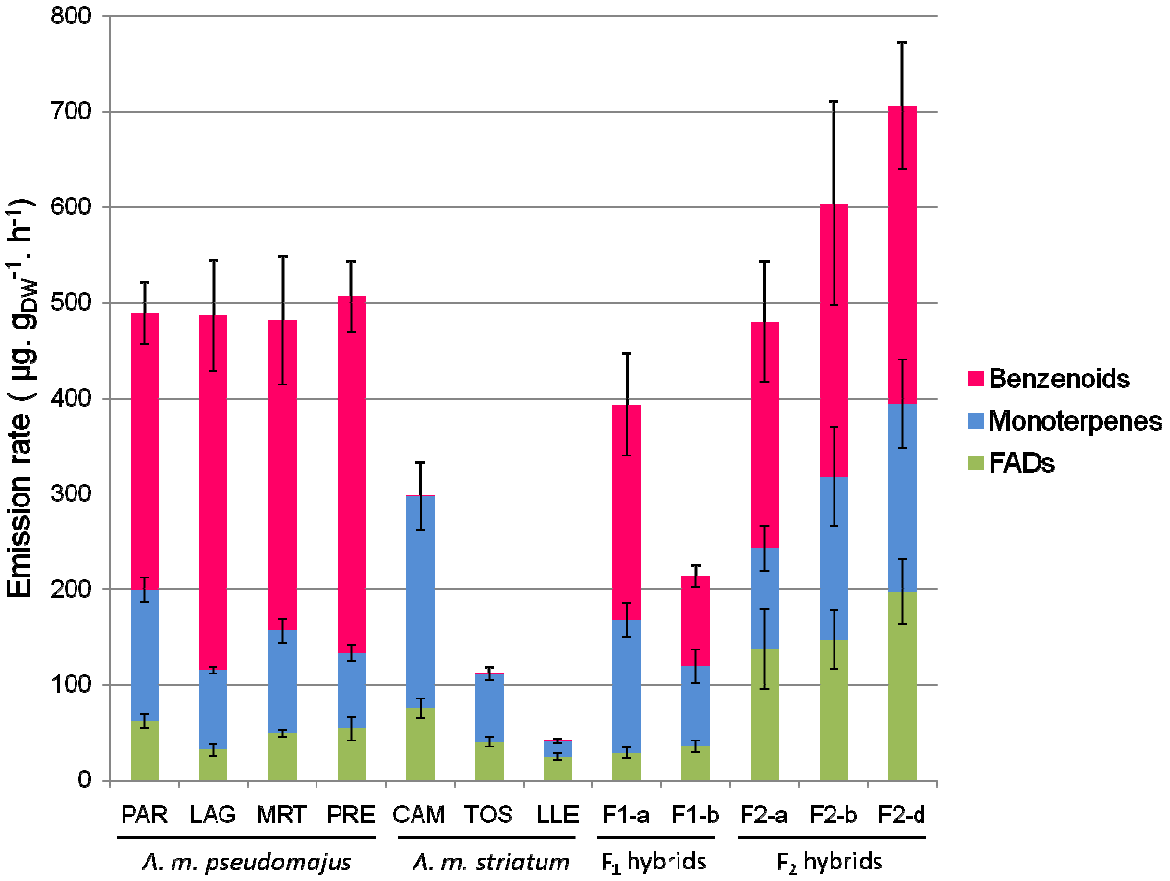

Figure 4: Proportions and standard error of the emission rate of the three classes of floral VOCs emitted by the populations of *A. m. pseudomajus* and *A. m. striatum*, and by the lineages of the F_1 and F_2 hybrids in greenhouse conditions (the N-containing compound is included in the FADs).

Appendix1: Correlation coefficients of the 43 VOCs of the *A. majus* floral scent to the three discriminant functions of the DFA relative to *A. m. pseudomajus*, *A. m. striatum*, and their hybrids F_1 and F_2. DF1 discriminates F_2 hybrids to the parents (coefficients are arranged by declining order), DF2 discriminates the two subspecies, DF3 the F_1 hybrids to the parents.

VOC	DF1	DF2	DF3
Decanal	0.70	0.03	-0.08
(E,E)-2,6-dimethyl-1,3,5,7-octatriene	0.63	-0.06	-0.04
2,6-dimethyl-2,4,6-octatriene	0.60	-0.09	-0.23
Dodecane	0.59	-0.01	-0.14
Nonanal	0.56	-0.06	-0.08
Methyl benzoate	0.55	-0.15	0.04
Octane	0.47	-0.04	-0.03
2-Butanone	0.46	0.02	-0.06
α-pinene	0.44	0.04	-0.11
methylester-2-methyl-butanoic acid	0.42	-0.05	0.05
Decane	0.37	-0.07	-0.24
Mesitylene	0.37	0.02	-0.09
1,3,5-Cycloheptatriene	0.34	0.06	-0.08
γ-terpene	0.34	0.06	-0.06
1-nonene	0.33	-0.02	-0.02
1-octene	0.32	-0.07	-0.11
Linalool	0.29	-0.02	-0.02
Pentanal	0.29	-0.03	0.20
1,1-diethoxy-ethane	0.29	0.01	-0.02
2,4-dimethyl-heptene	0.28	-0.02	-0.02
Nonane	0.27	0.18	-0.19
Octanal	0.26	0.07	-0.05
Heptanal	0.26	0.08	-0.13
2,4-dimethyl-heptane	0.26	-0.02	-0.02
Hexanal	0.23	0.08	-0.11
Acetophenone	0.11	-0.54	-0.36
Benzaldehyde	0.10	-0.77	0.00
para-cymene	0.09	0.10	-0.15
(E)-β-ocimene	0.08	-0.02	0.08
1-pentanol	0.06	-0.06	-0.11
β-myrcene	0.04	0.09	-0.19
β-pinene	0.04	0.07	-0.08
6-methyl-5-hepten-2-one	-0.02	-0.36	0.11
Eucalyptol	-0.09	-0.08	-0.10
Hexyl acetate	-0.09	0.21	-0.07
Limonene	-0.09	0.30	-0.12
Hemimelitene	-0.11	0.09	-0.12
Ethyl acetate	-0.11	0.31	-0.07
(Z)-β-ocimene	-0.12	0.00	0.00
2-methyl-propanal	-0.14	-0.04	-0.21
3-methyl-butanal	-0.15	-0.07	-0.12
syn-3-methyl-butyl-aldoxime	-0.22	-0.18	0.20
(Z)-3-hexenal	-0.24	0.22	-0.22

CHAPITRE 2

L’influence des odeurs florales d’*Antirrhinum majus* sur le comportement des pollinisateurs

Résumé

Dans ce chapitre, j'ai cherché à savoir si les pollinisateurs étaient influencés par les différences d'odeurs florales entre sous-espèces caractérisées dans le chapitre précédent. C'est une des hypothèses que j'ai favorisé afin de tester si cette différence phénotypique est le fruit de processus adaptatif ou non.

Sur le terrain, nous avons observé que les bourdons représentaient le pollinisateur principal, tout particulièrement *Bombus terrestris*. J'ai donc utilisé, dans un premier temps, des bourdons commercialisés (*B. terrestris*), c'est-à-dire vierges de toutes expériences avec des odeurs florales. J'ai effectué des études d'électro-antennographie (EAG) et d'olfactométrie. J'ai travaillé avec des odeurs artificielles recomposées à partir de molécules synthétiques ce qui permet de manipuler les COV un à un. L'EAG a permis de mettre en évidence que les composés principaux sont tous détectés par les bourdons, et l'olfactométrie que les bourdons préfèrent, et ce donc de manière innée, un mélange de composés volatils d'*A. m. striatum,* dû à un effet aversif d'un composé, l'acétophénone, présent chez *A. m. pseudomajus*, mais absent chez *A. m. striatum*.

Dans une deuxième étape, cette étude a été répliquée en conditions naturelles. Les odeurs florales naturelles ont été échantillonnées et des pollinisateurs sauvages ont été prélevés dans des populations d'*A. m. pseudomajus* et de *A. m. striatum*. Notre étude montre que les pollinisateurs sont significativement plus attirés par les odeurs florales de leur sous-espèce d'origine. On en déduit donc que l'odeur florale influence nettement le comportement des pollinisateurs dans le système de pollinisation d'*A. majus* et que l'effet aversif de l'acétophénone chez *A. m. pseudomajus* est outrepassé. L'hypothèse explicative est que les pollinisateurs ont appris à associer l'odeur florale à une récompense, le nectar. Parce que des bourdons expérimentés envers un signal odorant d'une des deux sous-espèces soient significativement attirés par celui-ci est en accord avec l'hypothèse de constance de visite de pollinisateur, ce signal pourrait limiter les flux de gènes entre les deux sous-espèces dans les zone de contact.

Article 3

Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve bumblebees

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Abstract Two wild subspecies of snapdragon, *Antirrhinum majus*, subspecies *pseudomajus* and *striatum*, differ in floral color and can be visually discriminated by insect visitors. The extent to which olfactory cues derived from floral scents contribute to discrimination between snapdragon subspecies is however unknown.

We tested whether these two subspecies differ in floral scent, and whether these olfactory differences are used by bumblebees (*Bombus terrestris*) to discriminate between them. We grew individuals of both subspecies, collected from a total of 7 wild populations, under controlled conditions. We quantified the volatile organic compounds (VOCs) emitted by the flowers using gas-chromatography/mass-spectrometry/flame-ionization-detection (GC-MS-FID). We studied antennal detection of VOCs by bumblebees, by means of electroantennogram study (EAG). We also performed behavioral experiments in a Y-maze to determine the innate response of bumblebees to the main floral VOCs emitted by our snapdragon subspecies.

The floral scent of *A.m. pseudomajus* contained three volatile benzenoids absent in the floral scent of *A.m. striatum*. One of them, acetophenone, contributed over 69% of the absolute emissions of *A.m. pseudomajus*. These benzenoids elicited a significantly higher EAG response compared with other VOCs. In the Y-maze, bumblebees were significantly less attracted by acetophenone, suggesting an aversive effect of this VOC. Our findings indicate that bumblebees are able to discriminate between the two *Antirrhinum majus* subspecies. Differences in flower scent between these subspecies, and olfactory bumblebee preferences, are discussed in the light of biochemical constraints on VOCs synthesis and of the role of flower scent in the evolutionary ecology of *Antirrhinum majus.*

Key words: snapdragon, *Antirrhinum majus*, bumblebee, *Bombus terrestris*, flower scent, olfactory preference

Introduction

Plants have evolved a fascinating array of cues to attract animal pollinators and this has been a fertile ground for research in evolutionary ecology (Ricklefs and Renner 1994; Mitchell et al. 2009). The mechanisms of flower detection by pollinators are complex, and understanding them also is an active field of research (Giurfa and Vorobyev 1997; Chittka and Thomson 2001; Harder and Barrett 2006). Insect-pollinated flowers offer visual cues through flower size, shape and color, which are used by the pollinators to find and discriminate their resources (Giurfa and Menzel 1997). The volatile organic compounds (VOCs) of the flower scent also play an important role in plant-pollinator interactions (Stebbins 1970; Heinrich and Raven 1972; Raguso 2008), and new techniques in organic chemistry have shed a new light on the role of these VOCs (Tholl et al. 2006). For instance, pollinators may be innately attracted by floral VOCs (Omura and Honda 2005; Riffell et al. 2008) and also learn to associate a scent with a food reward (Komischke et al. 2002; Deisig et al. 2002; see Giurfa 2007 for a review).

In the mountain range of the Eastern Pyrenees, two wild subspecies of *Antirrhinum majus* (Scrophulariaceae) are encountered on roadsides and open habitats. The subspecies display characteristic flower colors: the flowers of *A. m. pseudomajus* are magenta, whereas *A. m. striatum* has yellow flowers (Fig. 1). The flowers are self-incompatible (an estimated 4% of selfing), and they are thought to be pollinated by relatively large Apidae, as these have strong enough bodies to open the closed corolla (Andalo et al. 2010). A nectar reward is offered to flower visitors. In a preliminary census of flowers visitors in our study site, we inventoried over 10 species of insect visitors in the genera *Bombus* and *Xylocopa* with *Bombus terrestris* being the most frequent species (Suchet et al. unpublished data). These insects were observed

as flower visitors in both snapdragon subspecies. The subspecies are usually allopatric or parapatric but they are occasionally found in contact, and there pollinators cross-fertilize the flowers, generating hybrids that display a wide array of coloration patterns (Whibley et al. 2006). Whibley et al. (2006) found that in the hybrid zone, flower color was under strong stabilizing selection, and they suggested that pollinators may counter-select the hybrids. In support of a pre-zygotic barrier to gene flow, bumblebees were shown to discriminate between the two colors of subspecies (Tastard et al. 2008). However, in experiments with artificial flowers with the colors of the two parent lines, the constancy of the visitor was only weakly associated with flower color (E. Tastard, C Andalo, C Thébaud et al., unpublished results). Thus no direct link between selection on the floral phenotypes and the color of these phenotypes was demonstrated.

In addition to color differences, it is also possible that VOCs differ between the flowers of the subspecies of *A. majus*, and if so, that pollinators use flower scent as a detection and recognition cue. In the present work, we thus study the role of floral VOCs in the detection of *A. majus* by bumblebees. The domesticated relatives of the wild *A. majus* (horticultural snapdragon) have long served as a model species to discover the chemical pathways of floral volatiles (Dudareva et al. 2000; 2003; 2006). By artificially manipulating floral scent of horticultural snapdragons in free-flying bumblebee studies, Odell et al. (1999) showed that it is the color that influences the bumblebee behavior in the tested floral color-scent combinations. However, Wright et al. (2005) showed that honeybees could discriminate slight changes in the composition of a mixture of VOCs using snapdragon cultivars. Focusing on horticultural snapdragons, these studies are therefore conflicting. However, the floral scent of horticultural snapdragon may differ from that of their wild relatives. We here study the

composition of the floral scent of the two wild *A. majus* subspecies. In addition, we assess how bumblebees may show innate preferences for one or the other scent.

In the present work, we combine VOCs identification, biologically active VOCs determination and behavioral tests with the interacting insect because they are important steps to understand chemical mediation in plant-insect interactions (Schatz et al. 2009). Our questions are the following: (i) Do the two subspecies *A. m. pseudomajus* and *A. m. striatum* produce distinct floral blends of VOCs under identical greenhouse conditions? (ii) Can naïve bumblebees perceive most of the VOCs of the two *A. majus* subspecies flower scent? (iii) When exposed to simulated floral scent of the two subspecies, do naïve bumblebees show a preference toward one scent?

Materials and Methods

Plant material growth and collection

Antirrhinum majus is a widespread semi-perennial plant (Scrophulariaceae), with annual axes of racemous inflorescences and zygomorphic flowers, growing at an elevation of 0-1600 m asl in the Eastern Pyrenees mountains. The distribution of the two subspecies is non-overlapping, except for a few hybrid zones (see map in Whibley et al. 2006). Flowers are closed and self-incompatible, and are visited mostly by Apidae (Andalo et al. 2010). Even though the pollinating efficiency of flower visitors has not been studied in detail, *Bombus* species (henceforth bumblebees) are pollen vectors for *A. majus* as we did observe fresh pollen deposited on their thorax and legs after flower visitation (C Suchet, personal observations).

We grew 10 seeds from each of four wild populations of *A. m. pseudomajus* near the villages of Lagrasse, La Preste, Le Martinet in France and near Pardines in Spain (for a total of Np=40 adults), and from each of three wild populations of *A. m. striatum* near the villages of Lles, Collada de Toses in Spain and Camurac in France (for a total of Ns=30 adults). The populations were selected so as to maximize their geographical range and elevation with respect to the other subspecies. Initially, a fourth population of *A. m. striatum* was included in our experimental design but germination showed a low rate of success, and it was subsequently discarded. Seeds were collected between 2000 and 2006 at the end of the growing season from mature fruits. Seeds from ten fruits from ten individual plants for each of the seven populations were grown in greenhouse conditions between November 2008 and May 2009 (16 h/day of light, at 25°C average temperature, in individual pots with universal compost and with no addition of nutrients). Ten adult plants were selected by population at the flowering time for their similar phenotypes and their diversified genetic sources. We minimized the number of sampled plants grown from the seeds of the same fruit. Overall, 5 to 7 maternal lineages are represented among the ten selected plants by population.

Determination of floral scents

Sampling of floral scent. The VOCs emitted by the flowers were sampled between February and May 2009 in the greenhouse. Preliminary analyses showed that diurnal variations in emission were similar with those described by Dudareva et al. (2000; 2003). Therefore, sampling was conducted during the peak of emission intensity, between 11:00 am and 4:00 pm, and on whole inflorescences. To minimize biases due to flower developmental stages

(Dudareva et al. 2000; 2003; Goodwin et al. 2003), VOCs were sampled when the inflorescence had at least four open flowers with dehiscent anthers.

To sample floral emissions, a dynamic headspace method was used (Tholl et al. 2006). We enclosed each inflorescence *in vivo* into a 2 L glass chamber, and the VOCs were adsorbed on a TenaxTA 60/80 (100 mg) trap connected to a battery-operated vacuum pump operated at 200 mL min^{-1}. This design optimizes the signal/threshold ratio without exceeding the breakthrough volumes of each VOC (Kesselmeier et al. 1996; Simon et al. 2005a; 2005b). The flow rate that purges air from the headspace was maintained at 600 mL min^{-1}. Sampling duration was fixed at 10 min. To control for possible environmental contamination, ambient air was also trapped during each sampling session. Sample tubes were stored in the dark at 0-4°C before analysis.

Floral VOC emissions depend on light intensity and temperature (Guenther et al. 1995; Dudareva et al. 2000; 2003; 2006). Hence we measured both variables from the headspace of the inflorescence for each sampling session. Temperature was measured with an EL-WIN-USB datalogger (Lascar Electronics LTD., United Kingdom). Photon flux was measured with a LI250A light meter connected to a LI190SA Quantum Sensor (LI-COR Biosciences, Lincoln, USA). The intrafloral temperature may differ among flowers of different color in *A. majus* (Comba et al. 2000), but we did not measure this parameter because of technical difficulties. To normalize the emissions among plants, we cut the inflorescences after VOC sampling, and we measured their oven-dry weight (inflorescences were dried at 100°C for 48h).

Analyses of volatile compounds The VOC samples were thermodesorbed using a Turbomatrix TD desorber (Perkin Elmer, US), and were analyzed using a gas chromatograph coupled with a mass spectrometer and a flame ionization detector (FID) (Clarus 500, Perkin Elmer, US). The separation of VOCs was performed using a DB-5 non-polar capillary column (30m x 0.25 mm ID x 0.25 µm film thickness). Oven temperature was held at 35°C for 5 min, heated to 160°C at 5° min^{-1} and then up to 220°C at 15°min^{-1}. The carrier gas was helium. Mass spectra were recorded in the electron impact mode at an ionization voltage of 70eV, and scanned from m/z = 33 to 450.

The identification of VOCs was based on their Kovats index relative to C_5 – C_{18} n-alkanes and mass spectra which were matched with those from the NIST library (2005) and those reported in literature (Adams 2001).

The quantification of the compounds was made based on their FID peak area. Ocimene (TCI Chemical®, Stockholm, Sweden, 90.0%) and nonanal (Extrasynthese SAS®, Genay, France, pure) were used as external standards. The calibration was carried out in laboratory conditions by injecting a liquid volume of standard solutions directly into the sample tube. A linearity range from 2 x 10^{-5} to 9.2 x 10^{-4} µg was observed for the two external standards (R^2=0.99 for both compounds). The theoretical response factor of the studied compounds was computed using the theory of the effective carbon number (Jorgensen et al. 1990). To quantify the VOCs that were not calibrated individually, we applied corrections to the mean response factors (Komenda et al. 2001).

The emission rate of each VOC was obtained from the difference between the quantity of compounds recorded inside and outside the glass chamber. The emission rate E (µg. g (dry flowers weight)$^{-1}$. h^{-1}) was computed using the following equation:

$$E = \left(\frac{m_2}{q_2} - \frac{m_1}{q_1} \right) \frac{Q}{Mt}$$

where m_2 and m_1 are the mass of the compound in the outlet and inlet flow rates (µg), and q_2 and q_1 are the outlet and inlet flow rates (mL min^{-1}). Q is the flow rate of the enclosure purge air (mL min^{-1}), M is the dry weight of the enclosed flowers (g) and t the sampling time (h) (Sabillon and Cremades 2001). This quantification method makes it possible to compare among the plants in our study because it normalizes the amount of VOCs to the dry flower weight, regardless of the difference in flower number per inflorescence. A uniform sampling and analytical uncertainty of ca. 30% is associated with the chamber design (Moukhtar et al. 2005).

Bumblebees' olfactory tests

Bombus terrestris was found to be the most frequent flower visitor of *A. majus* in the wild (Appendix 1). Commercial colonies of this species are available so that physiological and behavioral measures could be performed in the laboratory. Naïve individuals were chosen to perform both electroantenograms and behavioral tests with chosen chemical compounds (one colony purchased from Koppert®, Berkel en Rodenrijs, Netherlands). We used only workers in our tests.

Electroantennography We determined whether VOCs present in floral emissions could be detected by the olfactory receptors located on the bumblebees' antennae. To this end, we

performed electroantennogram recordings. Electroantennograms measure the summed response of olfactory receptors on an insect's antenna to a given olfactory stimulus (Roelofs 1984). Hence, they indicate whether an insect has the ability to detect such a stimulus.

A single bumblebee antenna was cut and fitted both ways into two glass pipettes filled with KCl solution, and connected to the silver electrodes of an electroantennogram detector (SYNTECH®, Kirchzarten, Germany). The antenna was then stimulated with a VOC, and responses (in Vm) were measured by means of a volt-meter *via* the electrodes as described and illustrated in Thiéry and Marion-Poll (1998). Ten synthetic VOCs detected in the floral scents of *A. majus* were tested: three benzenoids (benzaldehyde, methyl benzoate and acetophenone, Sigma- Aldrich®, US, Bellefonte), the VOCs contributing the most to the differences between the two snapdragon subspecies (see below). We also included seven VOCs that were found to be either the most frequent and/or the most abundant in the two *A. majus* subspecies: *cis*-ocimene, limonene, nonanal, 2-butanone, myrcene, pentanal, and hexanal, (Sigma- Aldrich®, US, Bellefonte; TCI Chemical®, Stockholm, Sweden; and Extrasynthese®, Genay, France). We decided to use synthetic VOCs rather than scents from real flowers so as to minimize the variability among the tests.

For each stimulus, 1 µl of pure solution was deposited on a strip of filter paper and left for evaporation during 30 min in ambient conditions in a separate laboratory room. We initially used pure solutions because we aimed to compare the amplitudes of the antennal activities among the perceived VOCs, but we also carried out controls with compounds diluted at 0.1%, 1%, 10% and 50%. The antenna was stimulated with pulses of 0.5 s each, using a purified and moistened airflow of 11.3 mL s^{-1} across a Pasteur pipette containing the filter paper.

Each of the ten VOCs was delivered once; VOCs were presented in the same order so that the 10 tested antennae were submitted to a similar stimulus sequence, thus allowing comparisons between antennal signals. Stimuli were separated by a 40-s interval, to avoid saturation of the olfactory receptors. A control was performed at the beginning of each experimental series by measuring the antenna response to clean air. To check the sensitivity of antennal responses throughout the sequence of stimuli, the VOCs for which the response was the most intense were assayed again at the end of the sequence. For each bumblebee antenna, the response amplitude was normalized to the maximum response recorded.

Spontaneous olfactory preferences in a behavioral assay To determine whether bumblebees exhibit spontaneous preferences when confronted with the VOCs of the two *A. majus* subspecies, we tested their choice in a Y-maze presenting a dual olfactory stimulation (Dupuy et al. 2006). In these experiments, we used naïve bumblebees from a colony raised in the laboratory, so their choices reflect innate preferences for olfactory stimuli.

The maze presented a main channel and two bifurcating arms. The main channel was 7 cm long and the two arms were 14 cm long; all parts were 4 cm in height. Air flow moistened and neutralized by means of active carbon was delivered at the two extremities of the arms (flow rate: 150 mL min^{-1}). To favor a constant directional air flow through the maze, air was also pumped out at the entrance of the maze (flow rate: 200 mL min^{-1}).

To familiarize the bumblebees with the experimental set-up, we pre-trained them to the maze before testing them with VOCs. Bumblebees stayed six hours in the dark, without food in ventilated individual plastic tubes. Each individual was then released in the entrance arm of the maze. During the pre-training stage, 20 µl of 50% sugar solution was offered at the

intersection point of the maze. The bumblebee could freely move within the maze, and it easily found the drop of sucrose solution and consumed it. After this training, the bumblebee was replaced into its plastic tube and taken out from the maze. Between tests, the Y-maze was carefully cleaned using an ethanol solution. After a second pre-training visit, the experienced bumblebee was motivated to search for sucrose solution within the maze, and the olfactory tests could then begin. Both pre-training and test sessions were carried out in the dark, to favor the bumblebees' use of olfactory cues. To be able to observe the bumblebee behavior in real time, these tests were performed under red light. Because of the darkness bumblebees were walking in the Y-maze.

In the olfactory tests, two olfactory stimuli were delivered, each coming from one of the two arms of the Y-maze. The VOCs were deposited on a 1cm² piece of filter paper, and they were allowed to evaporate for one hour in a separate laboratory room. The filter paper was then inserted in a 10µL micropipette tip. In each arm of the Y-maze, the micropipette tip was inserted in a hole in the floor created for this purpose (Dupuy et al. 2006). Air filtered by active charcoal and moistened was pulsed at 150 mL min^{-1} from the dead end of each arm through teflon tubes, allowing the olfactory stimuli to flow towards the decision area, defined as the area where the bumblebee had to make a choice. The bumblebee did not find any reward at the intersection of the arms during the test.

The olfactory stimuli presented to the bumblebees were either single synthetic VOC or mixtures of pure synthetic VOCs. Synthetic mixtures were used because VOC concentration could then be easily manipulated, so as to identify which VOC mostly influences bumblebee behavior. We defined a "monoterpene mixture", a mixture of 50% *cis*-ocimene, 17% limonene (90% purity 3:4 ocimene ratio, Sigma- Aldrich®, US, Bellefonte) and 33% myrcene

(90% purity, Sigma- Aldrich®, US, Bellefonte). We also defined a "benzenoid mixture", a mixture of 66% acetophenone (99.5% purity, Sigma- Aldrich®, US, Bellefonte), 17% benzaldehyde (99.5% purity, Sigma- Aldrich®, US, Bellefonte), and 17% methyl benzoate (98%, Sigma- Aldrich®, US, Bellefonte). We also checked that during these tests the bumblebees were exposed to an olfactory stimulus in the same range as one delivered by real snapdragon inflorescences (results not shown).

Bumblebees were exposed to four different tests. In the first test, bumblebees had to choose between the monoterpene mixture and the monoterpene mixture plus the benzenoid mixture. In the second test, bumblebees were exposed to the monoterpene mixture vs. the monoterpene mixture plus methyl-benzoate and benzaldehyde (that is, all benzenoids except acetophenone). In the third test, bumblebees were exposed to the monoterpenes mixture vs. a blank (clean air). In the last test, bumblebees had to choose between acetophenone alone and a blank. Twelve bumblebees were tested in each of these tests. Thus, a total of 48 bumblebees were used in this experiment.

For each bumblebee, we recorded its first choice (i.e. which arm was chosen first) and the proportion of time spent in each arm of the maze during two minutes of observation (time spent in one arm divided by the total time spent in both arms of the maze). Each test was duplicated with the same bumblebee, swapping the presentation side of the olfactory stimuli. Between the observation sessions, the Y-maze was carefully cleaned with an ethanol solution.

Statistical analyses

Differences in VOCs between the blends of the two subspecies were determined based on presence/absence of VOCs and also on the emission rate of each VOC. VOCs that represented less than 0.01% of the total emissions were excluded from the analysis. All statistical analyses were carried out with the R statistical software, version 2.9.2 (http://cran.r-project.org/).

To determine whether some compounds occurred significantly more frequently in one or the other subspecies, a null model of VOC composition was generated for the presence/absence data. Significant difference per VOC was determined using the difference between the randomized sequences of presence/absence generated and the observed difference computed by: $p=(np/Np)-(ns/Ns)$, where, for a given VOC, *np* is the number of times the VOC was observed in *A. m. pseudomajus*, and *ns* is the number of times it was observed in *A. m. striatum*. The total sample sizes were Np=40 in *A. m. pseudomajus*, and Ns=30 in *A. m. striatum*. Significance was tested at 5% level using a two-way test. If $0<p<0.025$, the VOC was significantly more frequent in *A. m. pseudomajus* than in *A. m. striatum* and if $0.975<p<1$ it was significantly more frequent in *A. m. striatum.*

We used an analysis of variance (ANOVA) to test whether the VOC emission intensity was greater in one subspecies than in the other. In this analysis, we included only the floral chemicals that constituted more than 20% of the total emission of a subspecies to avoid a spurious significance effect due to occasionally emitted VOCs.

For the EAG analyses, the significance of differences of bumblebees' responses was tested using a Mann-Whitney test at 0.05 level. A Bonferroni correction, as modified by Holm (1979), was computed to adjust the *p*-value of the multiple tests carried out among the

bumblebees' responses of the ten VOCs tested in EAG. The classic Bonferroni correction is often considered as being too conservative and Holm's (1979) correction avoids this problem.

For the Y-maze test, a binomial test based on the first choice was computed to determine if the bumblebees exhibit a preference for one of the two volatile chemical signals. Differences in the cumulated time spent in the two arms were tested using a Student t-test. We also tested whether swapping the two same volatile chemical signals between the two arms of the Y maze yielded the same result for the two recorded variables. To this end, we applied a binomial test for the first choice and a Fisher test for the residence time.

Results

Floral scent composition in the *A. majus* subspecies

The flower scent of the wild snapdragon was composed of a total of 37 VOCs in *A. m. pseudomajus* and 34 VOCs in *A. m. striatum* (Table 1). We detected 20 fatty-acid-derivatives (FADs), including green leaf volatiles, one nitrogen-containing compound (the Syn-3-methyl-butyl-aldoxime), 11 monoterpenes, and 5 benzenoids.

The two subspecies had a significantly different scent. Three benzenoids (acetophenone, benzaldehyde, and methyl benzoate) were only emitted by *A. m. pseudomajus* and were totally absent in *A. m. striatum* (Fig. 2 and Table 1). This difference in flower scent composition explained the difference in absolute emission rates between *A. m. pseudomajus* and *A. m. striatum* (490 and 150 $\mu g\ g^{-1}_{DW}\ h^{-1}$ respectively) because acetophenone had the highest emission rate of all VOCs (on average 337 $\mu g\ g^{-1}_{DW}\ h^{-1}$, Fig. 2 and Table 1). The

absolute emission rates of floral VOCs were large, as they exceeded 100 µg g^{-1}_{DW} h^{-1}. Note that we found no correlation between the emission rate and temperature or light intensity. Therefore, we did not correct the measured emission rates for either temperature or irradiance.

In addition to the three benzenoids specific to *A. m. pseudomajus*, eight VOCs were encountered more often in *A. m. pseudomajus* than in *A. m. striatum* (Table 1). *A. m. striatum* had no specific VOCs and only two VOCs were significantly more frequent in *A. m. striatum* than in *A. m. pseudomajus*. (ethyl acetate and *p*-cymene, Table 1). Of the six VOCs whose emission rate significantly differed between the two subspecies, two were more abundant in *A. m. striatum* (limonene and γ-terpinene), and four in *A. m. pseudomajus* (6-methyl-5-hepten-2-one and the three specific benzenoids). These results did not depend on the population from which the plants originated.

Bumblebees' responses to olfactory tests

Electroantennography All 10 tested VOCs, when used as pure standards, induced a significant antennal electric activity (henceforth depolarization), thus could be detected by the bumblebee antennae (Fig. 3). These VOCs were also detected by antennae when the solutions were diluted at 1%, 10% and 50%, but not systematically with solutions diluted at 0.1%. In addition, the amplitude of depolarization was maximal and constant with dilutions at 10%, 50%, and pure standards. The three benzenoids, emitted only by *A. m. pseudomajus*, induced a significantly larger maximal depolarization compared with all other VOCs (Fig. 3). In contrast the widespread monoterpenes ocimene/limonene and myrcene produced small, albeit significant, maximal depolarizations. The three aldehydes, pentanal, hexanal and nonanal, induced intermediate and highly variable maximal depolarizations (Fig. 3). The compound 2-

butanone induced the smallest depolarization in spite of its much higher vapor pressure than that of the three benzenoids (71 mmHg for 2-butanone versus 0.75 mmHg for acetophenone, 0.25 mmHg for methyl benzoate, and 1 mmHg, benzaldehyde; all vapor pressures measured at 20°C). Hence, the significantly larger depolarizations recorded in the case of the three benzenoids were not due to higher concentration of these compounds on the antennae. Rather, they were probably caused by a higher number of receptors for these compounds and/or by a higher sensitivity of these receptors.

Spontaneous olfactory preferences in a behavioral assay An analysis of the first choice in the Y-maze showed that there was no difference in performance depending on the side of stimulus presentation so that data for both tests in which the same volatile chemical signals were swapped were pooled. In the first test comparing monoterpene and monoterpene plus three benzenoids, bumblebees significantly preferred the monoterpene mixture over the monoterpene mixture plus the benzenoid mixture (92%, Z=2.89, *p*-value<0.001, Fig. 4). In the second test, the same as the first test but excluding acetophenone of the benzenoid mixture, the first choice of the bumblebees did not differ significantly between the alternatives (50%, Z=0.58, *p*-value>0.05, Fig. 4). This suggests that acetophenone induces the aversion observed in the first test. In the third test, bumblebees were confronted with the monoterpene mixture vs. a blank (clean air), and they significantly preferred the monoterpene mixture (83%, Z=2.31, *p*-value<0.001, Fig. 4). This result shows that the monoterpene mixture is attractive *per se*. In the last test, bumblebees were confronted with acetophenone vs. a blank (clean air) and the first choice of the bumblebees did not differ significantly between the alternatives (66%, Z=0, *p*-value>0.05, Fig. 4). This result shows that bumblebees were not repelled by acetophenone but they avoided it, hence our choice of the term “aversive” instead of “repellent”.

An analysis of the time spent in each arm showed that there was no difference in performance depending on the side of stimulus presentation. Hence, data for both tests in which the same volatile chemical signals were swapped, were also pooled for this variable. In the first three tests involving the monoterpene mixture, the time spent in each arm of the maze showed exactly the same trend than the first choice. Bumblebees spent more time in presence of the monoterpene mixture than in presence of the monoterpene mixture plus the benzenoid mixture ($t=44.4$, $df=46$, p-value<0.001, Fig. 4). They had no preference when acetophenone was removed from this treatment ($t=0.21$, $df=46$, p-value>0.05, Fig. 4). Finally, they spent more time in presence of the monoterpene mixture than in the 'blank' ($t=13.25$, $df=46$, p-value<0.001, Fig. 4). The fourth test (acetophenone vs 'blank') yielded an interesting new result. We observed a significant tendency to spend more time in the 'blank' arm than in the arm containing acetophenone ($t=7.26$, $df=46$, p-value<0.001, Fig 4), thus confirming the aversive effect of this compound.

Additionally, we quantified the time spent in the main arm of the maze, before entering in one of the arms presenting the olfactory stimuli. This variable reveals the readiness of the bumblebees to choose among stimuli. The decision time did not significantly differ among the first three tests involving monoterpenes, whereas it was significantly different in the last test, where acetophenone alone was emitted ($F=14.64$, $df=3$, p-value<0.001, Tukey post test). Indeed, in the three first tests involving monoterpenes, bumblebees took on average less than 10 s to make a choice (mean and standard error, 4.92 ± 1.44 s; 5.2 ± 1.26 s; 8.2 ± 3.66 s respectively) whereas they needed three times longer to make a decision in presence of acetophenone alone (27.3 ± 6.32 s).

Discussion

Scent composition of the wild species *Antirrhinum majus*

Characterizing the floral scents of *Antirrhinum majus*, we found that this species emitted up to 37 floral VOCs. In previous studies on horticultural snapdragon only 12 VOCs were reported (Odell et al. 1999; Wright et al. 2005). In addition, the dominant VOCs of these studies on cultivars were not detected at all in our study (*cis*- and *trans*-methyl-cinnamate, *cis*-3-hexenyl acetate, linalool, dimethoxytoluene, diphenyl ether and nerolidol). Furthermore, several benzenoids and monoterpenes found to be abundant in our samples were not previously reported (benzaldehyde, hemimelitene, mesitylene, *p*-cymene, α and β-pinene, limonene, γ-terpinene, 6-methyl-5-hepten-2-one, 3,4-dimethyl-1,3,5,7-octatriene and (E,E)-2,6-dimethyl-1,3,5,7-octatetraene). Thus, obvious differences exist between the floral scents of the wild snapdragon and that of the cultivars.

Our second main result was that the two wild subspecies differed strikingly in their flower scent. *A. m. pseudomajus* emitted on average 490 $\mu g\ g^{-1}_{DW}\ h^{-1}$ of flower scent whereas *A. m. striatum* emitted 150 $\mu g\ g^{-1}_{DW}\ h^{-1}$. All of the *A. m. pseudomajus* populations emitted three benzenoids (acetophenone, benzaldehyde and methyl benzoate), which were absent from the *A. m. striatum* populations. The benzenoid acetophenone was, by far, the main VOC in *A. m. pseudomajus* but was not detected in *A. m. striatum* (Fig. 2 and Table 1). Such striking differences in floral chemical emissions between subspecies have seldom been reported. Majetic et al. (2007) showed that the floral scent of two sympatric morphs of *Hesperis matronalis* (white and purple flower color) also varied consistently, but they also found a large variation in floral scent of the white morph in the two studied populations. In contrast,

the chemical composition of the scents detected in the two *A. majus* subspecies was consistent across populations (C Suchet et al. unpublished data).

Evolution of the floral scent-color association in *A. majus*

Why do the magenta-flowering phenotypes produce benzenoids, in particular massive amounts of acetophenone, while the yellow-flowering ones do not? One explanation is that these benzenoids are produced by a biosynthetic pathway related to that of anthocyanin, the flower pigment that produces the magenta coloration. These secondary metabolites may then be jointly regulated. A prerequisite of this scenario would be that the three benzenoids of *A. m. pseudomajus* are indeed synthesized in the same pathway. Benzaldehyde and methyl benzoate are known to be produced in the benzenoid branch of the shikimic acid pathway (Dudareva et al. 2006). Little is known on the biosynthesis of acetophenone, apart that it is reported in 14 of the 90 listed plant families by Knudsen et al. (2006). We suspect that acetophenone is produced in the phenylpropanoid branch of the shikimic acid pathway, as in bacteria (Cripps et al. 1978; Rabus et al. 2002). Further evidence that the biosynthetic pathways of benzenoids and anthocyanins may be linked is offered by the study of Zucker et al. (2002). Indeed, when Zucker et al. (2002) genetically suppressed the expression of a central enzyme of the anthocyanin biosynthesis in transgenic carnation plants (*Dianthus caryophyllus L.*), they observed an over-emission of methyl benzoate. In *A. m. pseudomajus*, both anthocyanins and benzenoids are conspicuously expressed (as it is genetically known in tobacco, Martin et al., 2001), whereas anthocynanins are only expressed in the vein flower cells of *A. m. striatum* (Schwinn et al. 2006), in which, no benzenoids were detected. Hence these differences may be explained by differential regulation of the biosynthetic pathways in the two subspecies.

The striking phenotypic difference and scent-color covariation between the two *A. majus* subspecies could also reflect evolutionary responses to multiple selective pressures, including abiotic environment (e.g. based on flower color, Warren and Mackenzie 2001) and the selective pressure of natural predators (Raguso 2009). Field studies that would take into account the local abiotic conditions and the ecological network of interactions are an exciting prospect in the case of the *A. majus* model.

Role of pollinators in maintaining plant species phenotypes

It has been speculated that pollinators are responsible for the maintenance of two distinct phenotypes within *A. majus* (Whibley et al. 2006). Here, we provide new results that shed light on this scenario. We first showed that the two phenotypes have a clearly distinct flower scent. Through EAG analyses, we then showed that the VOCs were detected by the bumblebee antennae, and that the three benzenoids emitted by one subspecies but not by the other induced the highest physiological response (Fig. 3). Hence, at least at the antennal level, bumblebees are tuned to detect those VOCs that discriminate between subspecies.

We complemented this analysis by a behavioral experiment, which provided further evidence that bumblebees are innately influenced by VOCs present in the floral scents of *A. majus*. In particular, we showed that bumblebees exhibited an aversion for acetophenone, the most abundant benzenoid, and the one for which the highest depolarization was found in EAG recordings (Fig. 3). Bumblebees were more attracted to the synthetic blend mimicking the *A. m. striatum* flower scent than to that of *A. m. pseudomajus* (Fig. 4a). This preference was not only due to the attractive nature of the monoterpene blend (Fig. 4c) but also to the aversive

effect of acetophenone present in *A. m. pseudomajus* (Fig. 4b and d). This is a surprising result because acetophenone was found to be innately attractive to the butterfly *Vanessa indica*, which pollinates plant species in the Asteraceae (Omura and Honda 2005). Despite this difference, our results suggest that VOC emissions should significantly influence the choice behavior of unexperienced *B. terrestris* when foraging in the field.

Such scent-induced discrimination may be enhanced by means of visual cues. We know that the strikingly different colors of the two phenotypes are discriminated by bumblebees (Tastard et al. 2008). Tastard et al. (unpublished results) studied whether *B. terrestris* shows a preference for one of the two colors of scentless artificial snapdragon flowers. They showed that bumblebees preferred the magenta or the yellow color when it was presented against the hybrid colors, but they were not constant in their choice when the magenta and yellow colors were the alternatives. This result tends to support Kunze and Gumbert (2001)'s finding, who showed that *B. terrestris* discriminate colors more efficiently when the flowers are scented than when they are scentless. Kulahci et al. (2008) also found that bumblebees in the species *Bombus impatiens* trained on flowers differing by their shape and scent learned the rewarding scented flowers faster than those trained on flowers that differed only with respect to visual cues. Hence, the combined action of olfactory and visual cues improves discrimination of the two wild snapdragon subspecies. We emphasize that we did not directly test this hypothesis, but hope to return to this issue in the future.

On the possible ecological role of acetophenone

One open question is why, if *A. m. pseudomajus* emits an aversive scent, it still persists in the wild? A possible explanation could be linked to the flower reward. The nectar of *A. m.*

pseudomajus could be of better quality than that of *A. m. striatum*. If so, some pollinators would choose the attractive flower scent (*A. m. striatum*) but gain little reward, while others would learn that the less attractive flower scent also entails a greater reward. Indeed, it has been convincingly shown that bumblebees learn that even compounds that innately induce aversion (e.g. alarm pheromones) may be associated with a reward and thus respond by an appetitive behavior to these compounds (Guerrieri et al. 2005). Variables such as sucrose concentration, amino acid content, alkaloids, etc, may as also be important in the bumblebees' choice (Gegear et al. 2007; Manson et al. 2010). In the future, we plan to quantify the production and the major constituents of the nectar to test whether or not, *A. m. pseudomajus* flowers compensate the aversive effect of acetophenone emission by offering richer or less deterrent nectar to pollinators.

Acetophenone may also act as a defensive compound. Indeed, acetophenone has been shown to deter the western pine wood-boring beetle *Dendroctonus brevicomis* (Erbilgen et al. 2008). The effect of acetophenone could be tested in other insects interacting with *A. majus*. Hence, acetophenone may potentially repel herbivores such as the weevil *Rhinusa vestita* (that lays its eggs into the fertilized ovaries) or the caterpillars of the butterfly *Mellicta deione* (that feeds exclusively on wild snapdragon leaves, C. Thébaud pers. comm.). Acetophenone would be clearly advantageous as a deterrent compound of *Rhinusa vestita*, as this weevil may use flower scent to localize its nursery flowers. Preliminary tests showed that snapdragon leaves do not emit acetophenone. If *Mellicta deione* is deterred by floral acetophenone this would explain why some plants are entirely defoliated but still maintain their flowers.

In conclusion, we have shown that wild *A. majus* differ from *A. majus* cultivars in the composition of flower scent. *A. m. pseudomajus* and *A. m. striatum*, the two natural

subspecies of *A. majus*, emitted different scents, which were discriminated by naive floral visitor such as *B. terrestris*. Bumblebees innately avoided acetophenone, the main VOC in *A. m. pseudomajus*, absent from the floral scent of *A. m. striatum*. These findings point to a crucial role of floral VOCs in the evolutionary ecology of *Antirrhinum majus*.

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Table 1: Occurrences (in %) and emission rates (mean and standard error, in $\mu g\, g^{-1}_{DW}\, h^{-1}$) of floral VOCs in the two *A. majus* subspecies. The last column shows the significance of a randomized two tail test on the occurrence of VOCs: if $p<0.025$ the VOC is more frequent in *A. m. pseudomajus* and if $p>0.975$ it is more frequent in *A. m. striatum* (NS non-significant test).

	***A. m. pseudomajus* (n=40)**		***A. m. striatum* (n=30)**		**Test on the occurrences**
	Occurrence (%)	Emission rate µg $g^{-1}_{DW}\, h^{-1}$	Occurrence (%)	Emission rate µg $g^{-1}_{DW}\, h^{-1}$	
Fatty acid derivatives		**47.16 ± 15.16**		**45.25 ± 14.57**	
Aldehydes					
2-methyl-propanal	25	11.31 ± 6.42	47	5.84 ± 2.35	NS
3-methyl-butanal	33	4.85 ± 2.78	20	2.22 ± 1.57	NS
Pentanal	100	4.60 ± 0.49	100	5.71 ± 0.75	NS
Z-3-hexenal	65	0.21 ± 0.06	83	0.33 ± 0.11	NS
hexanal	100	1.33 ± 0.20	100	1.88 ± 0.36	NS
heptanal	100	0.97 ± 0.17	97	1.19 ± 0.28	NS
Octanal	100	1.45 ± 0.17	83	2.10 ± 0.57	***p*<0.025**
Nonanal	100	9.68 ± 1.68	50	7.20 ± 2.99	***p*<0.025**
Decanal	58	0.99 ± 0.25	37	2.24 ± 1.17	NS
Alcool					
1-pentanol	83	0.58 ± 0.15	50	0.37 ± 0.11	***p*<0.025**
Alcenes					
1,3,5-cycloheptatriene	100	1.31 ± 0.30	100	1.65 ± 0.32	NS
1-octene	68	0.34 ± 0.11	23	0.17 ± 0.09	***p*<0.025**
Alkanes					
1,1-diethoxy-ethane	53	0.17 ± 0.05	33	0.84 ± 0.41	NS
Nonane	100	0.48 ± 0.13	97	0.84 ± 0.26	NS
Decane	45	0.38 ± 0.15	43	0.22 ± 0.07	NS
Dodecane	38	1.04 ± 0.31	37	1.07 ± 0.51	NS
Esters					
Ethyl acetate	8	0.03 ± 0.02	13	0.20 ± 0.10	***p*>0.975**
Hexyl acetate	8	0.11 ± 0.07	20	0.44 ± 0.32	NS
Ketones					
2-butanone	98	7.26 ± 1.74	100	10.72 ± 2.29	NS
Ether cyclic					
Eucalyptol	5	0.07 ± 0.06	3	0.02 ± 0.1	NS
Nitrogen-containing compounds		**1.90 ± 0.38**	**33**	**1.14 ± 0.63**	
syn-3-methyl-butyl-aldoxime	68	1.90 ± 0.38	33	1.14 ± 0.63	***p*<0.025**
Monoterpenes		**101.57 ± 13.39**		**103.67 ± 31.14**	
Cyclic					
α-pinene	58	0.25 ± 0.10	70	0.33 ± 0.07	NS
β-pinene	30	0.06 ± 0.02	27	0.08 ± 0.04	NS
p-cymene	8	0.25 ± 0.16	30	0.40 ± 0.18	***p*>0.975**
Limonene	95	1.22 ± 0.47	97	2.69 ± 0.69	NS
γ-terpene	33	0.20 ± 0.10	20	0.45 ± 0.21	NS
Non-cyclic					
β-myrcene	100	12.82 ± 1.58	100	14.27 ± 3.72	NS
(Z)-β-ocimene	100	1.54 ± 0.49	77	1.47 ± 0.39	***p*<0.025**
(E)-β-ocimene	100	78.91 ± 9.22	100	79.99 ± 24.02	NS
3,4-dimethyl-2,4,6-octatriene	75	3.27 ± 0.63	40	1.95 ± 0.85	***p*<0.025**
(E,E)-2,6-dimethyl-1,3,5,7-octatetraene	48	2.10 ± 0.53	20	1.54 ± 0.86	***p*<0.025**
Irregulars					
6-methyl-5-hepten-2-one	100	0.95 ± 0.09	97	0.50 ± 0.11	NS
Benzenoids		**339.86 ± 56.24**		**0.18 ± 0.08**	
Acetophenone	100	337.39 ± 55.44	0	0.00	***p=0***
Benzaldehyde	100	0.88 ± 0.08	0	0.00	***p=0***
Methyl benzoate	20	1.47 ± 0.65	0	0.00	***p*=0**
Hemimelitene	15	0.03 ± 0.02	10	0.05 ± 0.04	NS
Mesitylene	15	0.09 ± 0.05	27	0.13 ± 0.04	NS
Total		**490.49**		**150.24**	

Fig. 1 Floral phenotypes of the two subspecies of the wild *Antirrhinum majus* species, *A. m. striatum* (left) and *A. m. pseudomajus* (right)

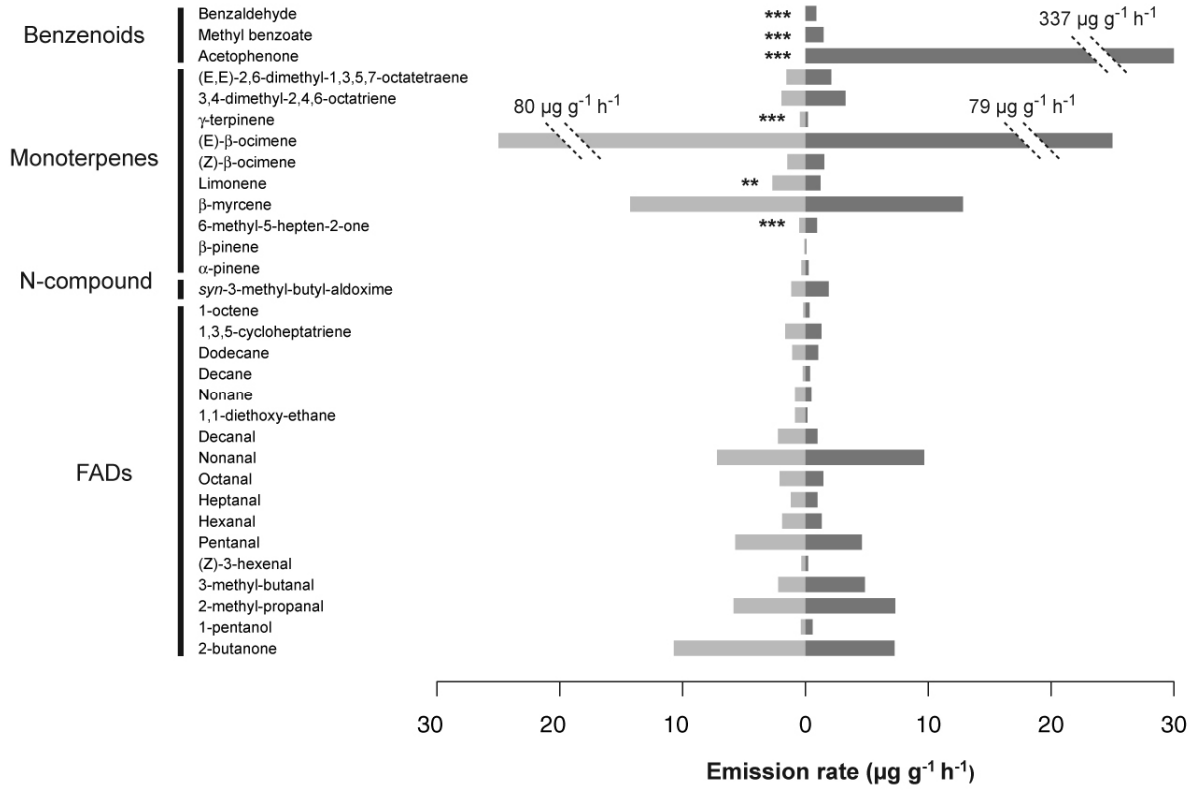

Fig. 2 Mean emission rates (µg g^{-1}_{DW} h^{-1}) of the floral VOCs emitted by *A. m. pseudomajus* (dark grey) and by *A. m. striatum* (light grey). Asterisks mark the six VOCs for which the emission rate significantly differed between the two subspecies.

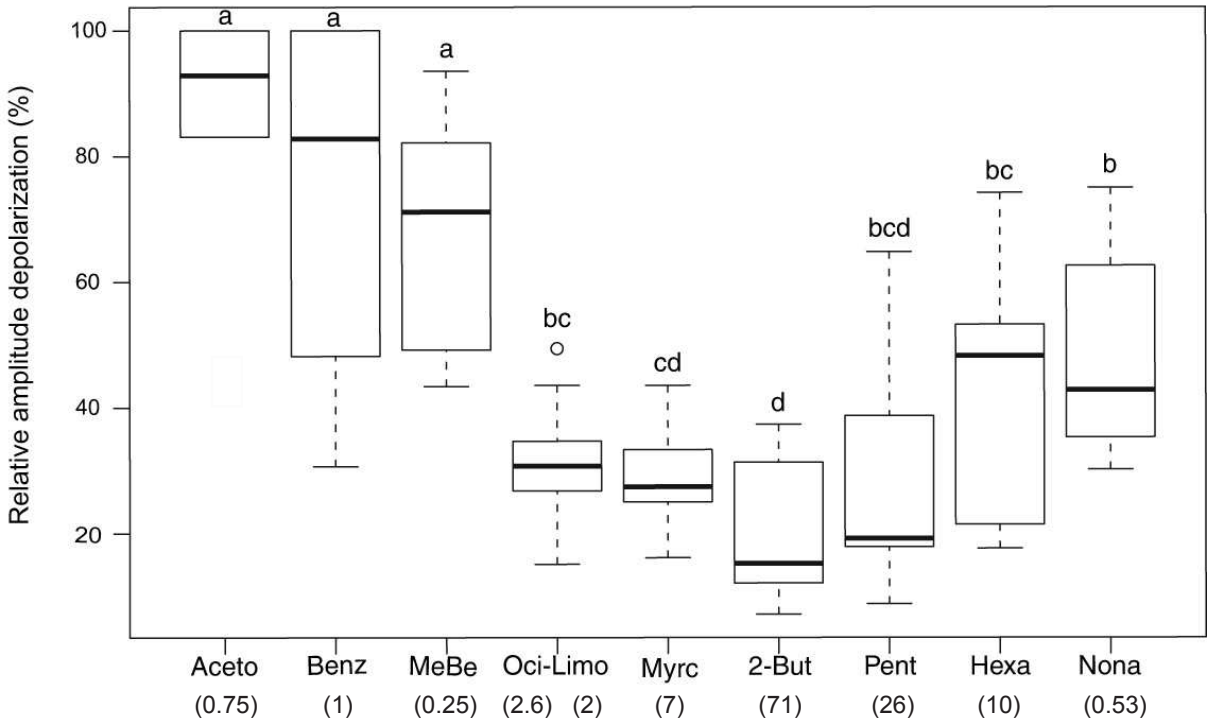

Fig. 3 Relative amplitude of antennal electric activity (i. e. depolarization) of a total of 10 bumblebee antennae for the ten synthetic VOCs analysed by electro-antennography method (EAG). The ten tested VOCs were: *Aceto* Acetophenone, *Benz* Benzaldehyde, *MeBe* Methyl benzoate, *Oci Lim cis*-ocimene and Limonene, *Myrc* Myrcene, *2-But* 2-butanone, *Pent* Pentanal, *Hexa* Hexanal, *Nona* Nonanal. Their respective value of vapor pressure are indicated between brackets (mmHg at 20°C, Perry and Green 1997). A pairwise comparison of the signal was performed: box plots with the same letter at the top were not significantly different.

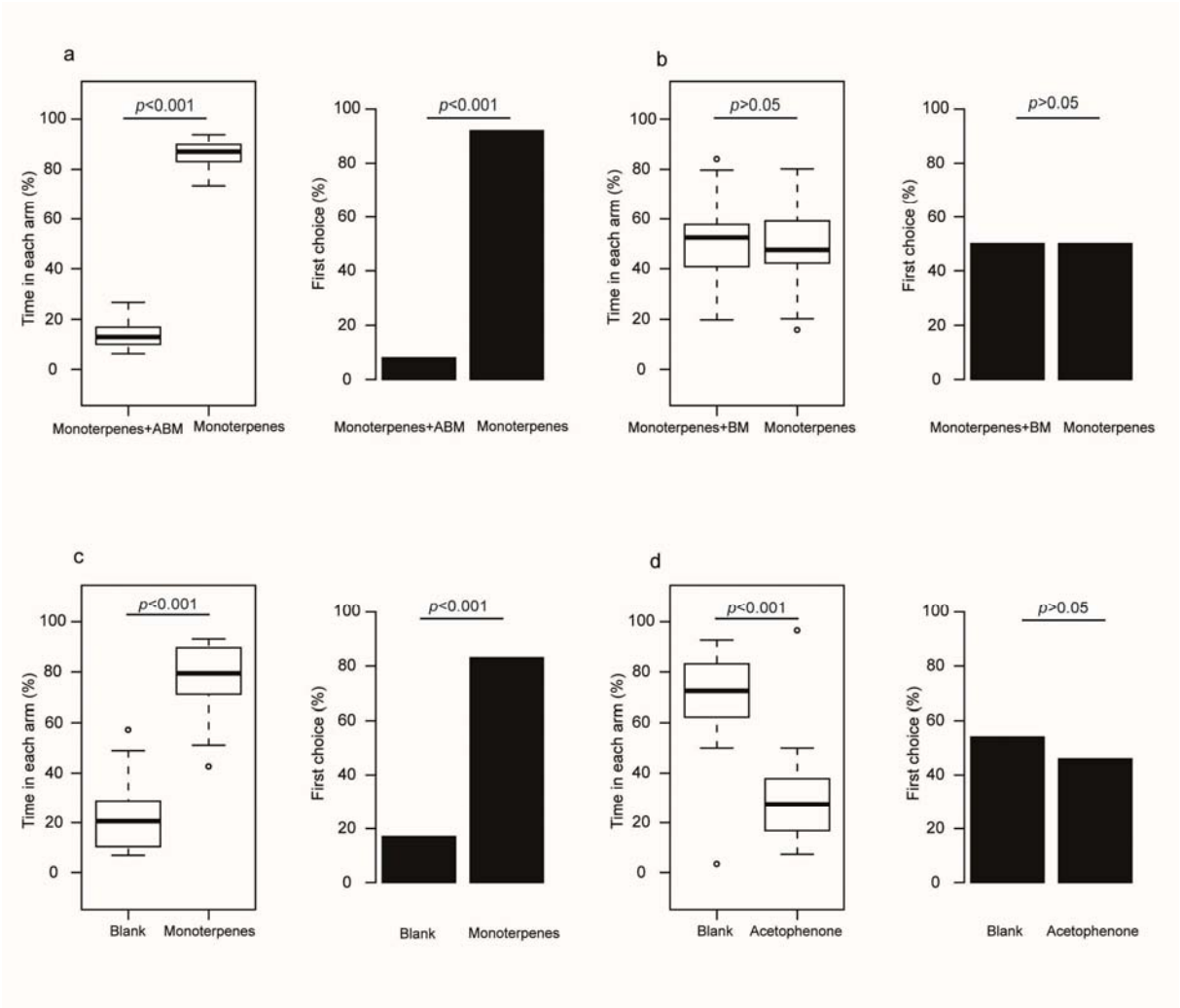

Fig. 4 Percent of time spent by bumblebees in each arm of the Y-maze (box plots) excluding the time spent in the entrance, and percent of the first choice (histograms) for the four pairs of volatile chemical signals tested. **a** First test contrasted three monoterpenes (*cis*-ocimene, limonene and myrcene, henceforth 'monoterpene mixture') to the same monoterpene mixture plus three benzenoids (A, acetophenone, B, benzaldehyde and M, methyl benzoate) (n=12) **b** Second test contrasted the monoterpene mixture to the monoterpene mixture plus methyl-benzoate and benzaldehyde (i.e. all benzenoids excepted acetophenone) (n=12) **c** Third test contrasted the monoterpene mixture to a blank (n=12); finally **d** Four test contrasted acetophenone to a blank (n=12).

Article 4

Wild pollinators learn the use of *Antirrhinum majus* flower scents

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In preparation

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Abstract

The present study aims to evaluate how variations of flower scent between two reproductively isolated subspecies of *Antirrhinum majus* can influence pollinator attraction. The natural flower scent of *A. m. striatum* and *A. m. pseudomajus*, distinct by their composition of floral volatile organic compounds (VOCs), were presented to insects in dual olfactory choice test using a Y-maze set-up. After determine which insect species were pollinators of both subspecies, we tested if naïve bumblebees (commercialized *Bombus terrestris*) display innate preference and, if yes, whether similar patterns can be observed with the two main classes of pollinators (*Bombus* sp. and Halictids) from the wild. We evidences that the naïve bumblebees innately preferred the flower scent of *A. m. striatum*. Wild pollinators of *A. majus* displayed the same olfactory preference when they were trapped in a population of *A. m. striatum* whereas they displayed a reversal preference when they came from a population of *A. m. pseudomajus*. Thus, wild pollinators are naturally conditioned to use the flower scent of their *A. majus* subspecies of environment despite the initially aversive effect of *A. m. pseudomajus* flower scent. The most parsimonious explanation is that pollinators learn using

these olfactory cues by associating it to the nectar found in the snapdragon flowers. These results represent indirect evidences that flower scents could be implicated in the reproductive isolation of the two *A. majus* subspecies by inducing a phenomenon of constancy of visit.

Key Word: flower scent, *Antirrhinum majus*, pollinator, odor preferences, reversal choice

Introduction

Among the floral cues for pollination, floral scents stand out for their complexity and their variation in volatile organic compounds (VOCs) (Jürgens 2009, Shiestl 2010). They act in diverse ecological interactions that plants keep with organisms in their environment (Raguso 2008), hence their multiple functions such as, pollinator attraction, fostering to pollinator fidelity and, defense against antagonists (Raguso 2009, Wright and Schiestl 2009). Even though the role of floral scent is well studied, the evolutionary factors that mediate the composition in VOCs in flowering plant are unclear compare to the other floral traits like color and form (Whitehead and Peakhall 2009). Some of the main current questions are: do pollinators influence the evolution of flower scent? Are pollinators predisposed to favor olfactory cue from flowers?

The characteristics of a specific flower scent, such as number, type of VOCs, and their relative proportions, depend on genetic (e.g. Raguso et al. 2006, Jürgens 2009) and environmental conditions (Majetic et al. 2009). Biosynthetic production of VOCs could be complex machinery that is subjected to pleiotropy (Kessler and Halitschke 2009). These non-adaptive processes, that do vary flower scent among conspecifics and populations, make it difficult to lead to a reliable olfactory signal needed for pollinator-mediated selection (Knudsen and Gershenzon 2006, Whright and Schiestl 2009). However, it currently begins to appear that occurrence and variation of floral VOCs can be correlated to pollination strategies suggesting that flower scent can be the target of selection (Jürgens 2009, Schiestl 2010).

Insects depend upon odors for vital activities such as feeding and mating. They have the capacities to detect and learn various VOCs because they are well adapted to deal with

odors (Chapman 1998). They can remember them for longer than visual cues (Kunze and Gumbert 2001). Generalist pollinators such as honeybees and bumblebees commonly associate odors with food and are renowned for their olfactory learning abilities (Giurfa 2007). Flower scent is therefore an important floral cue for pollinators that learn to associate it with rewards (Wright and Schiestl 2009). These two authors also argued "that a plant's emission of scent as a mean of advertising floral rewards and a pollinator's attendance to scent signals provide fitness advantages to both plant and pollinator which exceed those resulting from the use of visual signals alone".

Here we aim to test the relevance of flower scents of two wild subspecies of *Antirrhinum majus* in their relationship with pollinators. What makes ideal model, *A. majus*, for the present study, is that snapdragon display an unresolved reproductive isolation between *A. m. pseudomajus* and *A. m. striatum* for which the role of pollinators has been speculated as important by previous studies (Whibley et al. 2006, Tastard et al. 2008, Andalo et al. 2010). Moreover, these two subspecies differ in floral color and emit reproducible different composition of floral VOCs mainly due to acetophenone present only in *A. m. pseudomajus* (Suchet et al. submitted). This compound has been shown as aversive for naïve bumblebees (*Bombus terrestris*) using artificial blend of VOCs (Suchet et al. in press). *B. terrestris* were chosen because it was observed like the most frequent flower visitor of *A. majus* although any study characterized the snapdragon pollinator assemblage.

To test therefore whether the pollinators of *A. m. striatum* and *A. m. pseudomajus* use the two flower scents of these isolated subspecies, we ask the following questions: (i) What are the pollinators of each subspecies of *A. majus*? (ii) Are naïve bumblebees (*Bombus terrestris*) also innately attracted and influenced by the two flower scents naturally emitted by *A. majus*

subspecies? (iii) Are experienced pollinators recognized the natural flower scent of the *A. majus* subspecies of their origin, and do they prefer it between the two floral odors?

Material and Method

Plant species Antirrhinum majus is a semi-perennial plant (Scrophulariaceae) growing at an elevation of 0-1600 m asl in the Eastern Pyrenees Mountains between France and Spain. It starts to flower in May for population in low altitude and in early June for those of higher altitude until the end of June or early Jully. Its zygomorphic flowers are closed and self-incompatible (Andalo et al. 2010). They are highly smelted (Suchet et al. submitted) and produce nectar stocked at the bottom of the corolla without spur. The distribution of the two subspecies is non-overlapping. Both subspecies are therefore not sympatric but, as the distribution area of *A. m. striatum* is enclosed in the much larger distribution area of *A. m. pseudomajus*, few contact zones exist (Suchet et al. submitted). It leads sometimes to hybrid zones. One of them is particularly studied because in this stable hybrid zone the floral color has been speculated to be under selection (Whibley et al., 2006).

Pollinator censuses In May and June 2009, preliminary pollinator censuses were performed in eight *A. majus* populations (four for each subspecies) varying in altitude with one census per population of at least 500 minutes of observations each. Unfortunately, a logistic problem occurred at the insect determination stage, limiting the identification of these data at the insect genus level for each subspecies. Nevertheless, these censuses offering the first species list of insect visitors of *A. majus*. Using these data, we used estimators to determine the number of visitors necessary to obtain an adequate estimation of *A. majus* visitor assemblage (Gotelli

and Colwell 2001). In June 2010, we focus on the two populations around the most studied hybrid zone (Whibley et al. 2006) in Toses Valley in Spanish Pyrenees Mountains, one of *A. m. striatum* (Collada de Toses 42.36N, 1.92E, 1492m asl) and the other of *A. m. pseudomajus* (Pardines 42.31N, 2.19E, 1118m asl). Censuses were conducted under sunny conditions with no wind. As the distribution of *A. majus* populations is fragmented at this site, static observations were carried out in front of attainable patches of at least of 50 open flowers each. Two patches in the yellow flowered population and three patches in the red flowered population were followed for three censuses during the flowering season. At the first visit, censuses consisted to trap and referenced any insect that arrived to enter to an *A. majus* flower comprised in a patch and to bag budding inflorescences in order to observe virgin flowers of pollinator visit at the next visits. At the second and third visits, all insects visiting *A. majus* flowers were caught as well and the concerned flowers that were virgin were marked and bagged again. Insect determination was kindly carried out by Prof. Pierre Rasmond (Mons University, Belgium). After the flowering season, a last visit was spent to collect the fruit sets of the observed flowers to perform the efficiency of each species of visitors. Moreover to know which visitor species are pollen vector, a study of presence/absence of *A. majus* pollen grains on the back of the different visitor species was performed as well help to Prof Elise Van Campo and her team. Pollen grains were trapped from the back of the insects by swabbing them with jelly cubes according to the Dafni et al. (2005) method. To be able to observe pollen grain morphology under a microscope, these samples were then treated by acetolysis according to the Erdtman's method (1960).

Olfactory choice tests A Y-maze set-up coupled with natural flower scents were used to test if pollinators have olfactory preference between the two floral VOC compositions of the two *A. majus* subspecies. The Y-maze was in glass and cylindrical with a diameter of 4cm. The main

entrance of the Y-maze measured 7cm length and the two arms measured 16cm length each. Cap covered by an inert material (nalophan) were fixed at the extremities of the Y-maze. They were linked by inert tubes (teflon) to a battery-operated vacuum pump from the side of the main entrance and to two inert bags (nalofan) from the side of the two arms containing the inflorescences. Thus the two scents emitted by *A. m. pseudomajus* and *A. m. striatum* were pumped from the arms through the entrance of the Y-maze (with a flow rate of 600 $ml.min^{-1}$).

Two pairs of plants, each composed by the two *A. majus* subspecies, were chosen for their similar morphology in the field in June 2010 and put in pot 15 days before the experiment. They were kept in the same field site with special installation that prevents insect interaction, including pollinator visits. When these four plants were full flowering, we trapped the floral VOC emissions *via* the Y-maze set-up by using Tenax TA 60/80 traps between the main entrance and the pump. To control if the tested flower scents were characteristic of the *A. majus* subspecies the VOC samples were then analyzed by GC-MS based on Suchet et al. (submitted) analytical method.

Innate choice test A colony of worker bumblebees (*Bombus terrestris*) was purchased (from Koppert®, Berkel en Rodenrijs, Netherlands) and left 15 days in the field as well for acclimation. We tested 15 bumblebees in the Y-maze set-up, seven with the first pair of *A. majus* plants and eight with the second. To familiarize the bumblebees with the experimental set-up, we pre-trained them to the maze before testing them with VOCs. Bumblebees stayed between five to six hours in the dark, without food in ventilated individual plastic tubes. Each individual was then released in the entrance arm of the maze. During this pre-training stage, 20 µl of 50% sugar solution was offered at the intersection point of the maze. The bumblebee could freely move within the maze, and it easily found the drop of sucrose solution and

consumed it. After this training, the bumblebee was replaced into its plastic tube and taken out from the maze. Between any tests, the Y-maze was carefully cleaned using an ethanol solution. After a second pre-training visit, the experienced bumblebee was motivated to search for sucrose solution within the maze, and the olfactory tests could then begin. Both pre-training and test sessions were carried out in the dark (under red light), to favor the bumblebees' use of olfactory cues that consequently were walking.

For each bumblebee, we recorded its first choice (i.e. which arm was chosen first) and the proportion of time spent in each arm of the maze during two minutes of observation (time spent in one arm divided by the total time spent in both arms of the maze). Each test was duplicated with the same bumblebee, swapping the presentation side of the olfactory stimuli.

Learnt choice test To test if experienced pollinators use the flower scents of *A. majus*, wild pollinators were tested in the olfactory choice test as previously described at an advanced stage of flowering of the wild populations. The same plants than before were used for their flower scent stimuli in the Y-maze. Pollinators were tested immediately after their trapping on wild *A. majus* flowers. No pre-training was carried out because of the aggressiveness of wild insects. *Bombus* sp. and halictids from a population of *A. m. pseudomajus* and also from a population of *A. m. striatum* were tested. Other genera of the pollinator assemblage were too infrequent or difficult to trap to robustly test them.

Statistical analyses A binomial test based on the first choice of the Y-maze test was computed to determine if the bumblebees exhibit a preference for one of the two flower scents. Differences in the cumulated time spent in the two arms were tested using a Student t-test. We also tested whether swapping the two same flower scents between the two arms of the Y-maze yielded the same result for the two recorded variables, and if the two pairs of plants as

well. To this end, we applied a binomial test for the first choice and a Fisher test for the residence time.

Results

Pollinator assemblage Censuses of flower visitors in 2009 and 2010 showed that the frequency of visit in *A. majus* is low because we observed in average 1.4 visitors per hour for 50 snapdragon open flowers (Table 1). It differed in function of the subspecies in 2009 with a higher frequency in *A. m. pseudomajus* (2 visitors per hour) than in *A. m. striatum* (1.4 visitors per hour) but not in 2010. Figure 1 shows that the genus *Bombus* and the clade of halictids are the most represented among the *A. majus* flower visitors. The two years of observations converged to the fact that bumblebees are more abundant in *A. m. pseudomajus* than in *A. m. striatum* and, inversely halictids are more abundant in *A. m. striatum* than in *A. m. pseudomajus* (Figure 1). We found a total of 15 species/subspecies of visitors of *A. majus* flowers more the halictids that were not identified until the species level (Table 2). According to the estimators 18 to 25 species of visitors overall would constitute the visitor assemblage of *A. majus*. Likely few infrequent visitor species have therefore not been observed.

The study of pollen load showed that the seven visitor species observed in 2010 loaded pollen grains of *A. majus* on their back; they can therefore be considerated as pollen vectors. Moreover, the collect of the fruit set after visits of bumblebees and halictids shows that these insects provoked fructification; they can be therefore considerate as pollinators. The information is not available for *Xylocopa violacea* (carpenter bees) and *Rhodanthidium sticticum* because they were not observed on virgin flowers. However carpenter bees are certainly pollinator because they were often full of pollen after visiting *A. majus* flowers and were observed to visit then other *A. majus* flowers.

Innate preference GC-MS analyses of the floral VOC emissions sampled in the Y-maze as controls of the tested olfactory stimuli validated the specific profiles of *A. m. pseudomajus* and *A. m. striatum*. An analysis of the first choice in the Y-maze showed that there was no difference in performance depending on the side of flower scents presentation and on the pair of plants so that data for these tests were pooled. When naïve bumblebees had the choice between the natural flower scents of *A. m. pseudomajus* and of *A. m. striatum*, they significantly chose first the flower scent of *A. m. striatum* (83%, *p*-value<0.01, Figure 2).

An analysis of the time spent in each arm showed that there was no difference in performance depending on the side of stimulus presentation and on the pair of plants. Hence, data for these tests were also pooled for this variable. As for the first choice, naïve bumblebees significantly spent more time in the arm of the Y-maze in presence of the *A. m. striatum* flower scent than in presence of the *A. m. pseudomajus* flower scent (t=11.9, df=58, *p*-value<0.01, Figure 2).

Learnt preference Behavior of the wild bumblebees and halictids in the Y-maze were not significantly biased by the swapping of subspecies flower scents and the pairs of plants used for their floral scent. Data of these tests were therefore pooled in the following statistical tests. When bumblebees and halictids were trapped in a population of *A. m. striatum* they significantly chose first in the Y-maze the flower scent of *A. m. striatum* as naïve bumblebees (respectively 80%, *p*-value<0.05 and 79%, *p*-value<0.05, Figure 3). In contrast, in a population of *A. m. pseudomajus*, bumblebees and halictids chose significantly first the flower scent of *A. m. pseudomajus* (respectively 79%, *p*-value<0.05 and 100%, *p*-value<0.001, Figure 3). The analyses of the residence time in the arms of the Y-maze followed the same reversal trends than the first choice. Indeed, bumblebees and halictids from a population of *A. m. striatum* spent significantly more time in presence of the flower scent of *A. m. striatum*

(respectively t=-6.1, df=18, *p*-value<0.001 and t=-10.5, df=26, *p*-value<0.001, Figure 3). Bumblebees and halictids from *A. m. pseudomajus* spent significantly more time in the arm of the Y-maze were the flower scent of *A. m. pseudomajus* is present (respectively t=8.8, df=26, *p*-value<0.001 and t=5.5, df=14, *p*-value<0.001, Figure 3).

Discussion

The present study evidences that the pollinators of *A. majus* are influenced by the distinct flower scents of *A. m. pseudomajus* and *A. m. striatum* and that the most parsimonious explanation of our result is that pollinators learn using these olfactory cues. Indeed, the innate preference of *A. m. striatum* fragrance for naïve bumblebees (*B. terrestris*) was primarily confirmed compare to the previous study using artificial VOC mixture (Suchet et al. submitted). In addition, when the two more abundant types of pollinators of *A. majus* that differed in relative frequency between the subspecies (*Bombus* sp. and Halictids) were also tested, they show to be naturally conditioned to use the flower scent of their subspecies of origin, despite the aversive effect of *A. m. pseudomajus* fragrance. These results represent indirect evidences that flower scents could be implicated in the reproductive isolation of the two *A. majus* subspecies by inducing a phenomenon of constancy of visit.

The fact that naïve bumblebees are already significantly influenced by *A. majus* flower scents whereas they never deal with them indicates that they are predisposed to be sensitive to these odors. It is known that bumblebees produce some VOCs that they use not only for reproduction but also for feeding and foraging (Mollet et al. 2009). For example, they basically mark the visited flowers using footprints to not spend time if they go back during their foraging to these flowers too recently depleted of nectar (Stout and Goulson 2001). *A. m.*

striatum flower scent is constitutes by 69% of monoterpenes represented by 77% of (E)-β-ocimene (Suchet et al. submitted). If it is innately more attractive for bumblebees, it can be due to the presence of ocimene because it is one of the compounds of bumblebee pheromone (Mollet et al. 2008). Commercialized bumblebees had potentially never experienced other odors than their congeners in the hive. Consequently, face to the olfactory choice imposed in the Y-maze set-up, it is probably intuitive to follow familiarized odors. However these monoterpenes are also present in similar proportions in the flower scent of *A. m. pseudomajus* (Suchet et al. submitted) and bumblebees significantly avoid them when they have the choice between the two floral scents. This avoidance is therefore well due to the additive VOCs in *A. m. pseudomajus* flower scent like demonstrated in the previous study with the aversive effect of acetophenone, the most abundant floral compound in *A. m. pseudomajus*.

If *A. m. pseudomajus* flower scent is suddenly attractive when tested with wild pollinators from *A. m. pseudomajus* population. It therefore suggests that bumblebees have learnt it. Although *A. m. striatum* flower scent is still more attractive for both wild *Bombus* sp. and halictids when they come from yellow-flowered population, wild *Bombus* sp. and halictids from magenta-flowered population significantly prefer the flower scent of *A. m. pseudomajus*. Such reversal preference has already been observed (e.g. Guerrieri et al. 2005, Riffell et al. 2008). It is encounter when insects are conditioned to associate odors with food. Pollinators may basically establish their strategy of foraging on this combination of innate preferences and learning processes to optimize their fitness (Riffell et al. 2008). Here, we hypothesize that pollinators display a shift of odor preference when they come from different *A. majus* subspecies because they experienced the reward associate to the snapdragons olfactory cues and they learnt to associate the flower scents to the nectar of *A. majus*. This opposite preferences of pollinators for flower scents of the two subspecies of *A. majus*

according to their origin tend to the idea that pollinator could display a behavior of constancy for one *A. majus* flower scent when they have the opportunity to switch between both subspecies. This lets thus think that flower scent could reinforce the reproductive isolation of the two *A. majus* subspecies. One way to test this hypothesis would implicate to condition two hives of worker bumblebees for each *A. majus* subspecies flower scents and then to record their sequence of visits in a free flying experiment with co-occurrence of *A. m. striatum* and *A. m. pseudomajus*. An analysis of the quality of nectar of these two subspecies would be also important to determine the associative patterns that could happen between floral color, scent and nectar.

Emitting flower scent appears of a first importance for *A. majus* since it is attractive for pollinators anyway. No doubt that this floral trait fills its function in the present model. It then may be hypothesized that flower scent conveys a good competitive value of *A. majus* for pollinators face to the services that are also offered by other flowers in the same plant community. However, *A. majus* success for pollination may be beyond its control because its success depends on not only the efficiency of its own floral advertising but also of the efficiency of the signals of co-existing plants (Chittka and Raine 2006). How much is adaptive floral scent, itself and in co-variation with color and nectar traits? Do floral VOCs of *A. majus* play other function than pollinator attraction, such as defensive function? Does mainly adaptation shape its variation or is it more mediated by neutral processes? This is such questions that open the present study.

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Table 1 Summary of pollinator censuses of *A. m. striatum* and *A. m. pseudomajus* in 2009 and 2010.

		Total number of minutes of observation	Total number of visitors	Frequency of visit (visitor per h)
2009	*A. m. striatum*	1814	43	1.4
	A. m. pseudomajus	2167	72	2.0
2010	*A. m. striatum*	2440	42	1.0
	A. m. pseudomajus	1752	29	1.0

Table 2 List of visitor species and pollinator species observed on the two *A. majus* subspecies. nd, means "no data" and corresponds to the data of 2009 where no study of pollen and fruit set were done.

List of *A. majus* visitor species	Pollen vector	Fructification after visit
Anthophora aestivalis	nd	nd
Bombus hortorum	X	X
Bombus humilis quasimuscorum	nd	nd
Bombus lucorum	nd	nd
Bombus pascuorum maculatus	nd	nd
Bombus ruderatus autumnalis	nd	nd
Bombus ruderatus ruderatus	nd	nd
Bombus rupestris vasco	nd	nd
Bombus terrestris dalmatinus	X	X
Bombus terrestris lusitanicus	nd	nd
Bombus terrestris terrestris	X	X
Bombus muscorum	X	X
Rhodanthidim septemdentatum	nd	nd
Rhodanthidium sticticum	X	nd
Xylocopa violacea	X	nd
Halictids	X	X

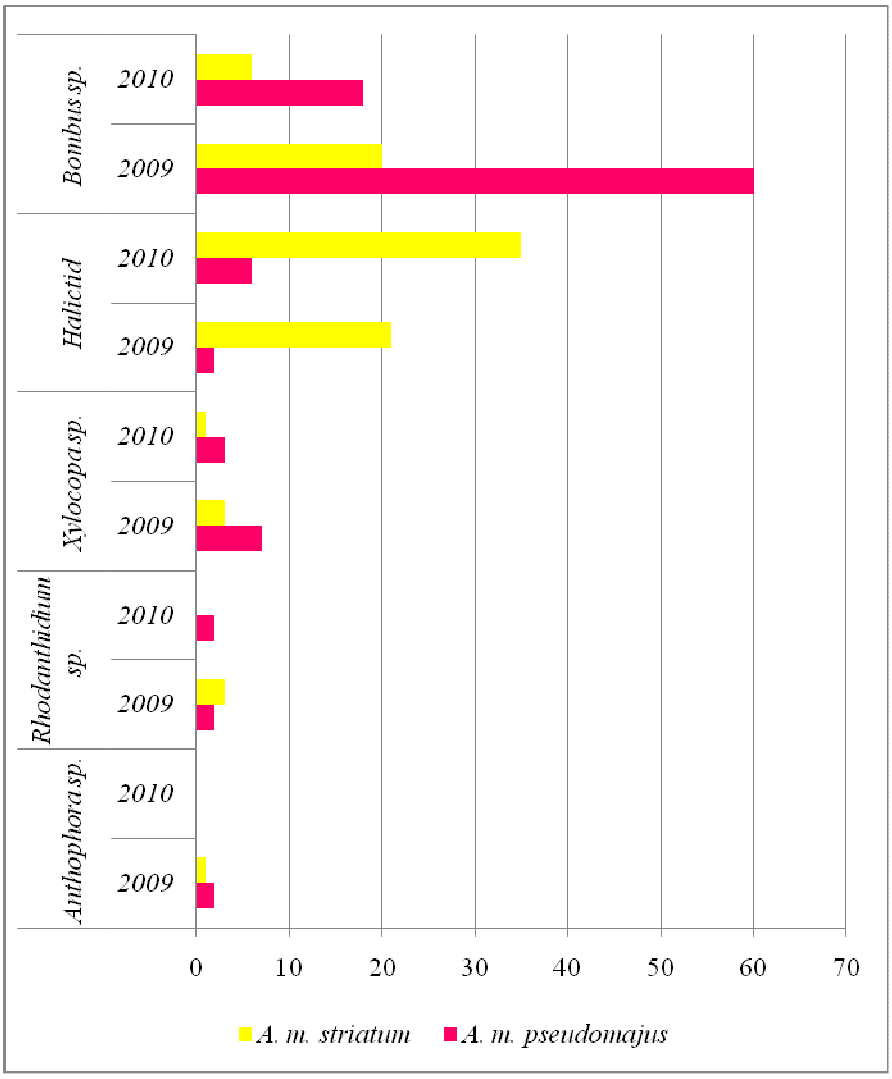

Figure 1 Abundance of flower visitor per insect genus/clade observed in *A. m. striatum* and *A. m. pseudomajus* in 2009 and 2010.

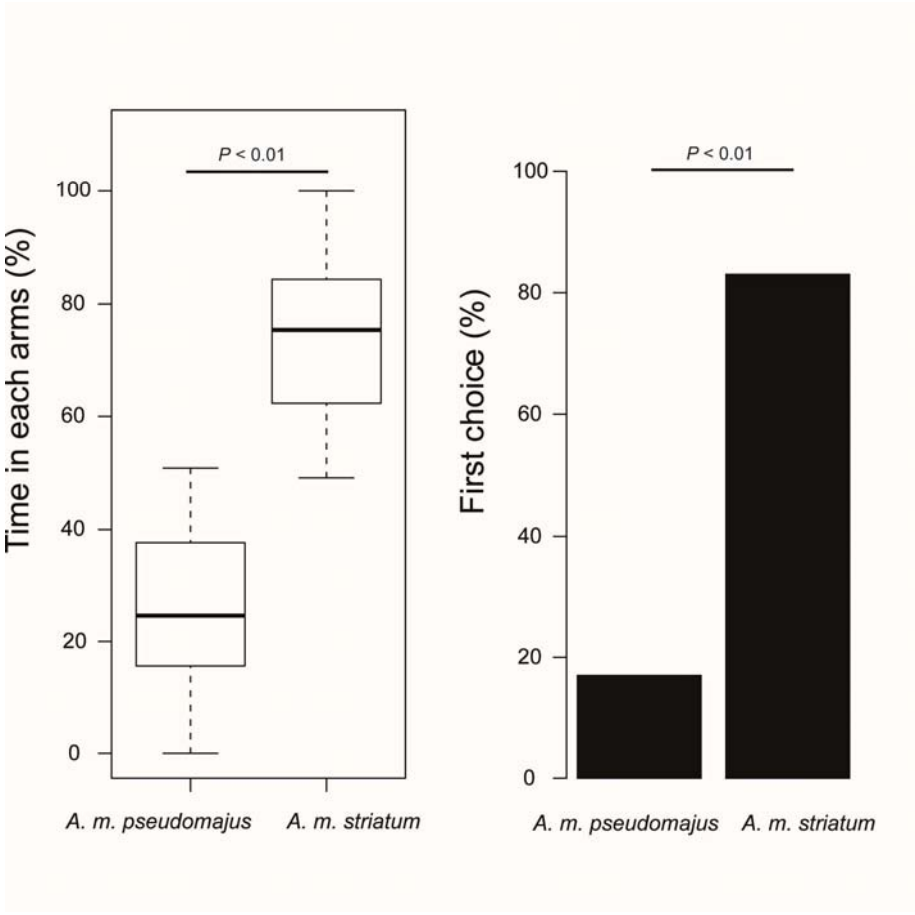

Figure 2 Percent of time (box plots) and first choice (bar plots) shown by naïve bumblebees (n=15) for each arm of the Y-maze when they have to make a choice between the flower scents of the two *A. majus* subspecies.

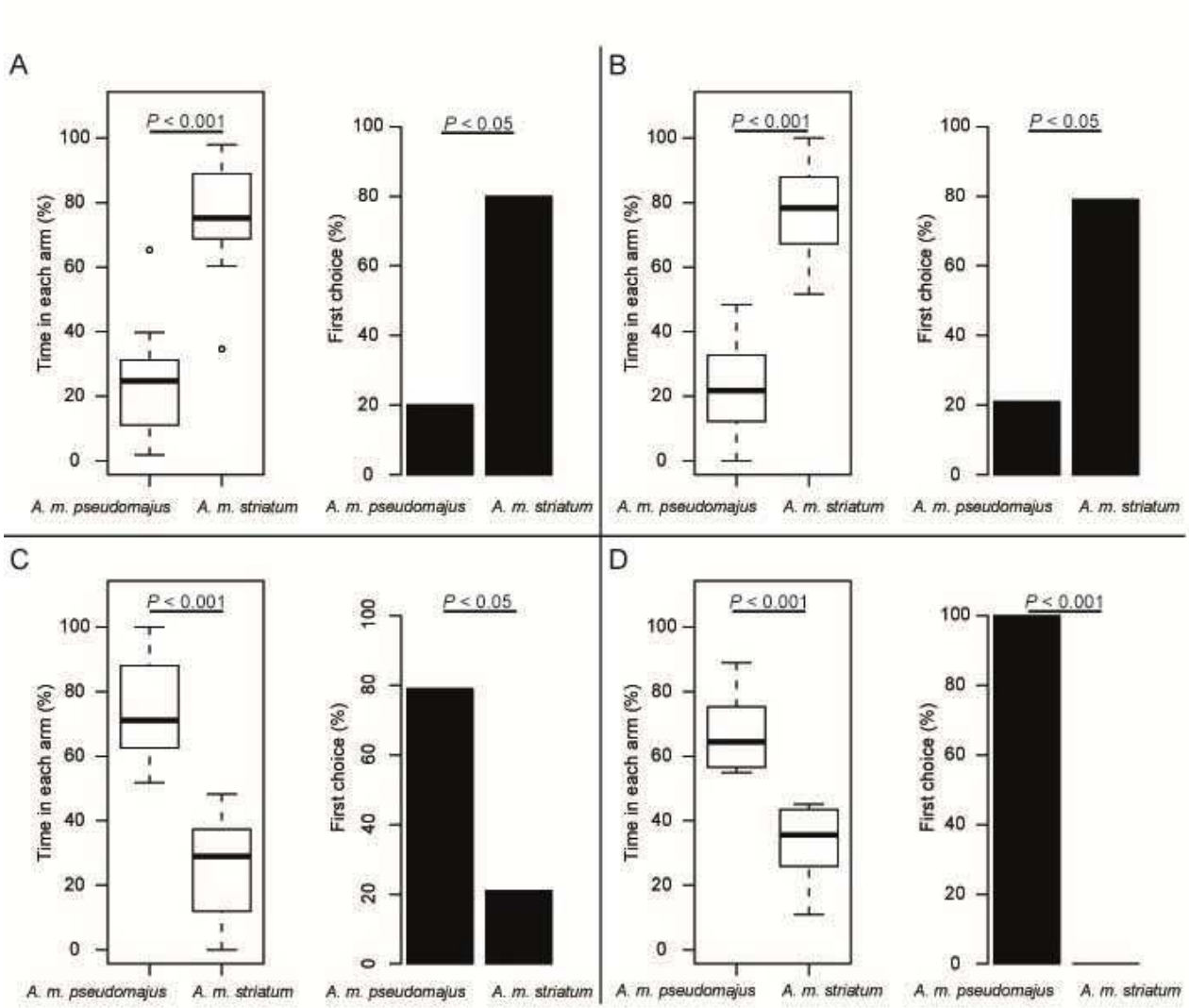

Figure 3 Percent of time (box plots) and first choice (bar plots) shown by bumblebees (A, n=5) and halictids (B, n=7) trapped in a population of *A. m. striatum*, and by bumblebees (C, n=7) and halictids (D, n=4) in a population of *A. m. pseudomajus*.

CHAPITRE 3

Les patrons associatifs entre le nectar et les odeurs florales chez *Antirrhinum majus*

Résumé

Parce que le nectar est la récompense majeure de la recherche de nourriture des pollinisateurs guidés par les signaux attractifs floraux, il conditionne l'interaction plante-pollinisateurs. Dans ce chapitre nous avons testé si les deux sous-espèces d'*A. majus* en isolement reproducteur rivalisent ou tendent à se spécialiser en offrant une quantité et une qualité de nectar différent en association avec les différences d'odeurs et de couleurs florales. Le nectar de toutes les fleurs cultivées en conditions contrôlées dans le chapitre 1 a été récolté et mesuré par capillarité à partir du bourrelet à nectar et de la base de la corolle. Une attention particulière a été mise œuvre pendant cette étape pour que le nectar ne s'évapore pas, et qu'aucune pollution ne vienne contaminer les échantillons afin d'analyser leur composés sucrés et protéinés. Les analyses HPLC ont révélé que la concentration en sucres et en acides-aminés ne différenciaient pas les deux sous-espèces alors que leurs volumes respectifs de nectar par fleur étaient différents. Nos résultats apportent de nouvelles informations et perspectives quant au caractère adaptatif de la différentiation florale des deux sous-espèces d'*A. majus*.

Article 5

Associative patterns between floral odor, color and nectar traits in two subspecies of snapdragon (*Antirrhinum majus*)

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In preparation

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Abstract

Nectar traits can condition the plant-pollinator interaction. Here we aimed to test whether two reproductively isolated subspecies of *Antirrhinum majus* from the wild compete for pollinators by offering different quantity and quality of nectar in association with their distinct floral odor and color. We measured the volume of floral nectar of *A. m. striatum* and *A. m. pseudomajus* grown in identical controlled conditions avoiding insect visits. Nectar samples were then analyses by HPLC analyses to detect any discriminant compositions and concentrations of sugar and amino-acid between the two subspecies. We found that the two *A. majus* subspecies significantly differed in volume of nectar per flower. However, sugar and amino-acid traits were not consistently different between the two subspecies. The observed difference of nectar production between *A. majus* subspecies induced several hypotheses

linked to floral CO2 emission and intrafloral temperature to test in favor of an adaptive process of this pattern.

Key Words: Nectar volume, nectar sugars, sugar concentration, nectar amino-acids, *Antirrhinum majus*, floral odor-nectar

Introduction

Most of the flowering plant species offer rewards to nectar-seeking pollinators to ensure their fecundation by fidelizing them (Mitchell 2004). Often highly concentrated in sugars, nectar is a major source of food for flower visitors. Glucose, fructose, hexose and sucrose are the most frequent sugars (Wykes 1951, Brandenburg et al. 2010). Nectar can also contain amino-acids and secondary metabolites such as alkaloids, phenolics or tannins (Baker and Baker 1975). Nectar sugars and amino-acids compositions and concentrations were often found to be representative of plant species based on a wide prospection of species (Wykes 1952, Baker and Baker 1975). Into the flower, the exhibition of nectaries that produce nectar is in respect of the plant species favoring access to specific pollinators (Brandenburg et al. 2010). However, the evolutionary significance of the floral nectar traits is poorly understood (Mitchell 2004). Nectar could be directly implicated in the complex selective pressures that shape flower phenotypes. Flowers display a multidimensional phenotype that addresses the challenge to attract pollinators, to compel visitors to an economic consumption of nectar, to repel the nectar-robbers and to defense against florivores and moistures (McCall and Irwin 2004, Kessler et al. 2008, Kessler et al. 2010). In the present work, we studied the nectar traits in the wild Pyrenean *Antirrhinum majus* species in order to test an evolutionary scenario hypothesis about the unresolved reproductive isolation mechanism of two snapdragon subspecies.

Sugars concentration has been related to pollinator type and plant family (Potts et al. 2003, Petanidou 2005). For instance, in 73 Mediterranean plant species the highest level of sucrose found in Lamiaceae is significantly preferred by bees and wasps whereas butterflies, syrphids, flies and beetles prefer lower sucrose concentrations in Liliaceae and Apiaceae

(Petanidou 2005). Both sugar composition and concentration of nectar are parameters that determine the number and type of pollinators among plant communities (Potts et al. 2003). Baker and Baker (1975) proposed that the amounts of amino acids in nectar are also connected to the pollination system because of proprieties of certain amino acids. More recently, Petanidou et al. (2006) have indeed shown that nectar of dry Mediterranean plants species highly concentrated of phenylalanine were more visited by long tongued bees like Megachilids likely because phenylalanine is an strong phagostimulant. Thus, nectar traits are assumed to be optimized for pollinators, but non-pollinators may also imposed selection by exploiting the reward (Strauss and Whittall 2006). Conflicting selective pressures are imposed if nectar-robbers are frequent and induce a lower concentration of sugars opposite to both the non-pollinator and pollinator preferences (Irwin et al. 2004). In *Nicotiana attenuata,* herbivores can be repelled by nectar-containing nicotine but nicotine also deter pollinator and attractive flower scent is needed to compensate the pollinator deterrence and to success pollination (Kessler et al. 2008). Therefore, although pollinators are probably the primary selective agents, direction and strength of selection can be mediated by selective pressures from co-occuring species in interaction resulting in conflicting selections. Nevertheless, the review of Mitchell et al. (2004) suggests that the literature is extremely poor concerning the heritability of nectar traits and that their genetic variation can be biased because nectar is responsive to environmental variations. Knowledge about variation of more various nectar traits is needed especially about the variation of nectar components because past studies primarily focus on nectar production rate (Mitchell et al. 2004).

Here, we tested whether the two subspecies *A. m. pseudomajus* and *A. m. striatum* differ in nectar quantity and quality to understand how these subspecies are maintained. The non-sympatric *A. m. pseudomajus* and *A. m. striatum* have no post-zygotic barriers (Andalo et

al. 2010) but they do not systematically hybridize when they come into contact in the wild. They synchronously flower and are easily distinguishable based on the floral color because *A. m. pseudomajus* has magenta flowers and *A. m. striatum* has yellow flowers. Reproductive isolation was also determined by abrut clines of these two floral color and one of a locus coding for the magenta pigment throughout a transect of a hybrid zone located between two parental populations (Whibley et al. 2006). Andalo et al. (submitted) found that *Bombus terrestris*, a common pollinator to the two subspecies that is able to discriminate flower colors of *A. majus* (Tastard et al. 2008), significantly prefers visiting parental floral colors, yellow and magenta, than the various range of hybrid floral colors (from pink to white and orange). This confirms the counter-selection of hybrids. Moreover, an color-odor associative pattern has been evidenced since magenta flowers of *A. m. pseudomajus* emit three benzenoid volatile organic compounds (VOCs) that yellow flowers of *A. m. striatum* do not (Suchet et al. submitted). Ethological isolation defining by non-random pollinator behavioral choices that would limit gene flow between these two subspecies has therefore been speculated. In support to this hypothesis, *A. majus* flower scent has been found to differentially influence the bumblebee behavior between the two subspecies (Suchet et al. in press). Indeed, one of the three benzenoids specific to *A. m. pseudomajus* flower scent has been shown as an aversive VOC for naïve *B. terrestris* whereas the main function of flower scent is the pollinator attraction (Suchet et al. in press). If *A. m. pseudomajus* persists in the wild (it is even more widespread than *A. m. striatum*) despite this aversive compound, it could be because this subspecies compensates with a better quantity or quality of reward. If so, some pollinators would choose the attractive flower scent (*A. m. striatum*) but gain little and/or bad quality of nectar, while others would learn that the less attractive flower scent (*A. m. pseudomajus*) also entails a greater and/or better quality of nectar. Another hypothesis is that the two subspecies could specialize in pollinators. In fact, the two *A. majus* subspecies seems be pollinated by a

different frequencies of visits of two common types of pollinators: a higher abundance of halictids has been observed in *A. m. striatum* than in *A. m. pseudomajus* and inversely a higher abundance of *Bombus sp.* has been observed in *A. m. pseudomajus* than in *A. m. striatum* (Suchet et al. unpublished data). However, the determination of the cohorts of pollinators is challenging because pollinator assemblages vary in time (during the phenology and among years) and space (throughout the distribution area). Differences of nectar traits could then supply other clues to support a pollinator specialization in the two snapdragon subspecies.

We aimed to test whether the two reproductively isolated subspecies of *Antirrhinum majus* from the wild compete for pollinators by offering different quantity and/or quality of nectar in association with their distinct floral odor and color. Thus, our questions are following: i) Do *A. m. pseudomajus* and *A. m. striatum* produce the same volume of nectar? ii) Do these two subspecies differ in their composition and concentration of sugars and amino acids?

Materials and methods

Plant material

We grew 10 seeds from each of four wild populations of *A. m. pseudomajus* near the villages of Lagrasse, La Preste, Le Martinet in France and near Pardines in Spain (for a total of Np=40 adults), and from each of three wild populations of *A. m. striatum* near the villages of LLes, Collada de Toses in Spain and Camurac in France (for a total of Ns=30 adults). Seeds were grown in greenhouse conditions between November 2008 and May 2009 (16 h/day of light, at 25°C average temperature, in individual pots with universal compost and with no addition of nutrients) to prevent nectar removal and contamination by insect.

Floral nectar sampling.

Nectar was sampled from the 381 flowers of the seven wild cultivated populations of *A. majus* between April and June 2009 always between 4:00 and 6:00 pm. More precisely, the amount of nectar produced by the flower was extracted from the base of the tubular corolla by capillarity. The nectar sampling was carried out just after the inflorescences were cut to prevent evaporation. Particular care was taken to avoid any contamination of the nectar by pollen, fingers or other possible contaminating sources. The volume of nectar was then measured using the graduation on the capillary. Finally nectar samples were all diluted in 250 µL of methanol at 20% in micro-vial and stored to the freezer at -20°C.

Nectar sugar analyses.

Sugar analyses were carried out with high-performance liquid chromatography (HPLC) (Biochrom, Cambridge, Royaume-Uni). Twenty nectar samples, each coming from one different plant, were selected (i) for their origin: ten samples per *A. majus* subspecies were chosen by equalizing the number of sample per population and (ii) for their similar stage of flower provenance (the first or second day after dehiscence of anthers). Before analysis 100µL of each micro-vial was dissolved first in 1mL of distilled water, and 100µL of this first dilution was then dissolved in 1mL as well. Parameters of analyses were adapted to detect all type the monosaccharids. Analysis was made on a dionex PA1 column (Biochrom, Cambridge, Royaume-Uni), and quantification was made by a detector (Biochrom, Cambridge, Royaume-Uni). Flow rate was 1 ml min^{-1}. The elution conditions were 1mM KOH for 40 min, a linear gradient from 0 to 40 mM KOH in 100 mM KOH over 10 min, a linear gradient of 1mM KOH in over 10 min. The column was regenerated with 1 M NaOH for 2hr and equilibrated for 10 min with starting buffer after every run. We investigated the presence of three monosaccharides: glucose, sucrose and fructose because they were reported

in *A. majus* nectar (Wykes 1952). Two runs were made per nectar sample, and the mean area of this runs was then used. Sugar quantification was performed on the peak areas by comparison with external standards.

Nectar amino acid analyses.

Identification of amino acids consists first to the hydrolysis of proteins in order to obtain a solution of free molecules. 200µL of 20 diluted nectar samples (10 per subspecies) were mixed with 100µL of muriatic acid (5.7M HCl). The purged and sealed tubes were warmed to 103°C for 24 hours. After evaporation of muriatic acid by freeze-drying for 50 minutes, they were diluted with 1mL of buffer at pH 2.2 and filtered using a membrane of 0.2µm.

Secondly, HPLC analyses (Biochrom, Cambridge, Royaume-Uni) of 100µL hydrolysis products are runned to separate and then identify and quantify the amino acids. The amino acid derivatives were colorimetrically detected by excitation at 570nm and 440nm for proline after reaction with ninhydrine. Amino acids were quantified by automatic integration after calibration of the system with known amino acid quantities.

Statistical analyses.

All statistical analyses were carried out with the R statistical software, version 2.9.2 (http://cran.r-project.org/).

The difference in volume of nectar between the two *A. majus* subspecies was tested using an analysis of variances (ANOVA) on values obtained from the three older flowers of each plant. In this way, we avoided the bias caused by the negative correlation between the nectar volume and the age of flower (the younger the flower, the less nectar it contains).

The non-parametric Mann-Whitney test was used to test if the two *A. majus* subspecies differed in sugar and amino-acid compositions and concentrations because sample sizes were too low to test their normal distributions.

Results

Nectar volume in A. majus subspecies.

A significant difference of nectar volume was found between the two subspecies of *A. majus* (F=23.17, df=1, $P < 0.001$, Figure 1). On average, the volume of nectar per flower was twice as important in *A. m. pseudomajus* (n=111) as in *A. m. striatum* (n=96) (means and standard errors: 8.11±0.80 and 3.67±0.45 µl per flower, respectively).

Nectar sugar composition and concentration in A. majus subspecies.

Three monosaccharids were detected and identified: glucose, fructose and sucrose. The 20 nectar samples composed by 10 samples from each subspecies contained these three sugars. *A. m. pseudomajus* and *A. m. striatum* therefore did not differ in nectar composition of monosacharrids.

Nectar of *A. m. pseudomajus* and *A. m. striatum* produced the three sugars in similar proportions. The sucrose was the most abundant of sugars and it represented in average 75% of the sugar quantity. Fructose and glucose respectively represented in average 18% and 7% of the sugar quantity. The sugar concentrations also did not differ between the two subspecies of *A. majus* (sucrose W = 69, *p*-value = 0.43, fructose W = 86, *p*-value = 0.91, and glucose W = 54, *p*-value = 0.79, Figure 2). When volumes of nectar were higher in *A. m. pseudomajus*

than in *A. m. striatum*, concentrations of the three sugars were similar between them. The higher volume of nectar in *A. m. pseudomajus* was therefore due to a higher dilution of sugars.

Nectar amino-acid composition and concentration in A. majus subspecies.

HPLC analyses detected a total of 14 amino-acids (Table 1). Four of them were omnipresent (cysteine, valine, histidine and proline), seven showed variable occurrences (glutamine, glycine, alanine, methionine, leucine, tyrosine, phenylalanine) and three were rare (aspargine, threonine, serine). None of them were consistently found only in one *A. majus* subspecies.

A. m. pseudomajus and *A. m. striatum* did not significantly differ in total number of amino-acids (6 and 8.7 respective means, p-value < 0.05).

Discussion

The present study shows that the floral phenotypic differences between *A. m. pseudomajus* and *A. m. striatum* based on the color and the odor is associated with a difference of nectar volume per flower. In contrast, compositions, concentrations and proportions of sugars in nectar were similar between the two subspecies. We identified the same sucrose-glucose-fructose nectar composition than previously reported in *A. majus* by Wykes (1952). As in Petanidou's (2005) evolutionary patterns, this mainly bumblebee-consumed nectar is largely dominated by sucrose. Among 73 dry Mediterranean species, only three species (two Lamiaceae *Prasium majus* and *Salvia triloba*, and one Capparidaceae *Capparis spinosa*) that could co-exist with *A. majus*, produce such huge volume of highly concentrated nectar (Petanidou 2005). This suggests that *A. majus* is a good competitor plant species for pollinators. However, as the higher volume of nectar found in *A. m. pseudomajus*

were found to display similar sugar concentration than in *A. m. striatum*, we concluded that these two subspecies should equitably compete for pollinators based on sugar resources. Occurrences and concentrations of nectar amino-acids were also found to not significantly differ between the two subspecies. Therefore no *A. majus* subspecies can be here claimed as more adaptive for any particular different nectar-quality-based strategy of pollination than the other reproductively isolated subspecies. Nevertheless, amplified the sample size of the analyses of the nectar quality (n=10 for each subspecies) may be needed to ensure this conclusion, especially for sucrose that tended to be higher concentrated in *A. m. pseudomajus*.

What advantage *A. m. pseudomajus* would have to produce higher volume of nectar? Bumblebee species were observed to visit this magenta flowered subspecies in higher frequency than in *A. m. striatum* that is more frequently visited by much smaller pollinators: *halictini* species (Suchet et al. unpublished data). The larger the pollinator, the more the nectar consumed per visit, the larger the body surface for collecting and depositing pollen (Pacini et al. 2003). The higher volume of diluted nectar in *A. m. pseudomajus* might therefore be an adaptation to its bigger pollinators with the advantage to not pay the cost of a higher production of sugars that have been shown to be costly in terms of seed production and photoassimilate allocation (Brandenburg et al. 2010).

Recently, it has also been demonstrated that high volume of nectar can also amplify an important attractant: CO_2 emission which is correlated to the nectar quantity (Raguso 2004, Goyret et al. 2008). *Datura wrightii* emits large amounts of CO_2 from anthesis when nectar volume is highest, provoking a strong attraction of the pollinator hawkmoth *Manduca sexta* toward the carbon dioxide source (Goyret et al. 2008). In *A. majus*, if flowers with more nectar can also be distantly detectable with chemistry by pollinators, then *A. m. pseudomajus* gets an evolutionary advantage by optimizing the pollen transfert between their flowers

compare to *A. m. striatum*. This could partially explain the more extent distribution area of *A. m. pseudomajus.*

A completmentary hypothesis is that the difference of nectar production between the two *A. majus* subspecies is due to their difference of floral pigmentation that can influenced intrafloral temperature. In cultivars of in *A. majus* anthocyanin pigments tend to warm interne flower (Comba et al. 2000). Since nectar production is activated with increasing temperature (Pacini et al. 2003), the higher volume of nectar in *A. m. pseudomajus* may be caused by a higher intrafloral temperature induced by the magenta floral pigmentation. Measurement of the intrafloral temperature represents an important perspective for the understanding of the maintenance of the snapdragon floral phenotypic diversity because this floral trait is a further reward sought by bumblebees. Dyer et al. (2006) showed that *B. terrestris* searches more than nectar in flower, it also seeks for the warmest flowers that help it to maintain their body temperature. In this study, bumblebees learn to associate this other reward of flowers with their color (Dyer et al. 2006). Thus, if a difference of intrafloral temperature can influence the pollinator preference, then *A. majus* pollinators may forage adaptively by paying attention to temperature when choosing between yellow and magenta flowers of *A. m. striatum* and *A. m. pseudomajus* in contact zone.

Secondary metabolites in nectar have also been shown important in plant-pollinator interaction because they can regulate both duration of visit and frequency of foraging pollinators (Brandenburg et al. 2009). Nicotine in Floral nectar of *Nicotiana attenuata* reduces the pollinator visitation time per flower and increases the visited flower number when working in synergy with the major volatile attractant, benzyl acetone (Kessler et al. 2008). In horticultural *A. majus*, iridoids has been identified. These secondary metabolites are non-volatile defensive compounds that are toxic for generalist herbivores and linked to the plant

reproductive strategy (Beninger et al. 2007, 2008 and 2009). Indeed, our preliminary tests confirm the presence of certain of these defensive compounds in leaves in the wild snapdragon species but also certainly in nectar. They may be use by *A. majus* pollinators as *Bombus impatiens* use the alkaloid gelsemine from the nectar of *Gelsemium sempervirens* because this molecule reduces intestinal infection caused by the *Crithidia bombi* pathogen whereas it defenses plant against the non-obligate insects species by its deterrent effect (Manson et al. 2010). Iridoids in *A. majus* nectar would represent the missing key components to understand the maintenance of the two subspecies floral phenotypes managing the pollination strategies.

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Table 1: Occurrence of the 14 amino acids contained in the nectar of the two *A. majus* subspecies.

	A. m. pseudomajus	*A. m. striatum*
	Mean occurrence (%)	Mean occurrence (%)
Cysteine	100	100
Histidine	100	100
Proline	100	100
Valine	100	100
Glutamine	75	89
Phenilalanine	50	67
Alanine	55	78
Glycine	33	55
Leucine	33	44
Methionine	25	55
Tyrosine	25	44
Serine	22	25
Threonine	22	11
Asparine	11	11

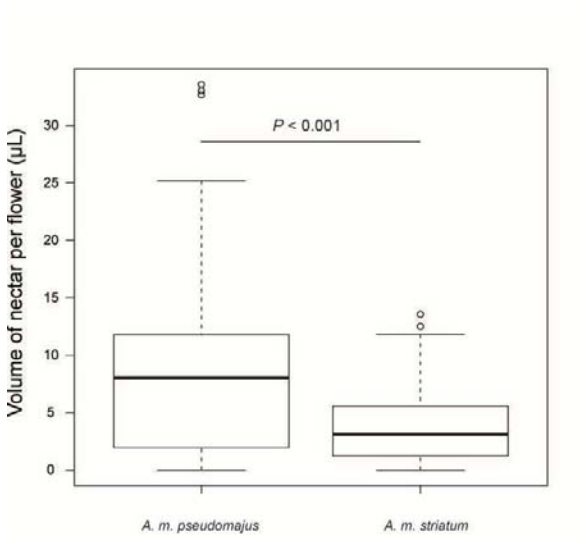

Figure 1: Difference in volume of nectar per flower (µL) between *A. m. pseudomajus* (n=111) and *A. m. striatum* (n=96) (ANOVA, F=23.17, df=1, $P < 0.001$)

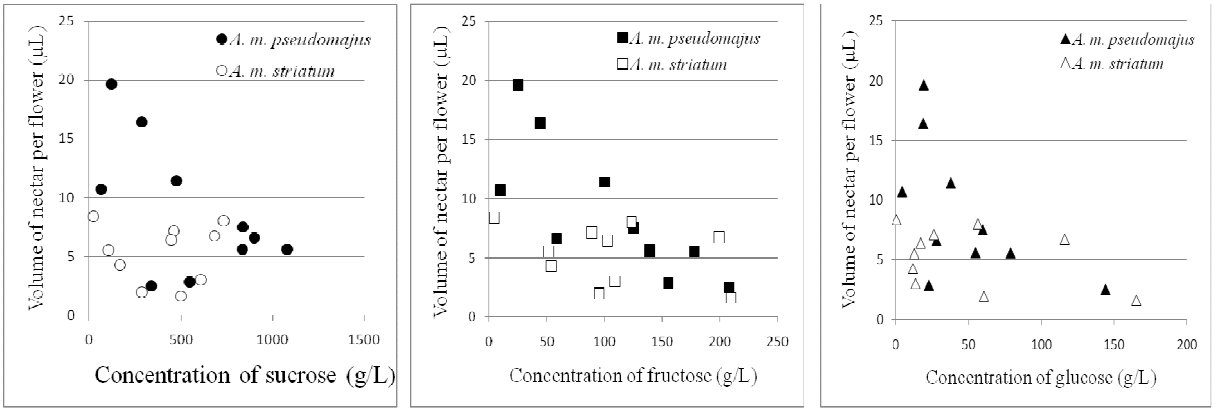

Figure 2: Concentration of sucrose (circles), fructose (squares) and glucose (triangles) relative to the volume of nectar per flower between the two subspecies *A. m. pseudomajus* (filled symbols) and *A. m. striatum* (empty symbols)

CONCLUSIONS ET PERSPECTIVES

Antirrhinum majus, la gueule de loup, s'est révélé être un modèle de choix pour accroître nos connaissances sur les fonctions des odeurs florales et leur évolution. En effet, des patrons significatifs, pour certains peu communs, ont été mis en évidence lors de mes travaux de thèse.

1. Conclusions

L'étude des profils d'odeurs florales a tout d'abord mis en évidence que les deux sous-espèces, *A. m. striatum* et *A. m. pseudomajus*, en plus d'être distinguables par la couleur florale, émettaient une diversité de composés volatils floraux qui leur étaient spécifiques. Le signal chimique floral d'*A. m. pseudomajus* était composé de trois benzénoïdes, dont majoritairement l'acétophénone, alors que les fleurs d'*A. m. striatum* n'en émettaient pas. Deux autres COV, le 6-methyl-5-hepten-2-one et le limonène, étaient aussi plus abondants l'un chez *A. m. pseudomajus* et l'autre chez *A. m. striatum* respectivement. Parce que ces différences entre sous-espèces ont été retrouvées systématiquement à travers les populations étudiées et les différents environnements de culture, un déterminisme génétique en est vraisemblablement la cause. Des patrons d'héritabilité des benzénoïdes chez les hybrides renforcent cette idée. Les hybrides de première génération, dont la lignée maternelle provenait d'*A. m. pseudomajus*, émettaient deux fois plus de benzénoïdes que les autres. Cependant, certaines difficultés de suivi d'individus lors des croisements en serre ont été rencontrées, et un trop faible nombre d'échantillons d'hybrides exploitables en milieu naturel ont limité les preuves pour confirmer la variabilité génétique des émissions en benzénoïdes chez *Antirrhinum majus*.

En milieu naturel, une plus grande variabilité des émissions de COV a été observée par rapport aux plantes cultivées en conditions contrôlées. Or, les protocoles étaient identiques et une attention particulière a été apportée sur le terrain afin de ne pas endommager les échantillons. De plus, il est exclu de penser que cette plus grande variabilité pourrait être due à une plus grande diversité génétique des individus échantillonnés sur le terrain puisque le nombre de lignées par population était comparable entre les deux études. Une part des variations d'odeurs florales en milieu naturel semble donc être sous contrôle de l'environnement, même si les différences systèmatiques entre les deux sous-espèces persistaient. Les causes précises de cette variabilité, qu'il s'agisse d'une influence des

conditions abiotiques et/ou biotiques, sont encore à déterminer. La plasticité phénotypique face à des agressions, par exemple, pourrait également être explicative.

L'ensemble de ces résultats met donc en évidence une multiplicité de causes à l'origine des variations d'odeurs florales. La part de la variabilité génétique semble être celle qui explique le plus les variations observées sous réserve qu'elles soient confirmées en tant que différences génétiques.

Pour discuter des causes des variations phénotypiques observées, il est important de prendre en compte qu'il s'agit là d'un patron associtif odeurs-couleur entre les deux sous-espèces d'*A. majus*. De tels patrons phénotypiques entre plantes phylogénétiquement proches, n'est pas courant dans la littérature. La Julienne des Dames (*Hesperis matronalis*, Brassicaceae), dans le Michigan, émet des odeurs florales différentes entre ses morphes violet et blanc qui coexistent (Majetic et al. 2007). Cependant, ces patrons de différenciation ne sont constants entre populations que chez les fleurs violettes alors que, chez les fleurs blanches, les émissions d'odeurs florales dépendent de leur localisation. A la Réunion, chez *Calanthe sylvatica* (Orchidaceae), un terpenoïde (le (E)-4,8-dimethylnona-1,3,7-triène, ou DMNT) est très abondant chez la variété violette, *C. sylvatica* var. *purpurea*, alors qu'il est peu ou pas émis par la variété jaune *C. sylvatica* var. *lilacina* (R Delle-Vedove et al. en préparation). Ces deux profils d'odeurs florales sont émis par une troisième variété *C. sylvatica* var. *alba* indépendamment de leurs populations d'origine. La gueule-de-loup présente donc des phénotypes autrement particuliers puisque des présences-absences et des ratios relatifs de COV sont spécifiques à chacune des deux couleurs des deux sous-espèces. La pléiotropie (lorsqu'une modification génétique controle plusieurs traits phénotypiques) ou l'épistasie (lorsqu'une modification d'un gène affecte d'autres gènes liés à celui-ci) affectant soit la voie de biosynthèse des benzénoïdes, soit celle des anthocyanines, sont des mécanismes probables pour expliquer la cause de ces différences (Figure 14). Mais cela reste des mécanismes complexes qui n'excluent pas l'apparition de modifications génétiques indépendantes controlant les odeurs et les couleurs florales (Figure 14). Même si tel est le cas, ces mécanismes évolutifs n'expliquent en rien le maintien des deux phénotypes les plus répandus alors qu'une infinité d'autres morphes sont intrinsèquement viables en zone hybride.

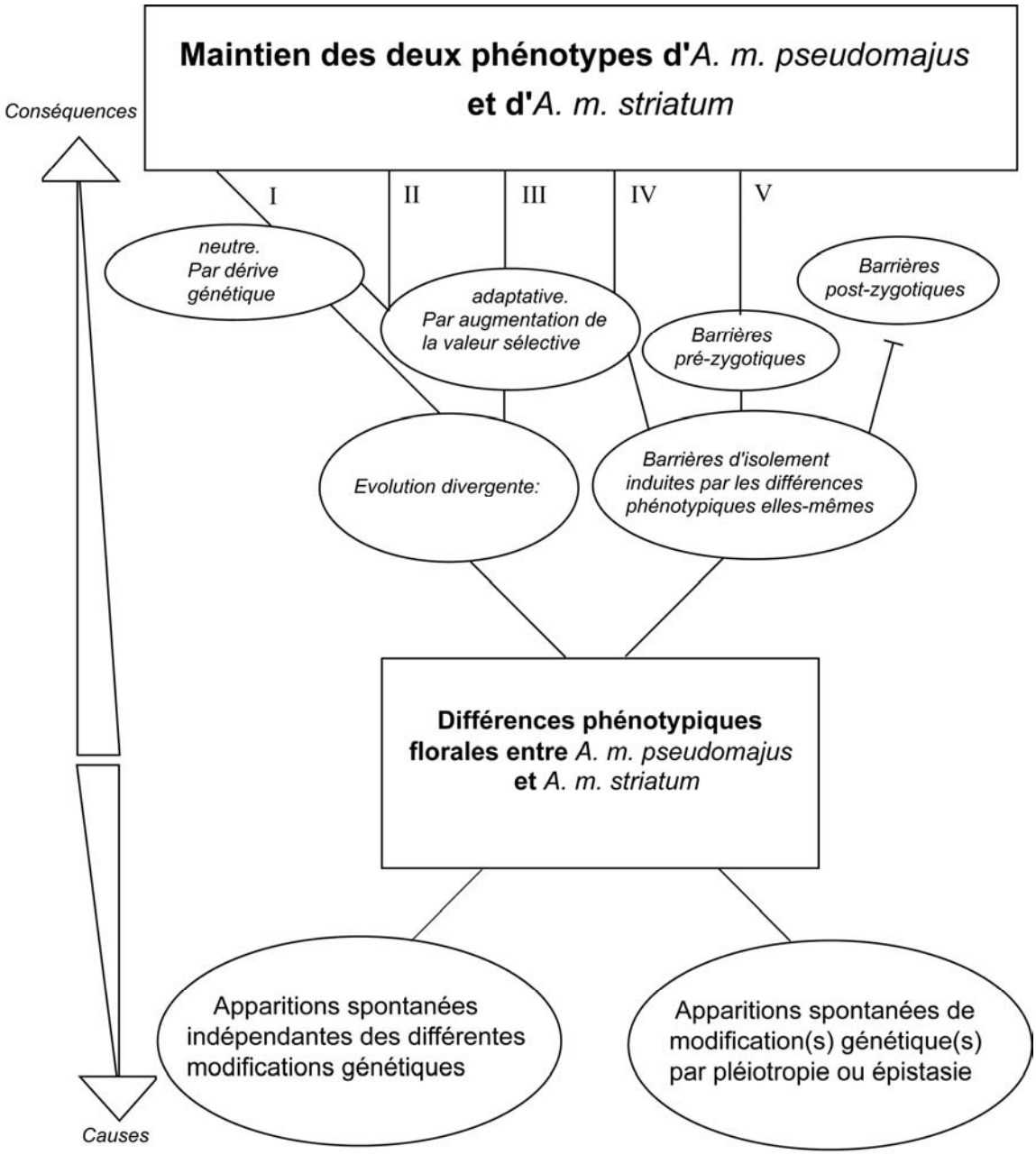

Figure14 : Synthése des hypothèses des causes et des conséquences des différences phénotypiques florales observées chez *Antirrhinum majus pseudomajus* et *A. m. striatum*. Les chiffres romains représentent les différents scénarii encore plausibles pour expliquer le maintien des phénotypes des deux sous-espèces

Toute la question est alors de savoir si les évolutions des deux sous-espèces divergent l'une de l'autre par dérive génétique (scénario I, Figure 14) ou par adaptation à leurs environnements différents par exemple (scénario II et IV, Figure 14), ou bien si les différences phénotypiques entre les deux sous-espèces induisent en elles-mêmes une barrière aux flux de gènes (scénario III, Figure 14). C'est une question qui reste centrale, de manière générale, en biologie évolutive des plantes (Johnson 2006). La réponse est probablement une

combinaison des deux : isolement et divergence combinés (scenario V, Figure 14). On sait par exemple, que des barrières d'isolement peuvent facilement amener à des conséquences pleiotropiques résultant en une divergence adaptative (Coyne et Orr 2004).

Plusieurs facteurs nous ont amené à essayer de tester l'hypothèse d'un isolement éthologique (scénario III, Figure 14). L'étude de Andalo et al. (2010) qui démontre qu'il n'y a pas de barrière post-zygotique entre les deux sous-espèces (Figure 14), combinée au fait que nous ayons rapidement mis en évidence une différence de récompense pour le pollinisateur entre les deux sous-espèces (le volume de nectar par fleur), est venu alimenter l'hypothèse avancée dans les études précédentes (Whibley et al. 2006, Tastard et al. 2008) d'un rôle prédominant des pollinisateurs dans l'isolement reproducteur d'*A. m. pseudomajus* et *A. m. striatum*. Ces intuitions ont mis à jour des résultats intéressants d'assemblage de pollinisateurs et de leurs comportements. Suite aux relevés de pollinisateurs effectués sur deux années consécutives, il s'est avéré que les espèces de pollinisateurs étaient partagées entre les deux sous-espèces d'*A. majus* mais que les bourdons (*Bombus sp.*) et le deuxième type de pollinisateur le plus abondant, les abeilles solitaires du clade des halictides (Halictini) présentaient des fréquences différentes en fonction des sous-espèces. Les bourdons ont été plus fréquemment rencontrés chez *A. m. pseudomajus* alors que les abeilles solitaires étaient plus fréquentes chez *A. m. striatum.* Ce résultat est en faveur d'une adaptation sous-spécifique à une cohorte différente de vecteurs de pollen. Cependant, il mérite d'être plus approfondit pour le confirmer et ceci représente un véritable challenge. Par expérience, ce sont des recherches qui sont fortement consommatrices de temps et de moyens humains et qui sont dépendantes des conditions météorologiques. Pourtant, un suivi plus étendu dans l'aire de répartition du modèle et dans le temps serait de première nécessité.

D'autre part, les différences d'odeurs florales influencaient le comportement des bourdons et des halictides. Les tests comportementaux face aux odeurs artificielles ont tout d'abord montré l'effet aversif de l'acétophénone sur des bourdons naïfs (*Bombus terrestris*). Ceci a été confirmé par la préférence des odeurs florales naturelles d'*A. m. striatum* face à celles d'*A. m. pseudomajus* chez des bourdons naïfs. Et, surtout le même test comportemental avec des bourdons et des halictides sauvages capturés sur des fleurs d'*A. majus* a montré que leurs préférences olfactives dépendaient de leur populations d'origine. Les pollinisateurs provenant de populations d'*A. m. pseudoamjus* préfèraient les odeurs florales de cette sous-espèces alors que ceux capturés sur des fleurs d'*A. m. striatum* préfèraient l'odeur de ces fleurs jaunes. Ces patrons d'apprentissage qui contrent l'effet aversif précédemment observé,

sont en accord avec l'hypothèse d'isolement éthologique indépendamment des différences de cohorte de pollinisateurs. Si les pollinisateurs préfèrent les odeurs florales de la sous-espèce qu'ils ont déjà expérimentée, c'est qu'ils les ont associés à une récompense qui leur est bénéfique et qui les encourage à revisiter des fleurs aux signaux similaires. Si tel est le cas, alors le flux de gènes est bel et bien limité entre les sous-espèces.

Néanmoins, même si ces résultats sont très encourageants, d'autres études seront à mener pour confirmer l'existence d'une limitation en flux de gènes par les pollinisateurs. Ces comportements, en effet, n'ont été observés qu'au sein du dispositif de labyrinthe en « Y ». Or, cette approche reste assez expérimentale, dans le sens où les conditions environnantes sont différentes des conditions naturelles dans lesquelles les pollinisateurs font réellement preuve d'un choix de forme florale. Ceci dit, maintenant que l'influence des signaux chimiques floraux a été démontrée, et ce en parallèle des signaux visuels de couleur florale sur des fleurs artificielles (qui rappellons le n'ont pas montré de choix particulier entre la couleur jaune et magenta), il apparaît alors primordial de tester l'influence de la combinaison de ces signaux sur le comportement des pollinisateurs.

2. Perspectives

D'ambitieux projets seraient à mener concernant la recherche de l'origine de la différenciation phénotypique décrite dans cette thèse. Un projet de biologie moléculaire pourrait relever le défi de chercher à savoir si les associations sous-spécifiques odeurs-couleur chez *A. majus* sont dues à une contrainte biochimique. Une telle hypothèse évolutive prédit qu'un évènement évolutif implique le fait que l'état d'un des deux traits phénotypiques, l'odeur ou la couleur florale, a des répercussions sur l'autre trait. Imaginons que les productions biosynthétiques d'anthocyanines et de composés benzénoïdes interfèrent. Alors la différence de régulation de ces métabolites secondaires chez *A. m. striatum* par rapport à *A. m. pseudomajus* pourrait être due soit à une mutation de l'un des gènes impliqué dans l'expression d'anthocyanine ou de benzenoïdes, mais affectant les deux traits phénotypiques (pléiotropie), soit à une différence de régulation d'un gène impliqué dans l'expression de ces deux traits par d'autres gènes non partagés par les deux sous-espèces (épistasie). Les manipulations génétiques chez l'œillet (*Dianthus caryophyllus L.*), réalisées par Zucker et al. (2002) portent à croire que cette hypothèse est plausible puisqu'elle montre qu'il y a effectivement un lien biosynthétique entre les benzénoïdes et les anthocyanines. La

découverte du gène codant pour l'enzyme à l'origine de la production d'acétophénone (le composé qui contribue le plus à la différence sous-spécifique) serait probablement une étape incontournable. Etape qui devrait être facilitée par le séquençage du génome entier d'*Antirrhinum majus* qui sera très prochainement disponible (Enrico Coen, communication personnelle). Une collaboration avec l'équipe de recherche d'Enrico Coen, basée à Norwich, en Angleterre, permettrait aussi d'approfondir les patrons de variations significatives d'odeurs florales détectées chez les hybrides F_1 et F_2 étant donné qu'ils ont à leur disposition un grand nombre de lignées hybrides. Ceci alimenterait également les études de biologie moléculaire précédemment citées par une approche complémentaire de génétique quantitative.

Des nouveaux échantillonnages de terrain, qui prendraient en comptent les conditions abiotiques locales, seraient aussi d'excitants futurs projets sur le modèle *A. majus*. En effet, la co-variation odeur-couleur observée entre les deux sous-espèces pourrait être due à des réponses évolutives face à de multiples pressions de sélection, incluant ceux de l'environnement abiotique. Par exemple, le maintien du polymorphisme de la couleur florale basée sur les pigments d'anthocyanines de cinq espèces de plantes à fleurs en Grande-Bretagne a été présenté comme étant une adaptation face à l'hétérogénéité de l'environnement et à la tolérance au stress (Warren et Mackenzie 2001). Une approche parallèle de la thèse en cours d'Aurélie Khimoun en génétique des populations révèle, que les deux sous-espèces pourraient bien se différencier par leurs niches environnementales. Les populations d'*A. m. striatum* se situeraient davantage dans des endroits humides où les conditions climatiques sont stables alors qu'*A. m. pseudomajus* semble aussi adapté à des conditions climatiques plus variables et plus sêches. Les deux sous-espèces étaient d'ailleurs davantage différenciées sur la base de leurs odeurs florales en conditions naturelles qu'en conditions identiques et contrôlées. Cependant, pour savoir si les émissions de COV floraux sont avantageuses face à certaines conditions abiotiques, il faudrait tout d'abord déterminer comment varient les odeurs florales face aux fluctuations des conditions abiotiques et par là-même si l'environnement abiotique influence bien ces émissions. Une approche comparative sous des conditions contrôlées et diversifiées, comme par exemple grâce au dispositif du nouvel Ecotron européen à Montpellier. Cette infrastructure (Figure 15) en macrocosmes scellés sous verre est parfaitement contrôlée par les examinateurs et elle ouvre l'opportunité d'enregistrer les réponses en émissions de COV floraux des plantes face à des changements programmés de températures, d'humidité, d'intensité lumineuse, de composition du sol ou encore de

concentration en CO_2... Ainsi, à la question encore peu explorée (Majetic et al. 2009) : « est-ce que les odeurs florales sont façonnées par les conditions abiotiques et édaphiques ? » nous aurions une réponse d'une envergure sans précédent.

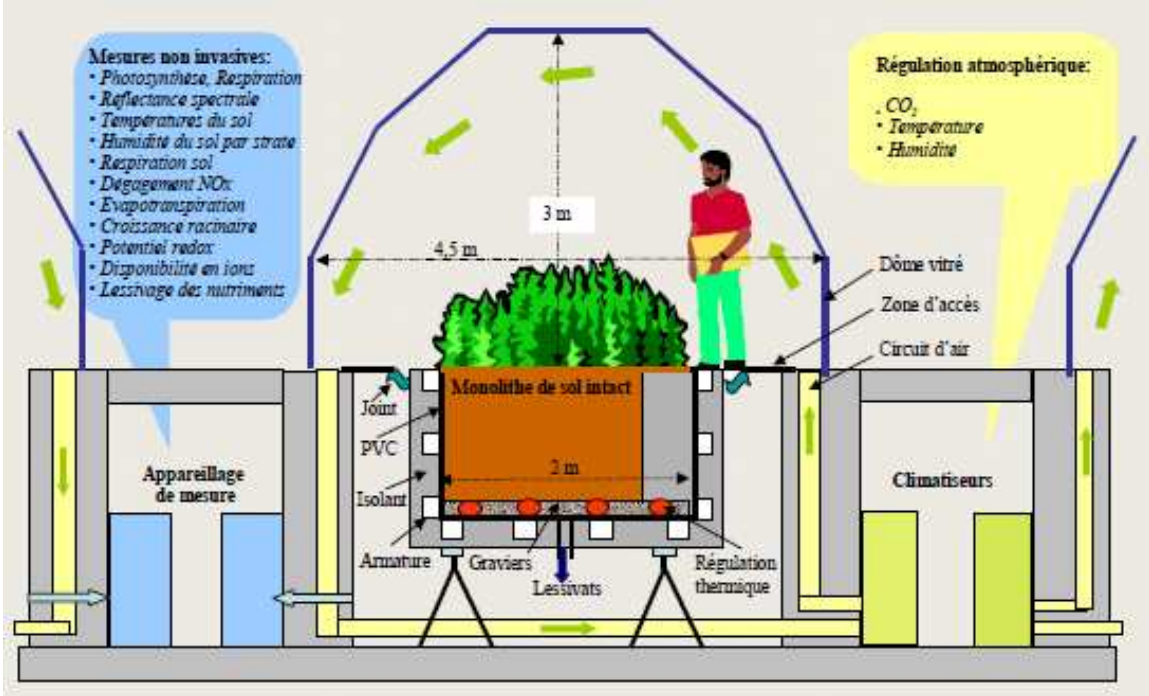

Figure 15 : Coupe schématique d'un macrocosme (http://www.ecotron.cnrs.fr/)

Enfin, les différences phénotypiques observées entre les deux sous-espèces d'*A. majus* pourraient être expliquées par un patron adaptatif répondant aux différentes pressions de sélection qu'imposent les différents partenaires biotiques en interaction. Cette hypothèse explicative demande un effort de travail considérable sur une longue échelle de temps pour avoir des données de terrain robuste. Elle n'a pu être que partiellement testée pendant ma thèse et les résultats sont pour autant plutôt encourageants. Les différences d'odeurs florales étant détectées et influençant le comportement des pollinisateurs principaux, on peut s'attendre à ce que le choix fait par ces insectes, qui assurent la reproduction de la gueule-de-loup, favorisent certains de ses phénotypes floraux. Autrement dit, le choix des pollinisateurs pourrait ne pas être aléatoire. Si tel est le cas, un phénomène de constance entre phénotype donc de limitation en flux de gène reste à démontrer pour expliquer le maintien de ces sous-espèces. En plus de la contre-sélection des phénotypes hybrides par les pollinisateurs, mise en évidence à plusieurs reprises par les travaux de la thèse d'Emmanuelle Tastard, les différentes espèces de pollinisateurs pourraient présenter de réelles préférences avant ou après

l'apprentissage des visites de ces deux phénotypes d'*A. majus*. Des expériences dites de « free flying », qui consistent à relever des visites de fleurs par les pollinisateurs libérés en cage, permettraient de tester ces mécanismes. Les phénotypes floraux des deux sous-espèces d'*A. majus*, les espèces de pollinisateurs ainsi que leur niveau d'apprentissage pourraient constituer les variables de l'expérience. Il serait alors nécessaire de compléter les relevés des espèces qui visitent et/ou qui fécondent les fleurs d'*A. majus* afin d'affiner nos connaissances sur les cohortes de pollinisateurs des deux sous-espèces, qui tendent du reste à différer au niveau de la fréquence de certaines espèces pollinisatrices. En effet, des données supplémentaires à ce sujet semblent incontournables, car la caractérisation de l'assemblage de pollinisateurs est une tâche complexe étant donné que celui-ci peut extrêmement varier dans le temps (lors de la phénologie et entre les années) et dans l'espace (au sein et entre les populations). Lors de ces nouveaux relevés il faudrait inclure les paramètres de la communauté de plantes à fleurs coexistant avec la gueule-de-loup étant donné que les visites des pollinisateurs d'*A. majus* ne sont pas exclusives à cette espèce mais que celle-ci rentre en compétition avec les autres espèces de plantes à fleurs environnantes. Le service de pollinisation des insectes est dépendant de leur mémoire à court et moyen terme (Raine et Chitkka 2006), les phénotypes floraux d'*A. majus* pourraient chacun être avantageux dans certaines communautés de plantes à fleurs. La fidélité des pollinisateurs est d'autant plus renforcée que les récompenses sont satisfaisantes. La co-variation odeur-couleur observée chez *A. majus* est associée à une production de volume de nectar presque doublée chez *A. m. pseudomajus*. Ce patron peut être directement expliqué par la pigmentation florale qui agit sur la température interne de la fleur qui, elle-même, agit sur la production de nectar. Des mesures de la température intra-florale entre les deux sous-espèces seraient non seulement intéressantes pour tester ce mécanisme mais aussi parce que la température de la fleur représente également une récompense recherchée par les pollinisateurs. Ils l'utilisent pour maintenir leur température corporelle (Dyer et al. 2006). Couplés aux relevés de température il serait alors intéressant de mesurer la durée des visites des visiteurs des fleurs des deux sous-espèces. Le temps passé par fleur peut être lié à la composition du nectar. Ici, on sait qu'elle ne diffère vraisemblablement pas entre les deux sous-espèces en terme de sucre et acides-aminés ; en revanche, la composition en métabolites secondaires n'a pas encore été testée. Des résultats préliminaires de ma thèse montrent que des composés iridoïdes de défense sont vraisemblablement retrouvés dans le nectar. Il semblerait que ces molécules ont un goût amer. Si les pollinisateurs y sont sensibles, la présence d'iridoïdes dans le nectar pourrait être un moyen de manipuler ses pollinisateurs

par la plante en réduisant leur temps passé par fleur, tout comme la présence de nicotine dans le nectar l'induit chez *Nicotiana attenuata* (Kessler et al. 2008).

En plus des pressions de sélection directes que peuvent imposer les pollinisateurs, les pressions de sélections indirectes des ennemies naturelles seraient à incorporer au modèle (Raguso 2009). La femelle de charançon *Rhinusa vestita* qui pond ses œufs dans les fleurs fécondées d'*A. majus* a tout intérêt de développer un moyen de détection de ces futurs fruits au risque de ne pas fournir le garde-manger et l'habitat sécurisé nécessaire à la croissance de sa progéniture. Les odeurs florales peuvent être un bon moyen de détection puisque l'on sait que celles-ci varient en période post-pollinisation chez *A. majus* (Negre et al. 2003). La question est surtout de savoir si les benzénoïdes émis par *A. m. pseudomajus* ont un rôle défensif. Au quel cas, *A. m. pseudomajus* présenterait un avantage sélectif considérable par rapport à *A. m. striatum*. L'acétophénone est le composé de répulsion utilisé pour contrer la phéromone d'agrégation du coléoptère ravageur, *Dendroctonus brevicomis,* du pin, *Pinus ponderosa,* aux Etats-Unis (Erbilgin et al. 2008). On peut prédire que l'hypothèse de composé de défense d'un ou des benzénoïde(s) est viable, et il faudrait donc vérifier si les ravages du charançon, spécifiques à *A. majus,* diffèrent entre les deux sous-espèces, notamment si elles sont supérieures chez *A. m. striatum*. Il en est de même avec l'autre antagoniste spécifique à *A. majus* : le papillon *Mellicta deione*. Sa chenille, qui se nourrit exclusivement des feuilles *d'A. majus*, a probablement développé un système de dégradation ou de stockage des composés de défense toxiques, les iridoïdes, que produit *A. majus* (Beninger et al. 2007, 2008, 2009, C Suchet et al. données non-publiées). Des relations pleiotropiques existent entre la production de pigments floraux et les composés de défenses (Solecka 1997, Strauss 1997, Fey 2004) Il reste à tester si les stratégies de défenses, sur la base de ces productions d'iridoïdes, diffèrent entre les deux sous-espèces.

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Glossaire

Allèle : Version d'un gène. Les gènes forment une suite de nucléotides, et sont pour la plupart en double exemplaire sur un même chromosome ; ce sont les allèles. Cet emplacement sur le chromosome des allèles d'un gène est fixe et s'appelle un locus. Mais les enchainements de nucléotides, eux, peuvent varier. Les mutations ou les recombinaisons génétiques sont à l'origine de ces différentes versions alléliques. Si les deux allèles d'un gène sur un chromosome sont identiques on dit que l'individu est homozygote, alors qu'il est hétérozygotz s'ils diffèrent.

Allopatrie vs. Sympatrie, Parapatrie : Deux espèces sont dit allopatriques si leurs aires de distribution sont non-chevauchantes, alors qu'elles sont en sympatrie lorsqu'elles coexistent ou en parapatrie si leurs aires sont adjacentes.

Angiospermes : (du grec « *angeion* » qui signifie vase et « *sperma* », graine) : plantes dont les fruits renferment des graines, à la différence des gymnospermes (« *gumnos* », nu) plantes à graines nues, tels les conifères.

Auto-incompatible : Se dit d'une fleur bisexuée, ou hermaphrodite, dont ses grains de pollen ne peuvent pas féconder ses propres ovules.

Dérive génétique : Moteur de l'évolution non-adaptatif qui induit une variation génétique par le fruit du hasard par le jeu de fluctuations de fréquences alléliques.

Mutation : Modification génétique irréversible et héréditaire d'un ou plusieurs nucléotides dans l'ensemble de l'ADN d'un être vivant, le génôme. Les mutations sont pour la plupart neutres, elles n'influencent pas la valeur sélective et peuvent être fixées ou disparaître dans une population par le jeu de la dérive génétique. En revanche, certaines mutations sont délétères à la survie de l'individu et sont éliminées par la sélection naturelle, qui favorise à l'inverse les mutations avantageuses, rares, qui tendent à se répandre. Les mutations sont une des sources de diversité transmissible de génération en génération, moteur de l'évolution.

Phénotype : (du grec « *phano* », paraître et « *typus* » type) : ensemble des caractères observables d'un individu.

Pollinisateurs : animal qui assure la pollinisation en transportant du pollen de fleur lorsqu'il visite des fleurs de la même espèce. Tous les visiteurs de fleurs ne sont pas forcément des pollinisateurs.

Sélection naturelle : processus évolutif par lequel les individus les plus adaptés survivent dans la nature et transmettre alors leurs capacités d'adaptation à leur descendance.

Fleurs hermaphrodites : fleurs qui présentent les deux sexes, présence d'étamines et de pistils.

Proboscis : chez les insectes, pièces buccales en siphon qui permettent d'aspirer une solution.

Espèces sœurs : espèces phylogénétiquement proches parce qu'elles partagent un ancêtre commun récent.

Annexes

Annexe 1 : Protocole d'échantillonnage des COV floraux (Figure 5)

Une cloche en verre englobait l'inflorescence, fermée par un tissu inodore en prenant soin d'inclure le minimum possible de parties végétatives et de ne pas ombrager l'inflorescence, afin de concentrer les COV dans le piège TenaxTA, la matière adsorbante,(le tube métallique indiqué par la flèche sur la Figure 5) émis en temps réels par l'inflorescence. Un système de double flux aspiratant, généré par deux pompes calibrées qui fonctionnent sur batterie, permettait à la fois de piéger les émissions en passant au travers du piège et à la fois d'homogénéiser le contenu de la cellule en orientant les émissions dans le sens de l'échantillonnage. Ces deux flux ainsi que le poids sec de l'inflorescence sont ensuite pris en compte dans le calcul des taux d'émission de COV floraux.

Annexe 2 : Protocole de test comportementaux en labyrinthe en « Y » (Figure 6)

Les tests comportementaux d'olfactométrie ont été menés avec des bourdons femelles de l'espèce *Bombus terrestris* commercialisés (bourdons naïfs) et sauvages (bourdons expérimentés) avec comme stimuli olfactifs soit des odeurs artificielles (reconstitution d'un mélange de COV à l'aide de molécules synthétisées), soit des odeurs naturelles provenant d'invidus d'*A. majus in vivo*. Des abeilles solitaires aussi ont été testées dans ce dispositif de labyrinthe en « Y » mais seulement avec des odeurs naturelles. Après deux sessions d'acclimatation par des explorations récompensées du labyrinthe sans stimulus olfactif, l'insecte dans la zone de décision doit faire un choix entre les deux émissions d'odeurs florales. Ces stimuli olfactifs sont soufflés vers le bourdon en pompant l'air autour des inflorescences contenu dans des sacs inodores en nalophane vers l'entrée du labyrinthe.