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THESE DE DOCTORAT

Traits morphologiques et biochimiques impliqués dans la spécialisation de *Trollius europaeus* sur les pollinisateurs prédateurs de graines *Chiastocheta spp*.

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RESUME

Traits morphologiques et biochimiques impliqués dans la spécialisation de *Trollius europaeus* sur les pollinisateurs prédateurs de graines *Chiastocheta spp.*

Résumé

Les interactions entre espèces sont un moteur d'évolution. Nous montrons ici quels sont les traits morphologiques et biochimiques du trolle d'Europe qui ont évolué au cours de sa spécialisation (*Trollius europaeus*) vis-à-vis des mouches pollinisatrices et prédatrices de graines (*Chiastocheta spp.*). La forme globulaire de la fleur est décisive dans l'attraction spécifique des chiastochètes. En comparaison avec une forme artificiellement ouverte, les fleurs globulaires, bien que souffrant plus de la prédation produisent plus de graines (4%), mais surtout elles exportent plus de pollen (85%). Un modèle de dynamique adaptative montre que l'évolution de la forme globulaire requiert non seulement une efficacité minimale de la pollinisation par les chiastochètes, par rapport à des pollinisateurs alternatifs qui ne consomment pas de graines, mais également une efficacité maximale : si les chiastochètes sont « trop » efficaces, en attirer beaucoup plutôt que quelques uns ne confère pas d'avantage. L'attraction des pollinisateurs se fait également par des signaux olfactifs. Plusieurs composés volatils émis par le trolle déclenchent une réponse électrophysiologique chez les chiastochètes (methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, linalool). Des observations de visites de chiastochètes en conditions naturelles ont montré que la variabilité des composés volatils présents dans les fleurs expliquait une part de la variabilité des visites reçues par ces fleurs, en comparaison avec des traits morphologiques et pigmentaires. Les interactions entre une plante et des prédateurs de graines sont conflictuelles : la plante à intérêt à soustraire les graines de l'appétit des larves. Un glycoside du flavonoïde lutéoline, l'adonivernith, s'accumule dans les parois des carpelles lorsque les dégâts causés par les larves augmentent, avec comme conséquence une baisse de l'intensité de prédation. Les six espèces du genre *Chiastocheta* étudiées induisent et réagissent différemment à l'adonivernith, cette molécule pourrait donc être impliquée dans la radiation sympatrique du genre. Les traits impliqués dans la spécialisation du trolle sur les chiastochètes sont donc à la fois mutualistes (morphologie globulaire et composés volatils de la fleur) et antagonistes (défense chimique contre les larves). Les contradictions de cette mosaïque de traits sont un moteur d'évolution.

Mots-clefs

mutualisme, coévolution, Trollius europeaus, Chiastocheta, dynamique adaptative, écologie chimique, pollinisation

ABSTRACT

Morphological and biochemical traits involved in the specialisation of *Trollius europaeus* on the seed-eating pollinators *Chiastocheta spp*.

Abstract
Interactions between species are a major driving force in evolution. We show here which morphological and biochemical traits evolved during the specialisation of the European globeflower (*Trollius europaeus)* on seed-eating pollinator flies (*Chiastocheta spp*). The globular shape is a key factor in the specific attraction of chiastochetes. Globular flowers produce more seeds (4%, they suffer higher predation but are better pollinated) and moreover export more pollen (85%) than artificially open flowers. An adaptive dynamics model shows that the evolution of the globular shape requires a minimal pollination efficiency by chiastochetes relatively to alternative pollinators that do not eat seeds, but also a maximal efficiency: if the chiastochetes are "too" efficient, to attract a lot of them rather than a few confers no advantage. The attraction of pollinators is also mediated by olfactive signals. Several volatile compounds emitted by the globeflower trigger an electrophysiological response in chiastochetes (methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, linalool). Field behavioural observations of chiastochetes visits have shown that the variability of the volatile compounds inside the flowers explains a part of the variability of the visits, together with morphological and pigmentation traits. Interactions between plants and seed predators are conflictual: the plants tend to reduce predation costs. A flavonoid close to luteolin, adonivernith, accumulates in the carpel walls when the damages caused by the larvae increase, leading to a reduction of predation intensity. The six *Chiastocheta* studied species have different exploitation patterns in the fruit, they induce and are affected by adonivernith in specific ways: this chemical defence could be involved in the sympatric speciation of the genus. The traits involved in the globeflower specialisation on chiastochetes are simultaneously mutualistic (globular floral morphology, floral colour and volatile compounds) and antagonistic (chemical defence against the larvae). The contradictions of this trait mosaic are a factor of evolution.

Key-words
mutualism, coevolution, Trollius europeaus, Chiastocheta, adaptive dynamics, chemical ecology, pollination

PUBLICATIONS & COMMUNICATIONS

PUBLICATIONS

Chapitre 1 (Ibanez et al., 2009)
Ibanez, S., Dujardin, G. & Després, L. 2009. Stability of floral specialization in Trollius europaeus in contrasting ecological environments. *Journal of Evolutionary Biology* **22**: 1183-1192.

Chapitre 2 (Ibanez & Després, 2009)
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Chapitre 3 (Ibanez et al., 2009)
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Annexe 1 (Despres et al., 2007)
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Annexe 2 (Gallet et al., 2007)
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COMMUNICATIONS ORALES

2005. 11th meeting of PhD students in Evolutionary Biology, Carcans-Maubuisson, France, 4-9 Septembre.
"Ecological stability of the *Trollius-Chiastocheta* mutualism."

2005. Réunion annuelle du GDR Ecologie Chimique, Tours, Octobre.
« Stabilité du mutualisme *Trollius-Chiastocheta* et interaction chimique entre les larves et le fruit. »

2006. Réunion annuelle du REID (Réseau Ecologie des Interactions Durables), Dijon, Janvier.
« Evolution de la spécialisation et de la généralisation dans une communauté mutualiste. »

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« Traits mutualistes et antagonistes dans l'interaction *Trollius-Chiastocheta* »

2008. 26th OIKOS meeting, Lund, 4-6 Février.
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Sommaire

INTRODUCTION : Des relations interspécifiques au système trolle-chiastochète.

1. Les relations interspécifiques

Un assemblage d'espèces en une communauté forme un réseau d'interactions. Une interaction entre deux espèces de cette communauté peut donner lieu à un flux d'énergie, de matière ou molécules d'eau ou d'éléments nutritifs ; à un transport (ou support) de gamètes ou d'individus ; à une modification de l'environnement perçue par les deux espèces. La combinaison de ces phénomènes écologiques peut alors « bénéficier » ou pas aux espèces considérées. Il est usuel de classifier les interactions interspécifiques en fonction du bénéfice de chacune des deux espèces :

Tableau 1. Classification des interactions interspécifiques en fonction des variations du taux d'accroissement de chacune des deux espèces. Le tableau 1 est symétrique. En gras, les trois types d'interactions les plus importants écologiquement.

Bénéfice	<0	0	>0
<0	**Compétition**		
0	Amensalisme	Pas d'interaction	
>0	**Exploitation**	Commensalisme	**Mutualisme**

Comment définir ce « bénéfice » ? Deux conceptions cohabitent à ce sujet (Boucher et al., 1982). La première définit le bénéfice au niveau individuel en comparant la fitness d'organismes qui participent versus ceux qui ne participent pas à l'interaction. Cette définition est bien adaptée à la micro-évolution qui considère l'individu comme principal niveau de sélection, mais elle est moins pertinente pour l'écologue qui s'intéresse par exemple à la conservation des populations. La deuxième définition se place alors au niveau de la population et considère son taux d'accroissement à court terme, ou sa taille à l'équilibre écologique (Abrams, 1987). Par convention, je retiens pour la suite du manuscrit la définition populationnelle pour les termes compétition, exploitation et mutualisme. Le décalage qui peut

se manifester avec la définition individuelle et le bien-fondé de ce type de classification seront discutés dans la conclusion.

2. Coévolution des relations interspécifiques : traits mutualistes et antagonistes.

Les relations interspécifiques sont un moteur majeur de l'évolution. L'espèce A peut évoluer face à l'influence de l'espèce B, sans que l'évolution de B ne soit affectée. Plus souvent, l'évolution de A influence également l'évolution de B, et réciproquement : on parle alors de coévolution. Les expressions phénotypiques de la coévolution sont des traits (ou caractères, ici nous emploierons le mot trait) coévolués. Lorsqu'un trait est modifié chez A (ou seulement dans une population de A) en réponse à B, c'est qu'il confère un avantage sélectif aux individus qui le portent (du moins dans les cas ou le niveau de sélection individuel est le plus pertinent, voir (Lewontin, 1970) pour une discussion détaillée de ce problème). La question est de savoir quel impact a ce trait sur la fitness des individus B : positif, négatif ou nul ? S'il est nul, il n'y a dans ce cas pas de coévolution. S'il est positif, les individus B seront sélectionnés pour évoluer un trait qui renforce le trait de A : la coévolution d'un couple de traits mutualistes est enclenchée. S'il est négatif, les individus B seront sélectionnés pour évoluer un trait qui contrecarre le trait de A : le couple de traits est alors antagoniste. Dans les interactions de compétition et d'exploitation, on peut supposer que les traits antagonistes prédominent, tandis que dans les interactions mutualistes, on s'attend à trouver essentiellement des traits mutualistes. Ces prédictions sont bien sûr fragiles : ce travail de thèse sera l'occasion de discuter cette fragilité et d'en tirer quelques conclusions sur la nature des relations interspécifiques.

3. Le mutualisme

Parmi les trois principales interactions interspécifiques (compétition, exploitation, mutualisme), le mutualisme a reçu relativement moins d'attention au cours du premier siècle de l'histoire de l'écologie (Risch & Boucher, 1976), mais depuis une trentaine d'années le nombre de travaux sur ce sujet augmente considérablement (Bronstein, 1994). A quoi est dû ce relatif oubli pendant un siècle ? L'ouvrage fondateur de (Darwin, 1859), L'origine des

espèces, et son insistance sur « la lutte pour l'existence » peut avoir joué mais l'histoire de la biologie évolutive et celle de l'écologie sont longtemps restées déconnectées (Acot, 1988). Les écologues contemporains de Darwin ne prenaient pas parti sur ses travaux, par exemple J. Vesque précise au début de son ouvrage (Vesque, 1882) qu'un lecteur fixiste peut le lire sans risquer la syncope. Les écologues du début du XX^{ème} siècle n'utilisent pas les travaux de Darwin (Acot, 1988). La frauduleuse interprétation sociale du darwinisme, combinée à la réalité sociale de la société occidentale dans laquelle la science écologique s'est développée, ont en revanche pu influencer les thèmes de recherche en faveur des interactions de compétition et d'exploitation.

Plus sûrement, c'est le développement de la science écologique elle-même qui est responsable de ce biais. L'étude des successions végétales au début du XX^{ème} siècle aux Etats-Unis conduit à se pencher sur le mécanisme de compétition qui permet, par exemple, à la strate ligneuse de se substituer à la strate herbacée. L'essor de l'écologie animale a été en bonne partie favorisée par le problème des ravageurs des cultures, ce qui conduit à se focaliser sur l'herbivorie et les parasitoïdes. L'avènement de la théorie des écosystèmes a été marqué par les notions de flux d'énergie et de pyramide trophique qui mettent l'accent sur la prédation. Les interactions mutualistes sont rarement trophiques dans les deux sens, souvent un lien est trophique et l'autre lié au transport, à la protection, à l'apport d'éléments nutritifs.
Enfin, l'écologie animale et l'écologie végétale sont restées longtemps déconnectées, alors qu'un grand nombre d'interactions mutualistes ont lieu précisément entre un animal et un végétal. C'est le cas, par exemple de 90% des mutualismes publiés entre 1986 et 1990 (Bronstein, 1994) : les interactions plante-pollinisateur et plante-disperseur de graines comptent pour 84%.

Tableau 2. Quelques-unes des principales interactions mutualistes et termes de l'échange mutualiste. Peu d'interactions sont trophiques dans les deux sens.

Partenaire 1	Partenaire 2	Ce que reçoit le partenaire 1	Ce que reçoit le partenaire 2
Plante	Pollinisateur	Transport des gamètes	Nectar, pollen, huile
Plante	Champignon	Eau, éléments nutritifs	Carbone organique
Plante	Frugivore	Dispersion des graines	Paroi du fruit, graine
Plante	Fourmi	Protection contre les herbivores	Nectar extrafloral, abris
Fabacées	Azotobacter	Azote	Carbone organique
Animal nettoyé	Animal nettoyeur	Élimination des parasites	Nourriture et protection
Animal	Symbiote digestif	Aliments assimilables	Aliments non assimilables par l'animal

Ce qui est reçu par un partenaire implique en général un coût pour l'autre : parmi les 7 types de systèmes mutualistes du tableau 2, c'est le cas des 5 premiers. Chaque partenaire a tendance à réduire les coûts que lui impose l'interaction tout en continuant à en bénéficier, c'est pourquoi les mutualismes sont considérés comme sujets à une instabilité évolutive (Herre et al., 1999, Bluthgen et al., 2007).

4. L'interaction plante-pollinisateur.

Le bénéfice recherché par la plante est toujours le transport du gamétophyte mâle, mais certains animaux sont capables de consommer le nectar sans entrer en contact avec les pièces fertiles, comme certains bourdons qui percent la corolle (Richardson, 2004, Inouye, 1983). La majorité des animaux pollinisateurs (insecte, oiseau ou mammifère) visitent les fleurs pour le nectar ou le pollen. La plupart consomment eux-mêmes nectar et/ou pollen, mais plusieurs milliers d'espèces de la super-famille des Apoidea récoltent nectar et pollen pour en nourrir leur progéniture. Certaines abeilles solitaires visitent des fleurs qui produisent des gouttelettes lipidiques (Buchmann, 1987). Des abeilles solitaires et des bourdons visitent également certaines orchidées en croyant y trouver un partenaire sexuel (Jersakova et al., 2006), dans ce cas l'interaction de pollinisation est coûteuse pour l'insecte.

5. L’interaction plante-consommateur de graines.

Les interactions plantes-prédateurs de graines font partie des interactions d’exploitations, comme les interactions hôte-parasite ou proie-prédateur ; elles sont un cas particulier des interactions plante-phytophage. Le prédateur de graines peut être un mammifère, un oiseau ou un insecte.

Lorsque le prédateur n’est pas un agent de dispersion, il existe une large gamme de défenses possibles pour la graine, tant physiques (enveloppe dure ou poilue) que chimiques (Janzen, 1971). Les défenses chimiques qui protègent les graines peuvent être localisées à l’intérieur de celle-ci, dans le tégument ou dans la paroi de l’ovaire chez les Angiospermes. Chez les Conifères, des résines liquides peuvent faire office de défense. Certaines familles comme les Poacées ou les Fagacées n’ont pas développé de défenses particulières.

6. L'interaction plante-pollinisateur et prédateur de graines.

Certaines interactions interspécifiques mettent en jeu des plantes et des insectes qui sont à la fois pollinisateurs et prédateurs de graines. Elles peuvent être comprises comme la combinaison de deux types de relations interspécifiques, une interaction plante-pollinisateur et une interaction plante-prédateur de graines. Le tableau 3 ci-dessous liste quelques-unes des interactions de ce type.

Tableau 3. Principales interactions plante – pollinisateur parasite de graines.

Plante	Insecte	Référence
Trollius europaeus (Ranunculaceae)	*Chiastocheta* (Anthomyidae)	Pellmyr, 1989
Ficus spp.(Moraceae)	Agaonidae	Anstett et al., 1997
Yucca spp. (Yuccaceae)	Prodoxidae	Pellmyr & Thompson, 1992
Lophocereus schottii (Cactaceae)	*Upiga* virescens (Pyralidae)	Holland & Fleming, 1999
Glochidion spp. (Phyllanthaceae)	Epicephala spp. (Gracillariidae)	Kato et al., 2003
Silene latifolia (Caryophyllaceae)	Hadena spp. (Noctuidae)	Bopp & Gottsberger, 2004
Lithophragma parviflorum (Saxifragaceae)	Greya politella (Prodoxidae)	Brown et al., 1997

7. L'interaction entre le trolle d'Europe et les mouches du genre *Chiastocheta*.

Le trolle d'Europe *Trollius europaeus* est une plante arctico-alpine de la famille des Renonculacées. Les individus sont pérennes et se développent dans les prairies humides de l'étage montagnard subalpin (voire à l'alpin) dans les Alpes, en plaine dans les régions scandinaves. Lorsque les conditions du milieu sont favorables le trolle peut constituer plus de 20% du couvert en début de saison. La floraison s'étend de Mai à Juillet en fonction de l'altitude, de l'orientation et du couvert neigeux. Le trolle a la particularité de posséder des sépales pétaloïdes formant un globe jaune. Les pétales sont réduits à des petites languettes nectarifères de même taille que les étamines.

La pollinisation est assurée par de petites mouches du genre *Chiastocheta* (famille des Anthomyiidées). Les chiastochètes adultes émergent pendant la floraison et séjournent en permanence à l'intérieur du globe, ils ne quittent un trolle que pour entrer dans un autre, à la recherche d'un partenaire sexuel ou d'un site d'oviposition. Les chiastochètes assurent 90% des visites que reçoit le trolle (Jaeger & Despres, 1998) et en sont entièrement dépendantes : l'interaction est hautement spécialisée. En effet, les femelles pondent des œufs sur les carpelles, les larves en percent la paroi et consomment une partie des graines en développement. Comme c'est le cas chez la plupart des interactions mutualistes, il existe entre le trolle et les chiastochètes un conflit d'intérêt pour l'accès aux graines, la plante ayant intérêt à disperser des graines saines tandis que les larves ne peuvent se développer qu'en les consommant (Jaeger, 1998, Jaeger et al., 2000).

Contrairement au système figuier-agaonide, pollinisation et oviposition ne sont pas des processus liés. Chez le trolle, les mâles à la recherche d'un partenaire sexuel participent autant que les femelles à la pollinisation (Despres, 2003). Il n'y a pas de corrélation positive entre le nombre d'œufs pondus et le taux de pollinisation d'une fleur (Despres et al., 2007), voir l'article en Annexe 1).

Six espèces de chiastochètes sont présentes dans les Alpes, les adultes émergent séquentiellement du début à la fin de la floraison dans l'ordre suivant : *C. rotundiventris*, *C. inermella*, *C. macropyga*, *C. setifera*, *C. trollii*, *C. dentifera*. *C. rotundiventris* visite et pond dans les fleurs dès qu'il est possible d'y rentrer, tandis que *C. dentifera* fréquente les fleurs qui sont en train de faner. Les espèces se distinguent également par le trajet de la larve dans le fruit :

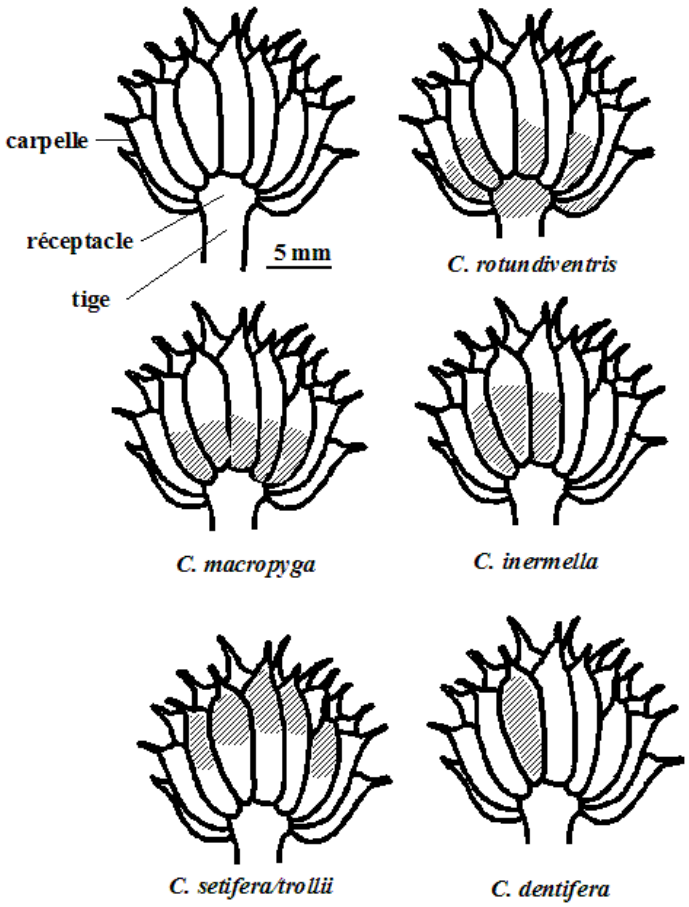

Figure 1. Structure du fruit et trajet des larves des différentes espèces de chiastochètes.

8. Pistes de recherche.

L'interaction spécialisée Trolle d'Europe - chiastochètes est duale : elle combine deux types de relations interspécifiques, une interaction plante-pollinisateur et une interaction plante-prédateur de graines. La première piste de recherche est que les traits impliqués dans la spécialisation de l'interaction devraient refléter cette dualité. Pour suivre cette piste, nous avons étudié successivement plusieurs traits morphologiques et biochimiques impliqués dans la spécialisation de l'interaction, en posant les questions suivantes :

Quelle est l'importance de la morphologie florale globulaire dans la spécialisation de l'interaction ? La forme globulaire est-elle évolutivement stable ? Dans quelles conditions a-t-elle pu apparaître au cours de l'évolution ? (chapitres 1 et 2)

Quel est le rôle des composés organiques volatiles dans l'attraction spécifique des chiastochètes ? (chapitre 3)

Peut-on mettre en évidence un trait chez le trolle qui soit le fruit du conflit évolutif qui l'oppose aux larves de chiastochètes ? (chapitre 4).

La seconde piste est que la dualité de l'interaction peut s'exprimer à la fois dans l'évolution de chacun des traits impliqués, et dans leur évolution conjointe. C'est essentiellement *a posteriori*, au regard de l'ensemble des résultats, que nous suivrons cette piste, dans la conclusion de ce travail.

CHAPITRE 1 : Stabilité évolutive de la spécialisation de la morphologie florale.

1. Introduction

Le service de pollinisation entomophile implique toujours un coût via par exemple la production de nectar ou d'huiles, ou la perte de pollen. Dans le cas de la pollinisation par des pollinisateurs prédateurs de graines, le coût semble particulièrement sévère puisqu'il s'agit de graines. Pourquoi, dans ce cas, se spécialiser sur de tels partenaires lorsque d'autres pollinisateurs moins coûteux sont présents dans le milieu ?

Afin de tester la stabilité évolutive de la spécialisation du trolle d'Europe sur les chiastochètes, nous avions besoin d'un trait impliqué dans la spécialisation et expérimentalement manipulable. Nous avons choisi un trait remarquable du Trolle d'Europe : la morphologie globulaire de la fleur. C'est cette caractéristique que l'on cite en premier lorsque l'on décrit la fleur au grand public. Dès que l'on évoque la « boule jaune », les personnes qui l'ont déjà rencontrée voient de quoi il s'agit. Si la forme globulaire est remarquable pour l'œil humain, qu'en est-il du point de vue de l'écologie évolutive ?

Dans le genre *Trollius*, les espèces *T. pumilus*, *T. ranunculinus* et *T. laxus* n'interagissent pas avec les chiastochètes ; leur calice est plat (Figure 2) et une large gamme d'insectes leur rend visite (Pellmyr, 1992).

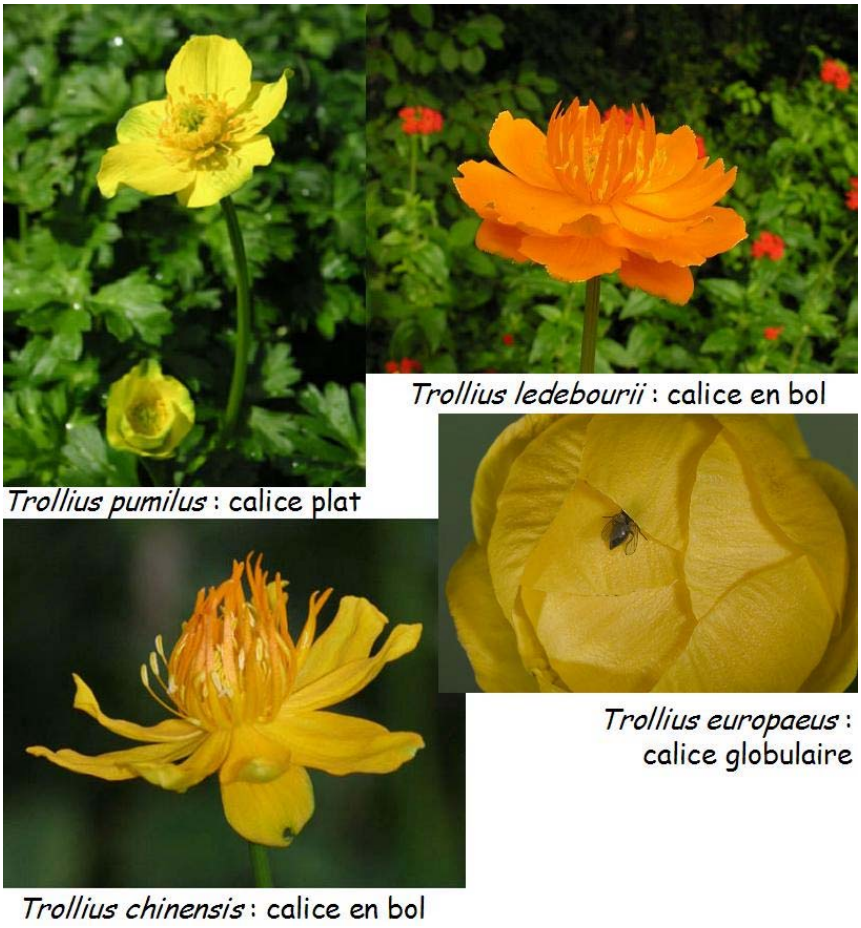

Figure 2. Diversité des morphologies florales dans le genre *Trollius*.

Une phylogénie moléculaire a montré que la morphologie florale ouverte est le caractère ancestral dans le genre *Trollius* (Despres et al., 2003) et que toutes les espèces visitées par les chiastochètes dans l'ensemble de leur aire de répartition forment un groupe monophylétique (*altaicus, europaeus, ledebouri, asiaticus, chinensis*, Figure 3). Ces espèces ont un calice en bol ou globulaire (Figure 2).

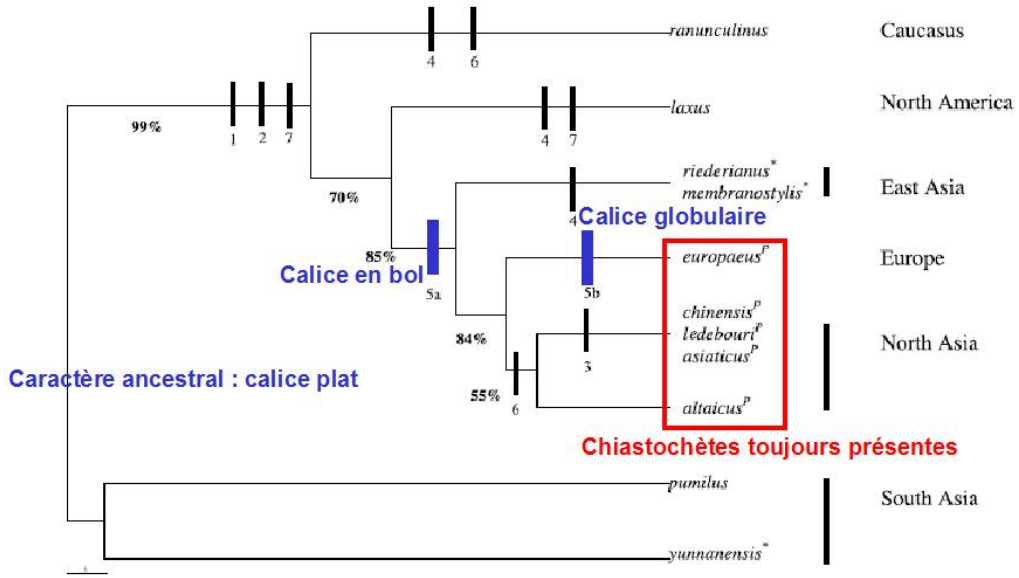

Figure 3. Phylogénie du genre *Trollius* d'après (Despres et al., 2003) et évolution de la morphologie florale. ^{P}: Chiastochètes présentes sur toute l'aire de distribution ; * seulement dans certaines populations.

Les *Chiastochètes* sont capables de se glisser entre les sépales, tandis que les autres pollinisateurs potentiels, notamment de plus gros diptères de la famille des Syrphidae et des bourdons, n'y parviennent pas la plupart du temps (Figure 4).

Figure 4. Bourdon tentant sans succès de rentrer dans un globe de trolle.

Dans l'article présenté dans ce chapitre, nous avons d'abord posé les deux questions suivantes :

- la morphologie florale est-elle effectivement impliquée dans la spécialisation ? La réponse à cette question semble claire, mais elle n'avait jamais été résolue expérimentalement.
- La spécialisation morphologique est-elle avantageuse, tant du point du vue de la fitness femelle (production de graines) que de la fitness mâle (export de pollen) ?

Nous avons étudié les deux fonctions mâle et femelle séparément, car elles peuvent répondre de manière différente aux interactions avec les pollinisateurs, soit dans le même sens mais dans des proportions différentes, soit dans des sens opposés. En présence de pollinisateurs alternatifs, il est possible qu'un trolle ouvert soit aussi bien pollinisé qu'un trolle fermé, tout en abritant moins de larves de chiastochètes : dans ce cas la forme ouverte augmente la fitness femelle. En même temps, on peut supposer que la fitness mâle soit plus élevée chez la forme globulaire, les chiastochètes étant de meilleurs vecteurs de pollen que les autres pollinisateurs

généralistes. Il en résulte un conflit évolutif entre les fonctions sexuelles (Lankinen et al., 2006).

Pour répondre aux questions posées ci-dessus, nous avons conduit sur le terrain des expériences basées sur une modification artificielle de la morphologie florale, décrite dans la section suivante. Pour aller plus loin, nous avons testé l'avantage de la spécialisation dans différentes situations écologiques et populationnelles. Les communautés des autres pollinisateurs potentiels varient quantitativement et qualitativement, notamment en fonction de l'altitude. Les syrphes, muscidés et bourdons sont abondants à l'étage montagnard mais pas à l'étage subalpin où seuls les muscidés sont observables régulièrement. En fonction de l'étage, il est donc possible que l'avantage ou le désavantage de la spécialisation soit variable, les expériences ci-dessous ont donc été conduites dans les deux étages. Enfin, nous avons pensé que la sélection qui s'exerce sur les phénotypes floraux ouverts et globulaires pouvait dépendre des abondances relatives des deux phénotypes. Nous avons formulé l'hypothèse qu'un phénotype localement abondant va être plus visité par les chiastochètes qu'un phénotype localement rare. Ce mécanisme pourrait permettre à des phénotypes ouverts localement abondants d'être sélectionnés positivement.

2. Méthodes

Sites d'étude.

Nous avons réalisé les expériences dans deux régions des alpes françaises, la Chartreuse, massif préalpin dominé par l'étage montagnard et la région du col du Lautaret ou dominent les pelouses subalpines. Dans la région du col du Lautaret, nous avons bénéficié du support de la Station Alpine Joseph Fourier (UMS 2925 UJF-CNRS), tant pour l'hébergement que pour le laboratoire.

En Chartreuse, trois populations ont été choisies dans le secteur du col de Portes (Sarcenas, 1101 m. ; Fontanil 1020 m. ; Cherlieu, 980 m. ; Figure 5)

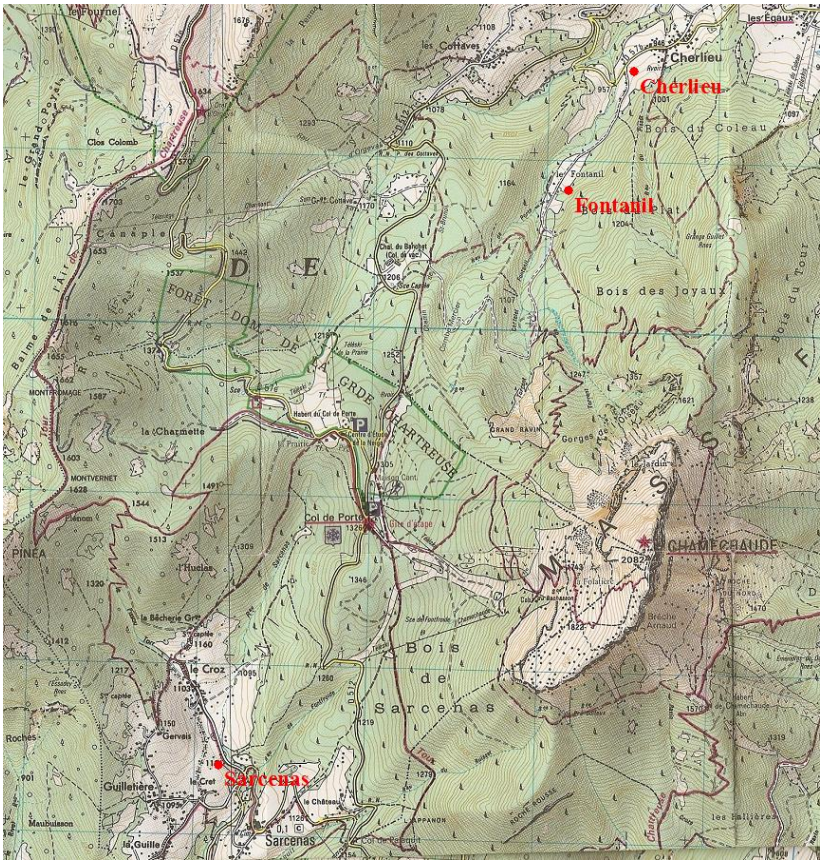

Figure 5. Sites d'étude en Chartreuse.

Dans le secteur col du Lautaret-col du Galibier, deux populations ont été choisies (Ruillas, 2025 m. ; Pré Gelé, 2374 m. ; Figure 6).

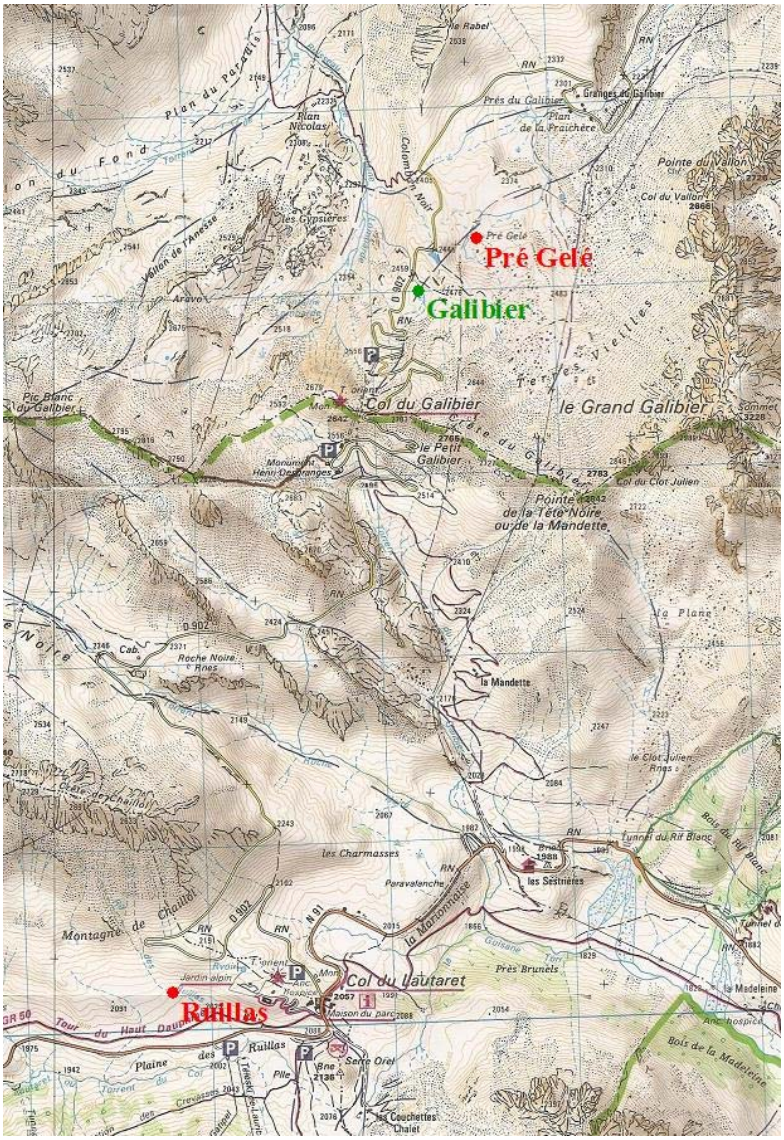

Figure 6. Sites d'étude dans la région du col du Lautaret. Le site en vert nommé « Galibier » a été utilisé dans une expérience présentée ultérieurement.

Modification expérimentale de la morphologie florale.

Nous avons évité de couper les sépales afin de ne pas créer de blessure qui pourrait modifier le comportement des insectes. Des anneaux en plastique de différentes tailles ont été utilisés pour ouvrir artificiellement le globe (Figure 7).

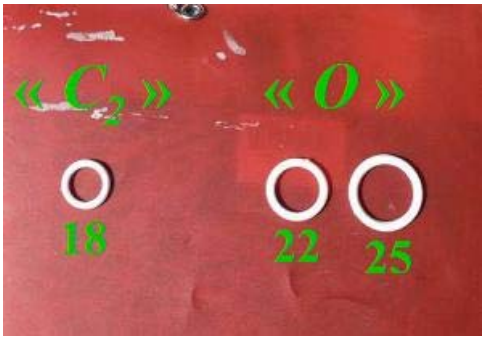

Figure 7. Diamètre des anneaux en plastiques utilisés.

Les fleurs étaient ouvertes avec les anneaux de diamètre 22 ou 25 mm, choisis en fonction de leur taille (traitement ouvert « O »). Le traitement de contrôle « C_1 » correspondait à une fleur non manipulée. Afin d'estimer l'effet de l'anneau en plastique sans modification de la morphologie, nous avons utilisé le petit anneau de diamètre 18 mm (traitement de contrôle « C_2 »). Le résultat de ces traitements sur la morphologie florale est illustré dans la Figure 8.

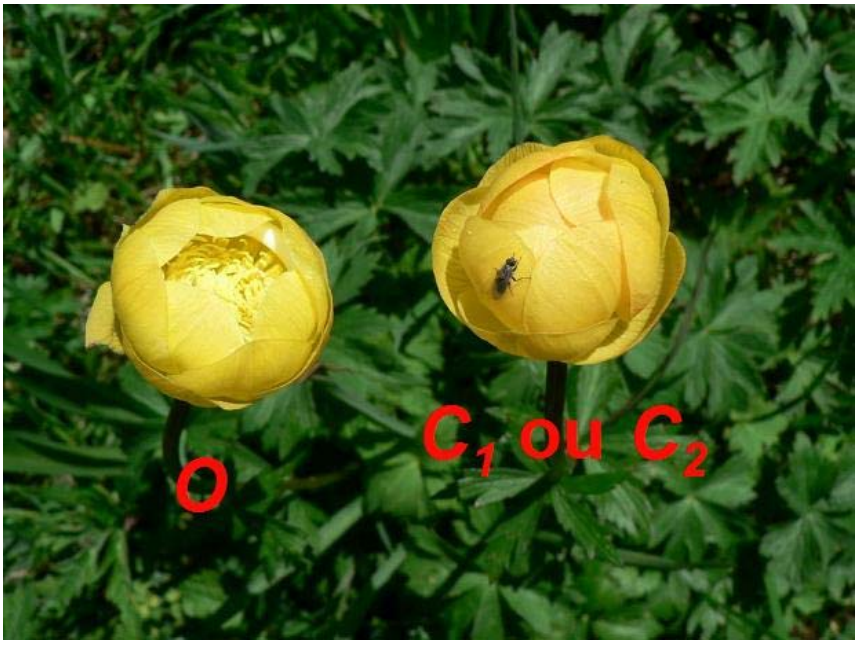

Figure 8. Morphologie florale artificiellement ouverte par un anneau que l'on distingue appliqué contre la face interne des sépales (« *O* ») et fermée, soit sans anneau (« C_1 »), soit avec un petit anneau (« C_2 »).

Observation de pollinisateurs.

Des séries d'observations dans les cinq sites étudiés ont été conduites. Un insecte était comptabilisé parmi les visiteurs lorsqu'il pénétrait le globe dans le cas d'une fleur fermée ou lorsqu'il entrait en contact avec les pièces fertiles d'une fleur ouverte (Figure 9).

Figue 9. Diptère syrphidé (*Scaeva pyrastri*) sur les étamines et carpelles d'un trolle artificiellement ouvert.

Fitness mâle : mesures des flux de poudre fluorescente.

Nous avons utilisé des particules de poudre fluorescente de couleur jaune et rouge comme un moyen de comparer l'export de pollen par des fleurs ouvertes et fermées. Le dispositif expérimental est schématisé ci-dessous et détaillé dans le corps de l'article.

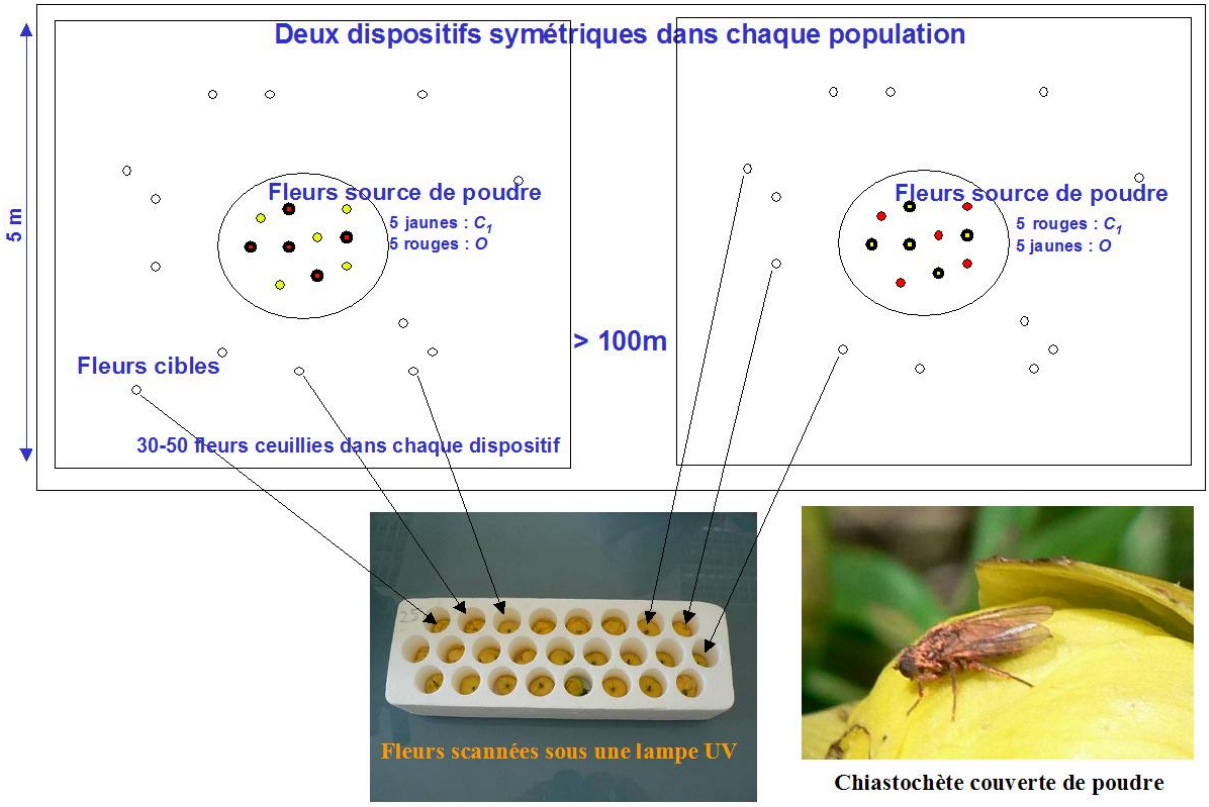

Figure 10. Dispositif expérimental utilisant des particules de poudre fluorescente pour l'export de pollen par des fleurs ouvertes et fermées.

Ce dispositif a été mis en place sur les sites de Sarcenas et Fontanil (Chartreuse) et Ruillas et Pré Gelé (Lautaret).

Fitness femelle : mesure de la production de graines.

Des fleurs immatures ont été ensachées avec un tissu en nylon (de type « voile de mariée ») pour éviter qu'elles ne reçoivent de visites avant le début de l'expérience (Figure 11a). Une fois matures, les fleurs reçoivent les traitements O, C_1 ou C_2 (Figure 11b). Après pollinisation, oviposition et chute des sépales, les fleurs sont ensachées afin d'empêcher les attaques d'herbivores et la dispersion des graines (Figure 11c). A l'issue de la maturation du fruit, lorsque les carpelles sont secs et commencent à s'ouvrir, les fruits sont récoltés et disséqués au laboratoire.

a. Fleurs immatures ensachées

b. Morphologie florale modifiée

c. Jeunes fruits ensachés

Figure 11. Séquence expérimentale pour la mesure de la production de graines par les phénotypes ouverts et fermés. Ce dispositif a été mis en place sur les cinq sites étudiés.

Manipulation de la fréquence des phénotypes ouverts et fermés.

Afin de savoir si la fréquence des phénotypes ouverts et fermés a un impact sur la sélection exercée par les pollinisateurs, nous l'avons manipulée en mettant en place deux types de blocs expérimentaux : un dans lequel le phénotype ouvert est fréquent et un dans lequel il est rare. Le plan d'expérience ci-dessous a été mis en place sur le site de Ruillas uniquement.

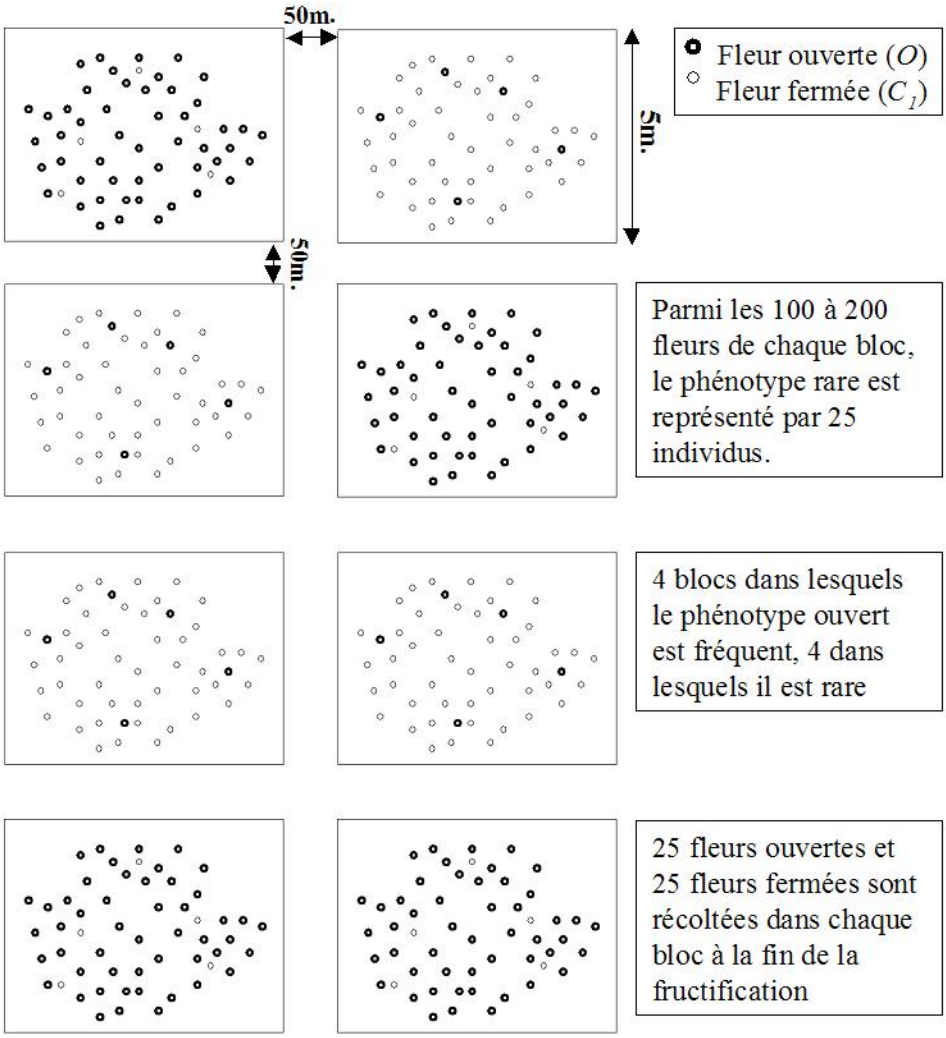

Figure 12. Dispositif expérimental.

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Stability of floral specialization in *Trollius europaeus* in contrasting ecological environments

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Running title: Stable floral specialization in globeflowers

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Abstract

Specialization of some plants on seed-eating pollinators is intriguing, especially when co-pollinators exclusively feeding on nectar are also present. We examined the stability of the morphological specialization of *Trollius europaeus* (L.) globeflowers with respect to *Chiastocheta* (Pokorny) flies by artificially opening the flowers. In the montane and subalpine environments studied other visitors contributed 2 and 28% of all the visits respectively and visited open flowers nearly eight times more often than closed flowers, but in both environments their contribution to pollination did not compensate for *Chiastocheta* aversion against open phenotypes. Net seed set (female success) was slightly higher (+4%) and pollen export (male success) was much higher (+85%) for closed than for open flowers. Selection in favour of the closed phenotype was even more intense in patches where open phenotypes were most common, precluding the evolution of open flowers in the study populations.

Key words: floral morphology, male and female fitness, most efficient pollinator, nursery pollination mutualism, pollinator-mediated selection.

Introduction

Over the last decade, our view of plant-pollinator interactions has shifted from the long-standing concept of "pollination syndromes" reflecting specialization as the common outcome of natural selection in those systems, to the widely accepted idea that generalization is widespread in pollination ecology, and that specialized plant-pollinators interactions might be the exception rather than the rule (Johnson & Steiner, 2000, Waser, 2006, Waser et al., 1996). If this is true, understanding the ecological factors and the evolutionary mechanisms that lead to specialization or generalization is of fundamental importance (*e.g.* Dilley et al., 2000, Gomez & Zamora, 1999, Mayfield et al., 2001, Thompson, 2001 reviewed in Fenster et al., 2004 and in Gomez & Zamora, 2006), as they could help to explain the emergence of diverse plant-pollinator communities through adaptive co-diversification (Aigner, 2005, Dilley et al., 2000, Hodges & Arnold, 1994).

Pollination typically implies a cost for the plant through nectar or pollen lost to feeding pollinators. The cost is particularly severe in seed-eating pollinator mutualisms where the offspring of pollinators feed on potential plant offspring. Plant specialization involving such partners has long been intriguing, especially when co-pollinators feeding only on nectar and not on developing seeds are also present, as is often the case (Holland & Fleming, 2002, Thompson & Cunningham, 2002). If seed-eating pollinators consume more seeds than fertilize ovules, they should be viewed as parasites rather than mutualists (Holland & Fleming, 2002, Thompson & Cunningham, 2002, Thompson & Fernandez, 2006). Plants may then be expected to develop traits that allow pollination by generalist co-pollinators and minimize the costs imposed by specialized seed-eating pollinators.

In the *Trollius europaeus* (L.) -*Chiastocheta spp.* (Pokorny) mutualism, *Chiastocheta* flies contribute to almost all pollination (Jaeger & Despres, 1998, Pellmyr, 1989), although other insects, mainly Diptera and Coleoptera, account for about 10% of plant visits (Jaeger & Despres, 1998). Among *Trollius* species and *Chiastocheta* flies, various levels of specialization can be found (Pellmyr, 1992). Some *Trollius* species are not visited at all by *Chiastocheta* flies, such as *T. pumilus*, *T. ranunculinus* and *T. laxus*. All of these present an open flat corolla and are likely to be pollinated by a wide range of insects (Pellmyr, 1992). Molecular phylogenetic analysis showed that an open flower is the ancestral state in the genus *Trollius* (Despres et al., 2003) and that all species visited by *Chiastocheta* flies throughout their range form a derived clade (*altaicus, europaeus, ledebouri, asiaticus, chinensis*). The only morphological character clearly linked to the presence of *Chiastocheta* is the bowl shape of the corolla, which reaches

its extreme in *T. europaeus* by forming a totally closed globe. This globular shape of *T. europaeus* is thus likely to be responsible for the exclusion of pollinators other than *Chiastocheta* flies. These small flies can navigate past the sepals to reach the inner part of the globe where larger insects are prevented from entering. We hypothesize that *T. europaeus* and *Chiastocheta* flies coevolved reciprocal specialization through a number of traits, one being the closed flower shape coevolving with the flower shape preference of the insect.

Other potential pollinators are present in communities where *T. europaeus* occurs, so that it is not obvious why the plant would be specialized on *Chiastocheta* flies. The shape of *T. europaeus* corolla in natural populations is always mostly globular, but in some populations open flowers can be found at very low frequency (less than 10%, S. Ibanez, personal observations), suggesting that this trait is potentially variable, and insects other than *Chiastocheta* have been reported inside the globe by various authors (Jaeger & Despres, 1998, Pellmyr, 1989). Previous observations have shown that the other visitors were more diversified at the montane than at the subalpine level, where mostly Dipterans are found (Jaeger & Despres 1998). It is possible that the presence of pollinators of different types would result in disruptive selection on floral morphology (*e.g.*, Medel et al., 2003). Given that *T. europaeus* populations are exposed to different insect communities in their montane and subalpine environments, they could be subject to variable selective pressures from male and/or female functions, hence creating a geographical mosaic with spatial variability in strength and direction of selective pressures on floral morphology (Thompson, 2005). In the *Trollius* case where the specific pollinator is also a seed predator, one might expect that female and male functions will respond differently to change in pollinator communities, the female function favouring the open morph when alternative pollinators are available, while male function still favours the closed phenotype. Finally, local densities of flower phenotypes could affect selection regimes, because insects often prefer common phenotypes to which they are accustomed and neglect rare phenotypes (Chittka et al., 1999). Open flowers might therefore be avoided when rare but regularly visited when locally abundant. To determine the direction of selection in favour of the specialized closed phenotype, in different community contexts, we manipulated the floral morphology of globeflowers in five populations (montane and subalpine environments), recorded visits from insects, evaluated pollen export, and counted final seed production in order to compare the relative fitness of closed and open flower phenotypes. More specifically, we addressed the following questions: (i) is floral morphology a key trait in globeflowers' specialization, (ii) do flowers always benefit from being specialized in present-

day populations, including contrasting ecological conditions, and (iii) is pollinator selection of open *versus* closed flower phenotypes frequency-dependent?

Material and methods

Study system

The European globeflower *Trollius europaeus* L. (Ranunculaceae) is a hermaphroditic, homogamous, arctic-alpine perennial species that grows in moist meadows (Despres et al., 2007). In the Alps, natural populations range from 700 to 2500 m above sea level (a.s.l.). We did field experiments around the "Station Alpine Joseph Fourier", col du Lautaret, France, in two populations at the subalpine level (above tree-line, Ruillas, 2025 m a.s.l. ; Pré Gelé, 2374 m a.s.l.) and in three populations located in the Chartreuse range at the montane level (below tree-line, Sarcenas, 1101 m a.s.l. ; Fontanil 1020 m a.s.l. ; Cherlieu, 980 m a.s.l.) from May until July in 2006 and 2007. Each flower contains about 30 multiovulate carpels dehiscing 3-4 weeks after the end of flowering. Flowering is synchronized within populations and typically lasts 2-3 weeks (Jaeger et al., 2000). Apart from *T. europaeus* which represented the most abundant flowering plant during the study period in all the sites, main co-flowering and insect-pollinated plants included *Geranium sylvestris* at the montane level, and *Narcissus poeticus* at the subalpine level. Other species were present at low densities (*Alchemilla vulgaris, Dactylorhiza maculata, Polygonum bistorta* at the montane level ; *Veratrum album, Polygonum viviparum, Bartsia alpina* and *Primula farinosa* at the subalpine level). In the Alps, *T. europaeus* is passively pollinated by six species of *Chiastocheta* flies (Anthomyiidae), but for this work we will consider these at the genus level because identification at the species level requires the dissection of genitalia and cannot be performed in the field (Michelsen, 1985, Collin, 1954). *Chiastocheta* larvae only feed on *T. europaeus* seeds; they are obligate associates of globeflowers. Both male and female flies exclusively visit the globe-shaped flower (we observed only once a *Chiastocheta* fly on *Ranunculus acris* during the whole study) where they eat nectar and pollen, and where they mate (Pellmyr, 1989). Both activities contribute to pollination (Despres, 2003). Females deposit one to several eggs, on or between the carpels. Seed maturation and larval development last 3-4 weeks (Jaeger et al., 2000). Each larva eats several seeds and falls to the soil to over-winter as a pupa.

Experimental modification of floral shape

Globeflowers were artificially opened ("*O*" flowers) similarly to the most naturally open flowers by placing a plastic ring inside the corolla, the diameter of which was chosen according to flower size (22 mm or 25 mm, see Aigner, 2004, Johnson et al., 1995 for similar experimental modification of floral morphology). The ring touches the sepals (and sometimes the filaments of the stamens) but neither the anthers nor the carpels. We did not cut any sepals

in order to avoid modification of the volatiles emitted by the flower. Control flowers were either untouched ("C_1" flowers) or harboured smaller plastic rings that did not modify the globular morphology (D = 18 mm, "C_2" flowers), in order to check for a *ring* effect. All the flowers we manipulated or surveyed were selected from vigorous plants. Flowers were of roughly the same age and height, with dehiscing stamens, abundant pollen and well-developed calyxes. The experiments were conducted when populations were at the peak of flowering in the five populations described above.

Visits census

Visits were recorded for observation periods ranging from 30 to 90 minutes in variable weather conditions between 9 a.m. and 4 p.m., corresponding to the peak of pollinator activity. During each period, 9 randomly chosen flowers were surveyed, three for each treatment *O*, C_1 and C_2. For each insect visit, we recorded if the insect entered the globe or not, if it belonged to the genus *Chiastocheta* or not, and if not, to which order it belonged. For each period, we pooled the number of visits received by the three flowers that received the same treatment in order to reduce the occurrence of zero values in the data set. The total number of observations was then 135, divided in 45 observation periods nested in five populations (3 in montane and 2 in subalpine environments). There were from 7 to 11 observation periods per population. We recorded a total of 1177 insect visits during a total of 151 h of observation. All statistical analyses were done with the software R 2.6.0 (Team, 2007). We compared the number of visits per given insect type using Generalized Linear Mixed Models, with the Penalized Quasi-Likelihood method (function "glmmPQL" in library MASS, Venables & Ripley, 2002). The fixed effects included the duration of observation, floral morphology (C_1 as reference level, *O* and C_2) and environment (subalpine as reference level, and montane), and excluded the interactions between these factors. The observation periods and the populations nested in environmental effects had normal random effects, and the model used the "Poisson" family (link function: log).

Measurement of fitness components.

Male fitness was measured using the movement of fluorescent dye particles as a surrogate for pollen export (Price & Waser, 1982). Ten flowers of the same age were chosen from the centre of a 5 m square patch; five of them were opened with a ring ("*O*") and their stamens dyed yellow using a paintbrush, five remained morphologically unmodified ("C_1") and their stamens dyed red. A hundred m away, the same experimental treatment was applied to a second patch, with the dye colours reversed, in order to check for a dye colour effect. Twenty-four h later,

60-65 previously untouched target flowers were collected from each patch and scanned under a UV lamp to reveal fluorescence. Preliminary experiments showed that during a period of 24 h the dye contained in a given flower was never found farther than 20 m away, precluding massive dye transfer between patches. We recorded the presence or absence of each dye colour on the stamens and the stigmas. This procedure was conducted in three montane populations and two subalpine populations to test for environmental variability. In each population, we recorded the proportion of target flowers marked with red and/or yellow in each patch (2 patches * 2 colours = 4 observations), so a total of 20 observations were used in the statistical analysis. We compared the proportion of flowers that received dye from *O* versus C_1 flowers using "glmmPQL" (binomial family, link function: logit). The fixed effects included dye colour, floral morphology (C_1 as reference level, and *O*) and environment (subalpine as reference level, and montane); interactions between morphology and dye colour, and between morphology and environment were also included. The populations nested in environmental effects had normal random effects.

Net seed production after pollination and predation, a measure of female fitness, was estimated by weighing the seeds of dehiscent fruits. Preliminary results showed a high correlation between this measure and the number of viable seeds (seed number = 2.11*total seed mass in mg, $R^2 = 0.96$, N = 31). A total of 120 flower buds were bagged to prevent pre-experimental pollination. At flower maturity (*i.e*. anther dehiscence), the bags were removed and treatments *O*, C_1 and C_2 were each applied to 40 flowers. Flowers remained untouched until carpel dehiscence (which was also the end of larval development), and were then collected. After hatching, the empty egg shell remains attached to the carpels, so that we were able to count egg number and to weigh seed mass. This experimental set-up was replicated in the five study sites. A large fraction of the 600 manipulated flowers were either lost or destroyed in the field, or were too damaged to be dissected in the lab, so only 291 and 294 were included in the statistical analysis of egg number and net seed production per flower respectively (from 28 to 93 per population). We compared seed production and egg deposition in flowers of different morphologies using "glmmPQL" ("Poisson" family). The fixed effects included floral morphology (C_1 as reference level, *O* and C_2), environment (subalpine as reference level, and montane) and carpel number; the interaction between morphology and environment was also included. The populations nested in environmental effects had normal random effects.

Manipulation of the local frequency of open phenotypes

We selected eight circular patches of *T. europaeus,* in a single large population near the Col du Lautaret. Each patch had a diameter of 5 m, contained approximately 100-200 flowers, and was separated from others by a distance of at least 50 m. Fifty target flowers in the centre of each patch were bagged until maturity, and either treatment *O*, or C_1 were each applied to 25 flowers. Additionally, we defined a low-frequency treatment in which all the flowers surrounding target flowers inside the patch were left untouched, so that only 25 flowers were locally open; and a high-frequency treatment where all the surrounding flowers were experimentally opened (*O* flowers), so that only 25 flowers were locally closed. Both treatments were each applied to 4 randomly chosen patches. High-frequency patches were visited every two days during ten days in order to open recently blooming flowers. As soon as the sepals of the target flowers had fallen, they were collected, and egg number and pollination rate were determined for each flower. Pollination rate was evaluated by randomly choosing five carpels and counting the number of initiated seeds *versus* the number of unfertilized ovules in each (15229 ovules counted in total). We did not wait for carpel dehiscence to weigh seed mass because seed initiation provides more immediate information about the selective regimes acting on floral morphology. Out of the 400 flowers manipulated, 234 were finally used in the analysis, the remaining being either lost or destroyed in the field, or too damaged to be dissected in the lab. We compared egg deposition and pollination rate of flowers of different morphologies in low and high-frequency patches using "glmmPQL" ("Poisson" and "binomial" families respectively). The fixed effects included floral morphology (C_1 as reference level, and *O*), frequency of open flowers in the focal patch (low frequency as the reference level, and high frequency) and carpel number; the interaction between morphology and the frequency of open flowers was also included. The patches nested in the frequency of open flowers treatment had normal random effects.

Results

Visits from Chiastocheta *and other insects*

Unsurprisingly, *Chiastocheta* and other insects' visits number increased with the length of the observation period (Table 1). *Chiastocheta* showed a preference for closed over open flowers, with an estimated 77% reduction of visits to open flowers. *Chiastocheta* were three times more frequent in the subalpine than in the montane environment (Fig. 1). Other insects belonged to Diptera, Coleoptera, Hymenoptera and Hemiptera and were rare compared to *Chiastocheta*, representing less than 15% of the visits. They were more frequent in the montane than in the subalpine environment, representing 28.4 and 2.3% of the visits respectively. In both environments, Syrphidae represented most of non-*Chiastocheta* visitors, followed by Hymenoptera. In the subalpine environment, only Diptera (mostly Syrphidae) and non-Apidae Hymenoptera (Tenthredinidae) were observed, while in montane environment, Hymenoptera were mostly Apidae, and Coleoptera (including *Odomera* genus) and Hemiptera were occasionally observed in addition to the numerous visits by syrphids and other Diptera. Non-*Chiastocheta* visitors showed a clear preference for open flowers (with nearly eight times more visits in open than in closed flowers). There were no differences between C_1 and C_2 flowers for visits of both *Chiastocheta* and other insects, indicating that the presence of a ring inside the flowers had no impact in itself on visitation rate.

Dye export

Although only pollen transfer to receptive stigmas contributes to pollination, we included in our analysis observations of dye deposited on stamens, because it indicates that flies not only efficiently transfer dye to stigma but also come into close contact with stamens. Red dye was less exported than yellow dye (Table 2), but this factor did not interact with the floral morphology: *Chiastocheta* flies are less attracted by reddish flowers, whether they are open or not. Dye export was more intense in subalpine populations, again without any regard to morphology. Open flowers exported dye toward many fewer flowers than closed ones, ***e.g.*** for the stigmas the observed decrease reached 85% (Fig. 2).

Egg load and net seed production

Unsurprisingly, both egg load and net seed production were positively correlated with carpel number (Table 3). Mean egg load was lower in montane populations compared to subalpine populations. Net seed production was also lower in montane than in subalpine environment but due to small sample size, the environment effect was not significant. Open flowers received

significantly fewer eggs (- 30%) and produced fewer seeds (- 4%) than closed flowers (Fig. 3). The differences between C_1 and C_2 flowers were not significant.

Egg load, pollination rate, and the frequency of open phenotypes

There was a positive correlation between carpel number and egg load, and a negative non-significant correlation between carpel number and pollination rate (Table 4). Mean egg load and pollination rate were lower in open than in closed flowers (Fig. 3), although these differences were not significant (Table 4). When open flowers were locally more frequent, there was a slight non-significant increase in mean egg load and pollination rate. The difference between egg load and pollination rate in closed and open flowers was larger when the open phenotype was frequent than when it was rare (Fig. 3), but the interactions between the floral morphology and the local frequency of open phenotypes for both egg load and pollination rate were non-significant (Table 4).

Discussion

Specialization and selection pressures on male and female functions

In all populations studied, the experimental opening of *T. europaeus* flower resulted in a decrease in both male and female fitness. We cannot make a formal comparison between male and female fitness because we used different proxies to estimate them (the proportion of target flowers marked with dye for male fitness, and seed weight for female fitness). The pattern of dye and pollen movement may vary between systems. For example, the correlation between the total amount of dye and pollen found on individual stigmas lies between 0.4 and 0.8 in systems using hummingbirds and bumblebees (Adler & Irwin, 2006, Thomson et al., 1986, Fenster et al., 1996, Waser & Price, 1982, Waser, 1988, Rademaker et al., 1997). Dye cannot be used to estimate absolute male fitness because dye particles are smaller than pollen grains and more particles are carried by insects than grains (Thomson et al., 1986); and because post-pollination mechanisms such as incompatibility can lead to a discrepancy between dye movement and gene flow estimated *via* paternity tests (Campbell, 1991). However, the use of dye is appropriate for comparisons on the relative dispersal of pollen from plants receiving different treatments because treatments affect dye and pollen carryover in similar ways (Thomson et al., 1986, Aigner, 2004).

Differences in seed production between floral phenotypes were small (4%) compared to the radical change in pollen export (85%). Despite the use of different proxies to estimate male and female fitness, the 20-fold difference is large enough to suggest that the main selective pressure on floral morphology is male function. Several other studies have shown that floral attractive traits evolve mainly through selection on male fitness (male-function hypothesis, reviewed in Burd & Callahan, 2000). The stability of highly specialized plant-pollinators mutualisms is often questioned in a different way: do insects provide a sufficient pollination service to account for the persistence of their host-plant populations (Anstett et al., 1997, Bloch et al., 2006, Ferriere et al., 2002, Morgan et al., 2005)? Our results show that in the *Trollius*-*Chiastocheta* interaction, seed production is not limited by pollen availability, so that competition between male gametes for seed siring is likely to be high, especially since globeflowers produce large amounts of pollen. More specifically, we show that the main effect of floral specialization on *Chiastocheta* flies is to increase pollen export rather than seed production: specialization of *T. europaeus* on *Chiastocheta* flies results more from the competition between male gametes, rather than from increasing overall effective pollination and seed set at the population level. (Cheptou & Schoen, 2007) emphasized the importance of

considering both female and male fitness in the study of the evolution of self-fertilization; here we show that considering both components is also necessary to understand the evolution of floral traits in response to pollinator selection pressures. Female and male fitness can respond differently to phenotypic changes (Aigner, 2004), sometimes in opposite ways (Lankinen et al., 2006). In the globeflower populations documented here, both male and female fitness decrease when the flower shape is open, ranging from a slight to a very sharp decrease.

Geographical variation of selection pressures

Chiastocheta visits were more frequent and dye export more intense in subalpine populations than in montane populations. Indeed, egg number per flower, which partly reflects fly population density (Johannesen & Loeschcke, 1996), was higher in subalpine populations. In contrast, other potential pollinators were much more abundant in montane populations, especially Syrphid flies able to enter open flowers: the pollinator community context dramatically changed with the environment. Geographically variable pollinator communities are a key feature of plant-pollinator interactions (Waser & Ollerton, 2006) and lead to selection mosaics and local coevolution (Thompson, 2005). Despite a highly variable community context in the montane and subalpine populations, we found no interaction between environment and floral morphology on either female or male fitness, which suggests that alternative pollinators play a minor role in on going coevolution of the specialization of the *T. europaeus – Chiastocheta spp.* interaction at the present time. Nevertheless, alternative pollinators may influence the morphological evolution of other *Trollius* species. For example, the Asiatic species *T. chinensis* that is visited both by *Chiastocheta* and alternative pollinators displays an intermediate floral morphology between a closed globe and a widely open corolla, while an open flat corolla is found in *T. laxus*, *T. pumilus* and *T. ranunculinus*, three species outside the range of *Chiastocheta*. Furthermore, the evolution of floral traits also involves species other than potential pollinators, such as pollen feeders, florivores and nectar robbers (Herrera et al., 2002). The closed morphology of *T. europaeus* might contribute to excluding these, leading to an additional selection pressure favouring closed phenotypes. Although some harmful insects might indeed be excluded thanks to the closed morphology, we did occasionally observe *Oedemera* (Coleoptera), small unidentified Coleoptera and Thysanoptera consuming pollen, and Lepidoptera larvae consuming developing anthers and carpels inside the globes after having pierced a hole through the sepals. These insects were not more frequently

observed in artificially opened flowers, and are unlikely to exert a strong selective pressure on *Trollius* floral morphology.

Negative frequency-dependent selection against open flowers

When open flowers were most common, the egg load was high in closed flowers and the pollination rate was low in open flowers (Fig. 3), although this was not significant (Table 4). The lack of statistical significance is due to a small number of replicates (8 patches) given the low magnitude of differences between treatments. The number of patches was small because it was impossible to design more than 8 distant patches in the population we studied. However, although non-significant, egg loads and pollination rates tended to be higher in closed flowers surrounded by open flowers than in closed flowers surrounded by closed flowers, suggesting that when closed flowers were rare, *Chiastocheta* flies tended to congregate in them, thereby advantaging even more the closed phenotype (negative frequency-dependent selection). Our initial hypothesis was that locally abundant open flowers would help flies learn to deal with the open phenotype (positive frequency-dependent selection). Patches where open flowers were locally abundant could appear by chance, and then would have been able to invade thanks to positive frequency-dependent selection (similar mechanisms might be at play in the evolution of altruism, *e.g.* Le Galliard et al., 2003). We found the opposite: the chances of locally abundant open flowers to invade are even further reduced as compared with rare open flowers.

Stability of the closed phenotype in present populations

Previous theoretical predictions suggested that the globe shape could enhance egg survival, increase larval competition and, eventually, reduce seed consumption because of contest competition between larvae, and would have evolved as a partner sanction to prevent overexploitation (Ferdy et al., 2002). This original explanation for the evolution of closed corolla is not supported by field observations: the survival of eggs harboured by artificially open *vs* globular flowers does not differ (L. Després, unpublished data). The empirical results presented here suggest instead that the evolution of a closed flower shape is involved in the pollination specialisation of globeflowers on *Chiastocheta*.

The mechanisms that ensure the current stability of a trait and those responsible for the evolution of this trait may well be different. In the case of coevolving mutualisms, one main reason is that both partners evolve (de Mazancourt et al., 2005): ancestral *Chiastocheta* flies were no doubt adapted to the open floral morphology they dealt with. The ecological conditions necessary for the persistence of *Trollius* specialization documented here (high

Chiastocheta pollination efficiency, *Chiastocheta* preference for closed corolla) are not necessarily the same as those required for specialization to evolve in the first place in a population of open flowers in a mixed, fluctuating, pollinator environment (Althoff et al., 2005, Thompson & Pellmyr, 1992). Nevertheless, the selective pressures against open flowers discussed above can explain the absence of high levels of variation in floral closure in present populations of globeflowers. Closure of the flower in *Trollius europaeus* is an effective way of filtering floral visitors and enhancing the efficiency of the most effective pollinator, as shown in other specialized systems (Aigner, 2005, Galen & Cuba, 2001, Schemske & Bradshaw, 1999), and specialization is not merely the reflect of depauperate pollinator communities (Johnson & Steiner, 2000). Evolution of traits through natural selection requires variable, heritable traits that are associated with differences in fitness. Corolla closure varies considerably among *Trollius* species, demonstrating that this trait has a genetic basis, and fully open phenotypes are found only in *Trollius* species out of the range of *Chiastocheta*. Other species such as *T. ledebouri* and *T. riederianus* are visited by both *Chiastocheta* and other pollinators, and, unlike *T. europaeus*, they have not evolved fully closed phenotypes, *i.e.* they did not specialize on *Chiastocheta* (Pellmyr, 1992). One may think that the open flower phenotypes occasionally observed in natural populations of *T. europaeus* did not emerge through mutation but only through environmental effects. While we argue that environmental effects cannot entirely account for flower polymorphism in *T. europaeus*, our findings demonstrate that if open flower mutants happened to emerge in natural populations, they would be counter-selected under a wide range of ecological conditions.

In this work we focused on the stability of the reciprocal specialization on the globeflowers' side. Specialization of a pollinator to a particular flower may be unstable, depending on the community context, but specific seed-eating pollinators that rely entirely on their host-plant for larval development require complex adaptations (*e.g.* Pellmyr & Krenn, 2002). Host shifts have been documented in specific seed-eating pollination mutualisms inside a given genus or between related genera (Kawakita et al., 2004, Michaloud et al., 2005, Molbo et al., 2003), but *T. europaeus* is the only member of its genus in Europe, so that *Chiastocheta* flies do not have another potential partner readily available, and they cannot get out from the specialized interaction. Finally, the stability of the morphological specialization of *T. europaeus* on *Chiastocheta* flies does not imply evolutionary stability for the whole interaction. Although the system studied here is considered to be mutualistic, agonistic traits can coexist with

antagonistic traits. For example, *Chiastocheta* larvae induce a plant chemical defence (Gallet et al., 2007), which acts as a potentially destabilizing factor in the specialized interaction.

Acknoledgements

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Table 1. Generalized Linear Mixed Model of the number of visits to flowers (either from *Chiastocheta* or from other insects) against the duration of observation, floral morphology and environment (fixed effects); and observation period (random effects, not shown). Interactions were not included. For floral morphology, C_1 is the reference level and the comparison between O and C_2 is also significant (not shown, $P<0.001$). For environment, subalpine is the reference level. Both numerator and denominator df are given. The number of observations is 135, the number of populations is 5 and the number of groups nested in populations (observation periods) is 45.

Effect		Chiastocheta entered		Other insects entered	
	df	Parameter value	t-value	Parameter value	t-value
Observation duration (min)	1;39	0.02	3.49**	0.01	0.68
O flowers	1;88	-1.46	-9.23***	2.05	4.69***
C2 flowers	1;88	-0.14	-1.34	0.41	0.76
Environment	1;3	-1.32	-5.92**	1.43	3.13.

. $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 2. Generalized Linear Mixed Model of the proportion of target flowers marked with dye (either on stigmas or on stamens) against dye colour, floral morphology (C_1 is the reference level) and environment where subalpine is the reference level (fixed effects); and population (random effects, not shown). Interactions between morphology and dye colour, and between morphology and environment were included. Both numerator and denominator df are given. There were 10 patches (with two observations per patch, depending on the origin of dye), and the number of populations was 5.

Effect		Stigmas		Stamens	
	df	Parameter value	t-value	Parameter value	t-value
Dye colour	1;11	-0.75	-2.32*	-1.02	-2.94*
Environment	1;3	-1.86	-2.83.	-2.39	-4.30*
Floral morphology	1;11	-2.09	-4.18**	-2.22	-4.72***
Dye colour x floral morphology	1;11	-0.19	-0.22	-0.96	-0.89
Environment x floral morphology	1;11	0.48	0.43	0.86	0.75

. $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 3. Generalized Linear Mixed Model of the egg number and net seed production per flower against floral morphology, environment (subalpine is the reference level), and carpel number (fixed effects); and population (random effects, not shown). For morphology, C_1 is the reference level and the comparisons between O and C_2 are significant (not shown, P=0.021 for egg number and P=0.008 for seed production). The interaction between morphology and environment was included. Both numerator and denominator df are given. The number of observations is 291 (egg number) and 294 (seed production), the number of groups (populations) is 5.

Effect	Egg number per flower			Net seed production per flower		
	df	Parameter value	t-value	df	Parameter value	t-value
O flowers	1;281	-0.38	-3,28**	1;284	-0.17	-1,88.
C2 flowers	1;281	-0.10	-0,92	1;284	0.06	0,74
Environment	1;3	-1.74	-3,22*	1;3	-0.72	-1,49
Carpel number	1;281	0.03	4,39***	1;284	0.04	7,64***
O flowers x environment	1;281	-0.07	-0,2	1;284	-0.05	-0,32
C2 flowers x environment	1;281	0.37	1,31	1;284	0.16	1

. $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 4. Generalized Linear Mixed Model of the pollination rate and egg number per flower against floral morphology (C_1 is the reference level), frequency of open flowers in the focal patch (low frequency is the reference level), carpel number (fixed effects); and patches (random effects, not shown). The interaction between morphology and frequency of open flowers in the focal patch was included. Both numerator and denominator df are given. The number of observations is 234, the number of groups (patches) is 8.

Effect		Pollination rate per flower		Egg number per flower	
	df	Parameter value	t-value	Parameter value	t-value
Floral morphology	1;223	-0.27	-1.89.	-0.17	-1.02
Open type frequency	1;6	0.11	0.75	0.32	1.49
Carpel number	1;223	-0.01	-1.41	0.03	5.99***
Open type frequency x floral morphology	1;233	-0.26	-1.31	-0.24	-1.09

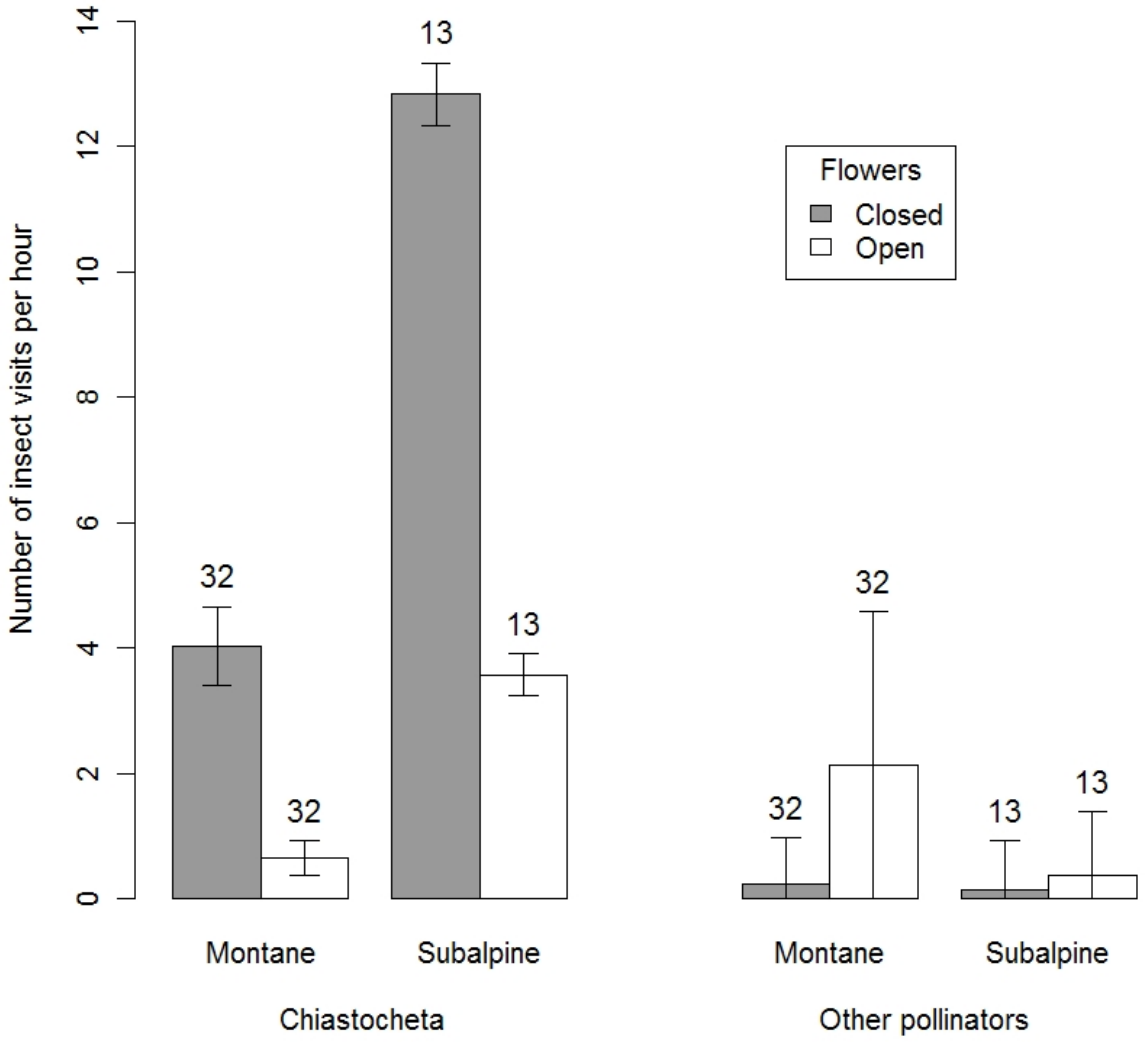
. $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

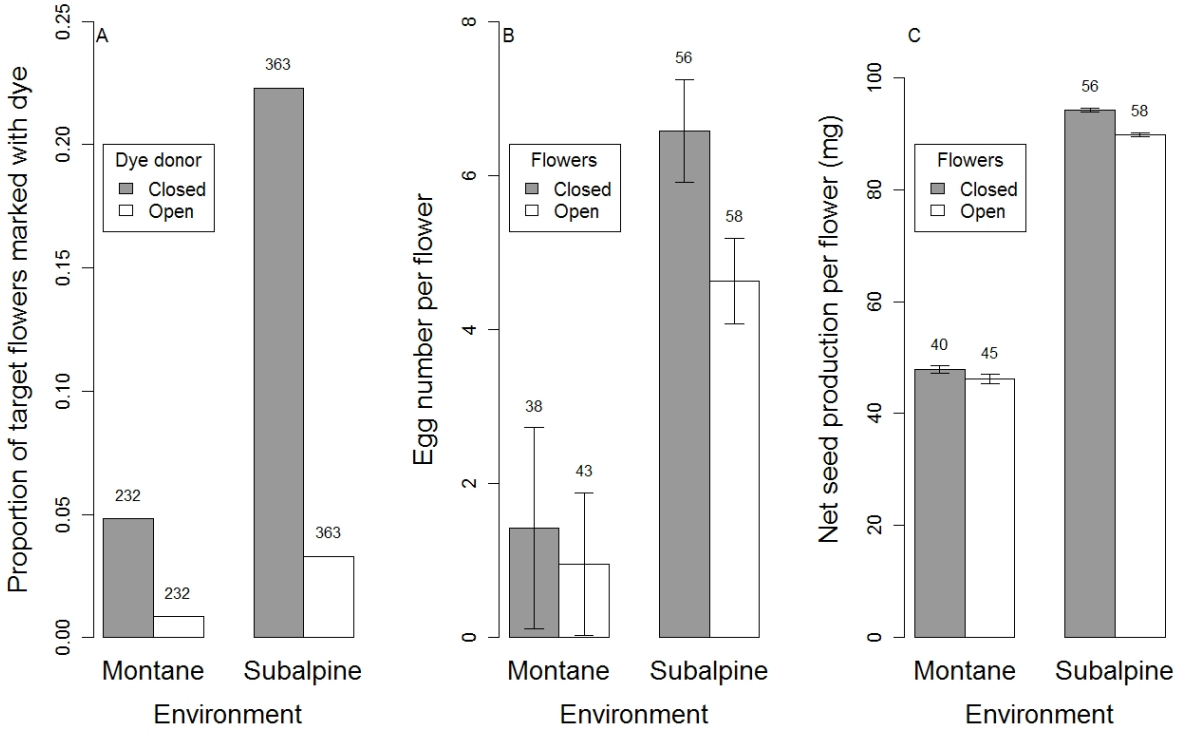
Legend to the figures

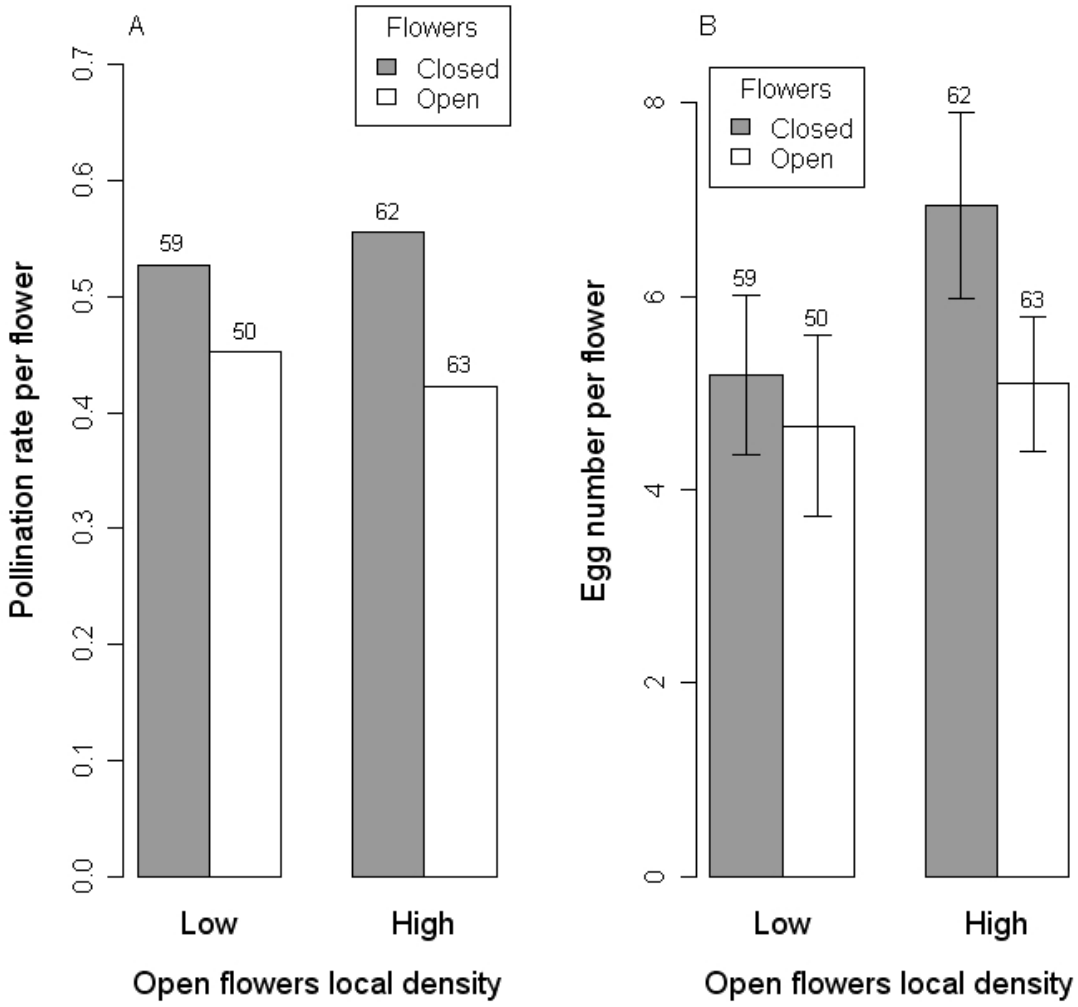
Figure 1. Effect of environment (montane or subalpine) and morphology (closed or open) of observed flowers on the number of visits per flower by *Chiastocheta* and other insects per hour of observation. Error bars indicate standard errors of the residuals of the number of visits explained (Generalized Linear Mixed Model) by the duration of observation (fixed effect) and observation period (random effect). The numbers above each bar correspond to the number of observation.

Figure 2. Effects of environment (montane or subalpine) and morphology (closed or open) of flowers (dye donor flowers in case of A) on (A) the percentage of target flowers marked by dye particles deposited on stigmas, (B) *Chiastocheta* egg number per flower and (C) net seed production per flower in mg. In B and C, error bars indicate standard errors of the residuals of the response variable explained (Generalized Linear Mixed Model) by carpel number (fixed effect) and population (random effect). The numbers above each bar correspond to the number of flowers used in the analysis.

Figure 3. Effect of local frequency of open floral types (low or high) and morphology of flowers (closed or open) on (A) pollination rate and (B) *Chiastocheta* egg number per flower. In B, error bars indicate standard errors of the residuals of the number of eggs explained (Generalized Linear Mixed Model) by carpel number (fixed effect) and patch (random effect). The numbers above each bar correspond to the number of flowers used in the analysis.


Number of insect visits per hour
0
2
4
6
8
10
12
14
Flowers
Closed
Open
32
32
13
13
32
32
13
13
Montane
Subalpine
Montane
Subalpine
Chiastocheta
Other pollinators


A
Proportion of target flowers marked with dye
0.00
0.05
0.10
0.15
0.20
0.25
Dye donor
Closed
Open
232
232
363
363
Montane
Subalpine
Environment
B
Egg number per flower
0
2
4
6
8
Flowers
Closed
Open
38
43
56
58
Montane
Subalpine
Environment
C
Net seed production per flower (mg)
0
20
40
60
80
100
Flowers
Closed
Open
40
45
56
58
Montane
Subalpine
Environment


A
Flowers
Closed
Open
Pollination rate per flower
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
59
50
62
63
Low
High
Open flowers local density
B
Flowers
Closed
Open
Egg number per flower
0
2
4
6
8
59
50
62
63
Low
High
Open flowers local density

4. Les principaux résultats

- **Un trolle globulaire a une stratégie spécialiste, un trolle ouvert est généraliste.**

- **La fitness femelle d'un trolle spécialiste est supérieure de 4% à celle d'un trolle généraliste.**

- **La fitness mâle d'un trolle spécialiste est supérieure de 85% à celle d'un trolle généraliste.**

- **Lorsque les trolles ouverts sont localement abondants, la sélection en leur défaveur est plus forte que lorsqu'ils sont rares (la différence est cependant non-significative).**

CHAPITRE 2 : Conditions écologiques à l'origine de la spécialisation de la morphologie florale.

1. Introduction

Dans le premier chapitre, nous avons montré que la morphologie florale globulaire est évolutivement stable tant à l'étage montagnard qu'au subalpin. Pour mieux comprendre l'évolution de la morphologie florale, je m'intéresse dans ce deuxième chapitre à la question de son origine évolutive, en utilisant un modèle théorique.

Le modèle suppose qu'initialement la morphologie florale est ouverte, comme dans les autres espèces du genre *Trollius*. Les chiastochètes sont 'habituées' (au sens évolutif) à cette morphologie et visitent les trolles ouverts. D'autres pollinisateurs sont présents et sont capables de visiter les trolles ouverts. Ils ne consomment pas de graines mais ont une efficacité de pollinisation variable, en fonction de la valeur des paramètres. L'objectif est de comprendre dans quelles conditions le trolle peut se spécialiser sur un pollinisateur coûteux, alors que d'autres sont présents. Seule la fitness femelle est déterminée, de cette manière c'est la situation la plus défavorable à la spécialisation sur les chiastochètes qui est considérée.

Le formalisme utilisé pour le modèle est celui de la Dynamique Adaptative (Adaptive Dynamics), développé au cours des années 90 par Hanz Metz, Ulf Dieckmann et Stefan Geritz notamment (Dieckmann & Law, 1996, Geritz, 1997, Metz et al., 1992). Ce formalisme combine dynamiques écologiques et évolutives. Un jeu d'équations différentielles décrit la dynamique écologique d'une population monomorphe pour le trait dont on va suivre l'évolution. La figure 13 ci-dessous donne une version imagée du bouclage éco-évolutif :

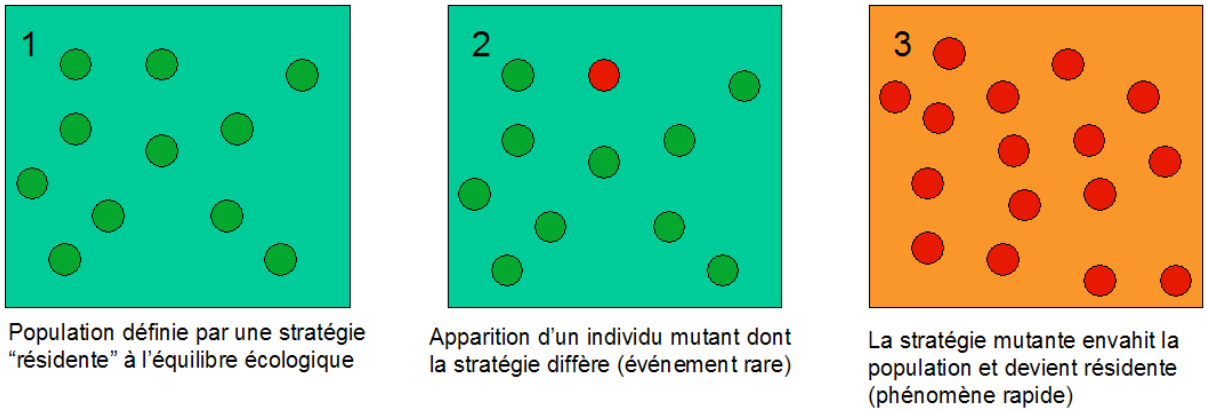


Figure 13. Bouclage éco-évolutif utilisé en dynamique adaptative.

La population est considérée à l'équilibre écologique (panneau 1) et un mutant dont la valeur phénotypique est proche de la stratégie de la population monomorphe apparaît (panneau 2). La fitness de ce mutant est définie comme son taux d'accroissement lorsqu'il est initialement rare. Si le mutant envahit la population, alors sa dynamique écologique est modifiée par la présence de ce nouveau phénotype (panneau 3). Par apparitions et invasions successives de mutants, la dynamique évolutive est enclenchée. La figure 13 donne une impression spatialisée et aléatoire, mais le modèle est non-spatialisé et déterministe.

Il est bien sûr possible de modéliser le bouclage éco-évolutif de plusieurs populations et plusieurs traits. Le modèle présenté dans ce chapitre considère la dynamique écologique d'une population de trolles en interaction avec une population de chiastochètes. La taille de la population des autres pollinisateurs généralistes est supposée constante. Le degré d'ouverture de la fleur ainsi que la préférence morphologique des chiastochètes sont sujets à évolution.

2. Manuscrit accepté dans Evolutionary Ecology Research

Ecological conditions promoting plant specialisation on a seed-eating pollinator differ from those stabilising the interaction.

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Running title*:* Specialisation on a seed-eating pollinator

Keywords: *Trollius europaeus- Chiastocheta* mutualism, floral morphology, adaptive dynamics, coevolution, pollination efficiency.

Total word count: 4250

Number of figures: 3

Number of tables: 1

Abstract

Question

What are the ecological conditions promoting plant specialisation on a seed-eating pollinator when less costly alternative pollinators are present?

Mathematical method

An adaptive dynamics model including the ecological dynamics of a plant/seed-eating pollinator mutualistic system.

Key assumptions

Plants are initially pollinated by specialist seed-eating pollinators and by generalist co-pollinators. Plant specialisation (floral morphology continuously ranging from closed to open) and seed-eating pollinator morphological preference coevolve, while co-pollinators always prefer open flowers. When seed-eating pollinators and co-pollinators have similar preferences, seed-eating pollinators are less effective. The functional relationship linking plants and seed-eating pollinators involves pollination efficiency, oviposition rate, the range of floral morphologies an insect is able to deal with (its degree of specialization), and the pollination and oviposition handling times.

Conclusions

Specialisation evolves only if pollinators interfere, and it is favoured when co-pollinators' efficiency is low, when seed-eating pollinators' oviposition rate is low, and when the range of floral morphology they deal with is greater for oviposition than for nectar- or mate-searching visits. Moreover, although high pollination efficiency of seed-eating pollinators is a key factor in the persistence of the specific mutualism nowadays, the first steps of the evolution of specialisation require an intermediate pollination efficiency of seed-eating pollinators.

Introduction

Over the last decade, our view of plant-pollinator interactions shifted from the long-standing concept of "pollination syndromes" reflecting extreme specialisation as the common outcome of natural selection in those systems, to the widely accepted idea that generalisation is widespread in pollination ecology, and that specialised plant-pollinators interactions might be the exception rather than the rule (Johnson & Steiner, 2000, Waser et al., 1996, Waser & Ollerton, 2006). Pollination always implies a cost for the plant, such as nectar or pollen lost to feed pollinators, but the cost is particularly severe in plant/seed-eating pollinator mutualisms where pollinator offspring feed on potential plant offspring (Bronstein, 2001, Jaeger et al., 2000, Pellmyr, 1989). It is therefore particularly intriguing that these mutualisms are among the few examples of extreme pollination specialisation. If specialisation on specific seed-predators is ubiquitous in all the species of *Yucca* and *Ficus*, this is not the case for the *Trollius* genus, where only the European species, *Trollius europaeus*, is exclusively pollinated by specialised seed-predators (*Chiastocheta* Pokorny, Anthomyiids). All *Trollius* species are perennial, 5-12cm tall herbs, growing in moist, boreal, montane or subalpine habitats throughout the temperate and arctic regions of Asia, Europe, and North America. Some *Trollius* species are not visited at all by *Chiastocheta* flies, such as *T. pumilus*, *T. ranunculinus* and *T. laxus*, all of which present an open flat corolla: these species are likely to be pollinated by a wide range of insects (Pellmyr, 1989). Other *Trollius* species, such as *T. altaicus, T. europaeus, T. ledebouri, T. asiaticus, T. chinensis* and *T. riederianus* are visited both by *Chiastocheta* and other pollinators. Molecular phylogenetic analysis showed that open flower is the ancestral state in the *Trollius* genus and that all species visited by *Chiastocheta* flies form a derived clade (Despres et al., 2003). The only morphological character clearly linked to the presence of *Chiastocheta* is the bowl shape of the corolla which reaches its extreme in *T. europaeus* by forming a totally closed globe. *T. europaeus* globular

shape is thus likely to be responsible for the exclusion of pollinators other than *Chiastocheta* flies. These small flies enter the globe by spirally crawling between sepals, whereas the sepals prevent larger insects from entering. Furthermore, a field experiment involving floral morphology manipulation has demonstrated that present-day *Chiastocheta* prefer to visit closed phenotypes (Ibanez et al., 2009). We hypothesise that *T. europaeus* and *Chiastocheta* flies coevolved reciprocal specialisation through various traits, one of them being the closed flower shape coevolving with the insect flower shape preference. According to this hypothesis, ancestral *Chiastocheta* flies were adapted to the open floral morphology they dealt with. Indeed, all the *Chiastocheta* species described so far (11 to 17 depending on authors, Despres et al., 2002, Pellmyr, 1992) lay eggs specifically on *Trollius* species regardless the floral morphology of the host-plant, their larvae feeding only on *Trollius* seeds, on globular flowers for European *Chiastocheta* species (6 species described), or on open flowers for Asiatic species. We therefore assume that ancestral *Chiastocheta* flies were dependent on an ancestral open-flower, generalist, *Trollius* species.

Plant specialisation on seed predators is especially intriguing when co-pollinators feeding only on nectar and not on developing seeds are also present in the community, which is often the case (Holland & Fleming, 2002, Thompson & Cunningham, 2002). If seed-eating pollinators consume more seeds than they fertilise ovules, they act as parasites rather than mutualists (Holland & Fleming, 2002, Thompson & Cunningham, 2002, Thompson & Fernandez, 2006). Plants are then expected to develop traits which allow pollination by generalist co-pollinators and to minimise the costs imposed by specialised seed-eating pollinators. Therefore, the presence of co-pollinators is predicted to prevent the evolution of plant specialisation on seed-predators, even when those are highly efficient pollinators, as observed for example in the *Lithophragma parviflorum- Greya politella* interaction (Pellmyr & Thompson, 1992). However, other potential pollinators are present in *Trollius europaeus*

populations and were presumably also present in past populations, raising the question of why specialisation on seed-predators evolved in the European globeflower, and not in other *Trollius* species. We built an adaptive dynamics model to understand how specialisation might have coevolved in the initial context of an open flower pollinated by both *Chiastocheta* and generalist pollinators. Our aim was to determine under which ecological conditions closure of the flower and the *Chiastocheta*'s preference for closed flowers could coevolve.

Model

Background

We consider a single community composed of a *Trollius* species labelled "*T*", the *Chiastocheta* genus labelled "*C*" and other potential pollinators, such as bumblebees and syrphids, labelled "*P*". *P* density is assumed to be constant, as these pollinators are generalists and mostly rely on flowers other than *T* which they visit only occasionally, whereas *C* density varies along the ecological timescale and entirely depends on *T* for reproduction. *T* is pollinated by both *C* and *P*, and only suffers from seed predation from *C*. Both *C* and *P* are assumed to be nectar consumers of equivalent intensity, so nectar producing costs are not incorporated in the model.

Interactions between species are assumed to be mediated by the degree of phenotypic matching between quantitative traits (Dieckmann et al., 1995, Nuismer & Doebeli, 2004). *T*, *C* and *P* traits are, respectively, *x*, *y* and *z*. We interpret *x* as the degree of flower opening, the values $x = 0$ and $x = 1$ correspond to completely closed and wide open flowers respectively, any morphologically possible value between 0 and 1. *y* and *z* are the insects' preferences when looking for a flower to visit. When traits *x* and *y* match (respectively *x* and *z*) *C* (respectively *P*) pollinate *T* at maximum efficiency, and when *y* and *z* match the negative effect of *P* on pollination by *C* reaches its maximum. Starting with all traits close to 1, the

model studies the coevolution of x and y, while z remains constant and equal to 1 because pollinators other than *Chiastocheta* are assumed to be generalists.

Ecological dynamics

The ecological dynamics are given by:

$$\frac{dT(x,t)}{dt} = T\left(poll(T,C,P,x,y,z)(1-pred(T,C,x,y))(1-T)-d\right) \quad (1a)$$

$$\frac{dC(y,t)}{dt} = C\left(b_c T poll(T,C,P,x,y,z)pred(T,C,x,y)\left(1-c_c\frac{C}{T}\right)-1\right) \quad (1b)$$

where $poll(T,C,P,x,y,z)$ is the pollination probability function, depending on T, C and P densities and their respective traits; and $pred(T,C,x,y)$ is the predation probability function, independent of P and z because generalist pollinators do not feed on seeds. The parameters d, b_c, and c_c represent respectively globeflower mortality, *Chiastocheta* fecundity and *Chiastocheta* intraspecific competition depending on the ratio C/T. Fixed parameters, ecological variables, evolutionary variables and their signification are summarised in Table 1. Equations (1) in this paper have some similarities with (5) and (6) of Holland et al. (2002) and (3) of Morris et al. (2003). In both equations, the right-hand sides correspond to population density multiplied by the per-capita growth rate, which is decomposed into birth and death rates. The T per capita birth rate equals the probability of being pollinated multiplied by the probability of escaping predation by larvae and by an intraspecific competition factor. The C per capita birth rate equals the amount of available seeds multiplied by the predation probability and by an intraspecific competition factor which depends on the number of flies per flower. Equations (1), as well as equations (2) below, have some degree of generality and could also describe the ecological dynamics of other plant-seed eating pollinator systems.

Pollination and predation functions are:

$$poll(T,C,x,y) = 1 - Exp\left(-\frac{\alpha(x-y,\sigma_{c_1})a_{c_1}C(1-c_p\alpha(y-z,\sigma_p)P)}{1+a_{c_1}h_1T} - \alpha(x-z,\sigma_p)a_pP\right) \quad (2a)$$

$$pred(T,C,x,y) = 1 - Exp\left(- \frac{\alpha(x-y,\sigma_{c_2})a_{c_2}C}{1 + a_{c_2}h_2T} \right) \quad (2b)$$

The functional responses of equations (2) are derived from DeAngelis & Holland (2006)'s equation (5) and have some similarity with the model of Morris et al. (2003). The term in the exponential function is the rate at which unpollinated ovules (respectively undamaged seeds) are fertilised (respectively eaten) during a short time scale; it corresponds to a standard Holling type 2 (prey-dependent) functional response (Begon et al., 1996). The pollination rate includes a ratio-dependent term corresponding to the *Chiastocheta* flies' contribution and a pollinator-dependent term corresponding to the other pollinators' effect. The predation rate only includes a ratio-dependent term corresponding to larval feeding. Equations *(2)* are obtained by integrating pollination and predation rates over the whole period of blooming. In the following, we detail the meaning of all the parameters used.

a_{c1} is the intensity of pollen transfer per visit ("quality" component *sensus* Herrera, 1987) and a_{c2} is the oviposition rate. h_1 and h_2 are the intensities of C/T ratio-dependence for pollination and predation; h_1 is the time spent by a fly handling each flower (DeAngelis & Holland, 2006) and h_2 is the time a female needs to lay eggs. If h_1 is low, a single fly is able to pollinate a large number of flowers and pollination efficiency in a globeflower population mainly depends on the density of *Chiastocheta* with globeflower population size having a limited impact, whereas if h_1 is high, pollination efficiency will depend on the pollinator to flower ratio. If h_2 is low, the intensity of predation is essentially dependent on the number of ovipositing females, whereas if h_2 is high, predation intensity depends on the C/T ratio. Extreme cases where h_1 (resp. h_2) are equal to zero correspond to pollinator- (resp. predator-) density dependence. For the analyses we chose $h_2 > h_1 > 0$ because field data suggest that the intensity of ratio-dependence is stronger for oviposition than for pollination (Despres et al.,

2007). c_p is the effect *P* exerts on *C* pollination through interference when bigger pollinators on the flower chase *Chiastocheta* fly away (S. Ibanez, pers. obs.) or through pollen waste by generalist pollinators ("ugly" pollinators, Thomson & Thomson, 1992), and/or stigma clogging with incompatible pollen grains. a_p is the pollination efficiency of *P* insects, it is expected to be low because generalist pollinators visit a wide range of flowers and therefore transfer many incompatible pollen grains to globeflowers.

Strength of the ecological interactions

The Gaussian function $\alpha(w,\sigma)$ is used to measure the interaction strength between two species (Egas et al., 2004, Egas et al., 2005, Doebeli & Dieckmann, 2000):

$$\alpha(w,\sigma) = \frac{1}{\sqrt{2\pi}} Exp\left(-\frac{w^2}{2\sigma^2}\right) \quad (3)$$

where w is the difference between two traits (the smaller the difference, the stronger the interaction) and σ is a variance-like parameter (when σ is large, the interaction is more tolerant to trait mismatch). Interaction strength concerns pollination and predation. When the pollinator trait (*y* or *z*) and the plant trait *x* match, the visitation rate of pollinating insects reaches its maximum ("quantity" component *sensus* Herrera, 1989). In the same way, when the *Chiastocheta* trait *y* and the plant trait *x* match, the visitation rate of ovipositing flies reaches its maximum. When traits *y* and *z* match, the other pollinators have the same morphological preference as *Chiastocheta* flies, so they exert maximum negative effect on their pollination ability.

σ_{c1} reflects the tolerance of *Chiastocheta* in their pollination behaviour as regards floral morphology: for high σ_{c1} values, visitation rate will be close to the maximum even if phenotypes do not match. Similarly, σ_{c2} is the tolerance of ovipositing flies in their visitation rate as regards floral morphology: for high σ_{c2} values, females will still visit flowers which morphology does not match with their preference. σ_p is the other pollinators' visitation

behaviour tolerance as regards floral morphology. We assume it is equal to the interaction strength between *Chiastocheta* and other pollinators with different floral morphology preferences: if a *P* pollinator matches the plant trait and occupies the inside of the globe, it will prevent *Chiastocheta* flies from doing so. Finally, we assume that trait match does not influence handling times, and that larval predation efficiency is independent from floral morphology and from adult morphological preference.

Evolutionary dynamics

Traits *x* and *y* are susceptible to evolutionary change, our aim is to analyse their coevolution using the Adaptive Dynamics' mathematical framework, a deterministic approximation of individual-based models, describing evolution in asexual populations under frequency-dependent selection (Dieckmann & Law, 1996, Geritz et al., 1998, Metz et al., 1992). Assuming that evolution is mutation-limited and that mutational steps are small, *P* and *C* fitness landscapes are determined by the invasion fitness of mutant phenotypes, defined as the long-term per capita growth rate of an initially rare mutant facing the resident population at its ecological equilibrium (noted $s(x'|x,y)$ and $s(y'|x,y)$, Metz et al., 1992). We used the canonical equation of adaptive dynamics (Dieckmann & Law, 1996, equations 4.12 and 6.1) which here is a set of two differential equations describing the coevolution of *x* and *y* traits (equations 4). The canonical equation takes into account the local selection gradients; the mutation rates and the *variance-covariance* matrix of both traits; and the density of each population at ecological equilibrium (noted $T^*(x,y)$ and $C^*(x,y)$). For simplicity mutation rates and variances were assumed equal to 2 and 1 respectively, and because traits *x* and *y* concern different species, they were considered to be independent. As a result, the canonical equations *A1* below are simple products of population densities by local fitness gradients:

$$\frac{dx}{dt} = T^*(x,y)\left.\frac{\partial s(x'|x,y)}{\partial x'}\right|_{x'=x} \qquad (4a)$$

$$\frac{dy}{dt} = C^*(x,y)\left.\frac{\partial s(y'|x,y)}{\partial y'}\right|_{y'=y} \quad (4b)$$

The canonical equations *4* were discretised using Runge-Kutta (RK2) method; at each time step population sizes and local fitness gradients around the resident strategies were determined.

We ran coevolutionary calculations until no further evolutionary change occurred: such steady states are coevolutionary singular strategies (CoESS), their property is to attract coevolutionary trajectories. CoESS can either be stable (coevolutionary continuously stable strategies, CoCSS) or unstable with disruptive selection acting on one or both traits (evolutionary or coevolutionary branching points, CoEBP). We checked stability of the CoESSs by determining the sign of the second partial derivative of $s(x'|x,y)$ and $s(y'|x,y)$ with respect to x' and y', evaluated at x and y respectively (Leimar, 2005).

We calculated deterministic coevolutionary runs with x and y initially set to 0.99, a value close enough to $z = 1$ so that the effects of other pollinators are close to their maxima, but slightly different from 1, in order to determine whether traits converge toward 1 or on the contrary diverge from it. Parameter values were chosen in order to obtain realistic values at the ecological equilibrium for pollination and predation rates, and for *C/T* ratio, i.e. within the range of those observed in natural populations (Despres et al., 2007, Jaeger & Despres, 1998, Jaeger et al., 2001). They were also chosen in order to maintain the system in the absence of *Chiastocheta* so that at the beginning of the coevolutionary process, the mutualism between *Trollius* and *Chiastocheta* is optional; indeed, ancestral open flower species in the *Trollius* genus persist without *Chiastocheta* (Pellmyr, 1992). Sensitivity analyses were conducted using all relevant parameters (the ranges used are as described in Table 1). All calculations were performed using the software Mathematica 5.1.1.0 (Wolfram Research)

Results

Short-term coevolutionary dynamics.

Short-term coevolutionary dynamics were used to understand the first steps of the specialisation *vs* generalisation evolution. At the beginning, trait *x* always evolved toward 1 while *y* evolved away from 1, and after a while, two types of short-term coevolutionary dynamics were observed (Figure 1): *a) y* stopped evolving away from 1 and reversed its evolutionary course, and *b) x* was driven away from 1 and evolved in the same direction as *y*. In both scenarios, the difference between *x* and *y* was always very small (<0.0005) compared to the tolerance parameters (ranging from 0.1 to 1).

Long enough after the short-term dynamics presented in Figure 1, the coevolutionary dynamics kept monotonous, so that long-term evolution toward specialisation or generalisation can be predicted from the gradient at the end of the short-term dynamics.

Long term coevolutionary dynamics.

Traits *x* and *y* always coevolved close to each other so in the long term coevolutionary trajectories appeared confounded (Figure 2). Two main coevolutionary trends were observed: either other pollinators had an attractive evolutionary effect and both *x* and *y* traits coevolved generalisation toward 1; or they had a repelling evolutionary effect and both traits coevolved away from 1 (Figure 2). In both cases second partial derivatives were negative (not shown), so the coevolutionary attractors were stable (coevolutionary continuously stable strategies, CoCSS). The speed of the coevolutionary process depended upon parameter values and could change by an order of magnitude (*e.g.* see figures 1.a and 1.c with $h_1 = 1$), reflecting the variable strengths of local selection gradients. When both traits coevolved away from 1, they could reach the phenotypic boundary (figure 1.a, 1. b with $h_1 = 1$) or stabilise at a CoCSS between 0 and 1 (figure 1.a with $h_1 = 0.8$); in both cases, *Trollius* and *Chiastocheta* traits matched and escaped from the other potential pollinators, and the result was reciprocal specialisation.

Sensitivity analysis.

We allowed most parameters of the model to vary (see Table 1) and conducted a sensitivity analysis, a sample of which is presented in Figure 3. Coevolution toward specialisation or generalisation was inferred from the short-term dynamics in order to reduce computation time. Specialisation never occurred when the other pollinators did not have any negative effect on *Chiastocheta* pollination: such an effect was a necessary condition for the coevolution of specialisation. Specialisation occurred only when the strong effect of other pollinators on *Chiastocheta* pollination was combined with an intermediate pollinating visits rate (Figure 3.a). Figure 3.b shows that specialisation occurred only above a given σ_{c2}/σ_{c1} ratio. Figure 3.c shows the effect of pollination parameters. As in Figure 3.a, specialisation occurred at intermediate *Chiastocheta* pollination efficiency. a_{c1} had to reach a given level for specialisation to evolve, and this level increased with higher handling time values. At the same time, a_{c1} had to stay below a given level for specialisation to evolve, and this level increased with higher h_2 values. Figure 3.d shows the effect of predation parameters. The ecological system collapsed due to very rare oviposition rate. Specialisation occurred for low oviposition rates, except within a narrow handling time range (around 0.4 with our parameter choice) in which the occurrence of specialisation increased.

Discussion

Mechanisms of the coevolution of specialisation

Trollius and *Chiastocheta* traits always coevolved close to each other, which is not surprising: *Chiastocheta* flies entirely rely on *Trollius* for the development of their larvae so they have no choice but keep specialised. The short-term coevolutionary dynamics show that *Chiastocheta* flies try to escape the other pollinators' negative impact. Once *Trollius* morphology lays between the other pollinators' and *Chiastocheta* flies' preferences, it has to choose between one of the partners. If *Trollius* chooses to specialise on *Chiastocheta*, the system will keep

coevolving away from the other pollinators because of their negative impact on *Chiastocheta*. If *Trollius* chooses to match the other pollinators' morphological preference, *Chiastocheta* flies will have no choice but follow this "unfortunate" decision, despite the negative impact of other pollinators. This is why we never observed disruptive selection and evolutionary branching. In short, either *Trollius* or *Chiastocheta* drives the coevolutionary process. Although we cannot speak about *reciprocal* specialisation because only *Trollius* can choose between different partners, we have to take into account the evolution of both partners' traits (de Mazancourt et al., 2005) and build a coevolutionary model.

Emergence of a trade-off

Specialisation often implies trade-offs (Aigner, 2001, Egas et al., 2004, Schemske & Bradshaw, 1999), although this is not always the case (Aigner, 2004). In our model, *Trollius* does not face any trade-off at the beginning of coevolution, since the differences between the traits are very small compared with pollination tolerance parameters (σ_{c1} and σ_p). When the system evolves generalisation the differences between traits become even smaller, but when specialisation evolves a trade-off emerges from the coevolutionary process. When the system reaches the CoCSS state, the difference between the other pollinators' and *Chiastocheta* flies' preferences is large; *Trollius* now faces a strong trade-off. At this point, the ecological conditions necessary to the initiation of the specialisation process can change without affecting the stability of the CoCSS. Field experiments conducted on *T. europaeus*, the only *Trollius* species which evolved specialisation, showed that the closed phenotype is highly stable and that the specialisation trade-off between *Chiastocheta* and other pollinators is very strong (Ibanez et al., 2009). Artificially opened globeflowers produced less seeds and exported far less pollen than controls, because of a dramatic decrease in visitation rate by *Chiastocheta* flies, which was not compensated by visits by other pollinators.

Ecological conditions for specialisation to evolve

In the following we detail four ecological conditions leading to specialisation.

First, specialisation only occurs when pollination services are not additive (Aigner, 2001, Lau & Galloway, 2004), *i.e.* when other pollinators prevent *Chiastocheta* flies from achieving their maximum pollination contribution. Additionally, specialisation is favoured when the other pollinators' efficiency is low. In present-day globeflower populations, interference between *Chiastocheta* and other insects was occasionally observed, mostly when large Diptera, Coleoptera, or bumblebees, land on globeflowers chasing away already present *Chiastocheta* (pers. obs.), and/or prevent *Chiastocheta* from entering the globe by occupying the space. In past open flower populations, such interferences were likely to be more frequent, and perhaps even stronger because flies could not hide inside the globe. Furthermore, pollen waste by generalist pollinators ("ugly" pollinators, Thomson & Thomson, 1992), and/or stigma clogging with incompatible pollen grains transferred by generalists, is a likely outcome of the co-occurrence of specialists and generalists on a plant. Transfer of pollen between different plant species reduces pollination efficiency of generalist pollinators.

Second, specialisation is favoured when the *Chiastocheta* flies oviposition rate is low. A high oviposition rate increases seed predation and therefore reduces the mutualistic benefit of the interaction. Seed consumption per larva is another factor increasing total seed predation, so we expect that low seed consumption per larva would favour specialisation. The production of adonivernith, a flavonoid close to luteolin, is enhanced when larval density is high in a single *T. europaeus* flower (Gallet et al., 2007), and an increase in this plant chemical compound results in a decrease in seed consumption per larva (Ibanez et al., 2009). Paradoxically, this plant defence mechanism acting on an antagonistic trait (fly predation) might have favoured mutualistic specialisation. Interestingly, adonivernith is absent from other *Trollius* species, that have not evolved specialisation on *Chiastocheta* (Gallet et al., 2007).

Third, flies must be less selective on floral morphology for oviposition than for nectar- or mate-searching visits. When oviposition is poorly selective, the plant pays the costs of the mutualistic interaction whatever its strategy is, and it pays to specialise on *Chiastocheta* through corolla closure, because the benefits gained from increased pollination will exceed the cost incurred due to increased predation.

Fourth, specialisation does not occur when *Chiastocheta* effectiveness is too low, nor when it is too high. The latter result is counterintuitive since specialisation is expected to occur with the most effective pollinator (according to the "most effective pollinator principle", Stebbins, 1970). In our model, when *Chiastocheta* are highly effective, pollination is fully achieved despite the negative effect of other pollinators on *Chiastocheta* flies and the plant does not specialise. The condition of intermediate pollination effectiveness is linked with the condition of a negative effect of the other pollinators on *Chiastocheta* pollination. If other pollinators do not prevent *Chiastocheta* from achieving pollination, there is no reason for the plant to select for their exclusion. This surprising finding might explain why *T. ledebouri* and *T. riederianus* which are visited by *Chiastocheta* flies in Japan did not evolve pollination specialisation on *Chiastocheta*. In these species, *Chiastocheta* pollination effectiveness is much higher than on *T. europaeus* species (Pellmyr, 1992), which might paradoxically prevent the evolution of specialisation.

Distinguishing between past and present mechanisms promoting specialisation

Field experiments show that *Chiastocheta* flies are highly effective pollinators in present-day globeflower populations (Ibanez et al., 2009), which is essential for the current stability of this highly specialised mutualism. However, the present model shows that both intermediate *Chiastocheta* pollination effectiveness together with negative interactions among co-pollinators, are required at the beginning of the interaction for specialisation to evolve. Present conditions for the stability of the interaction are the result of the specialisation

evolutionary history, and are contradictory with the past mechanisms acting at the beginning of specialisation. Following (Levins & Lewontin, 1985), “the conditions necessary to the initiation of some process may be destroyed by the process itself”.

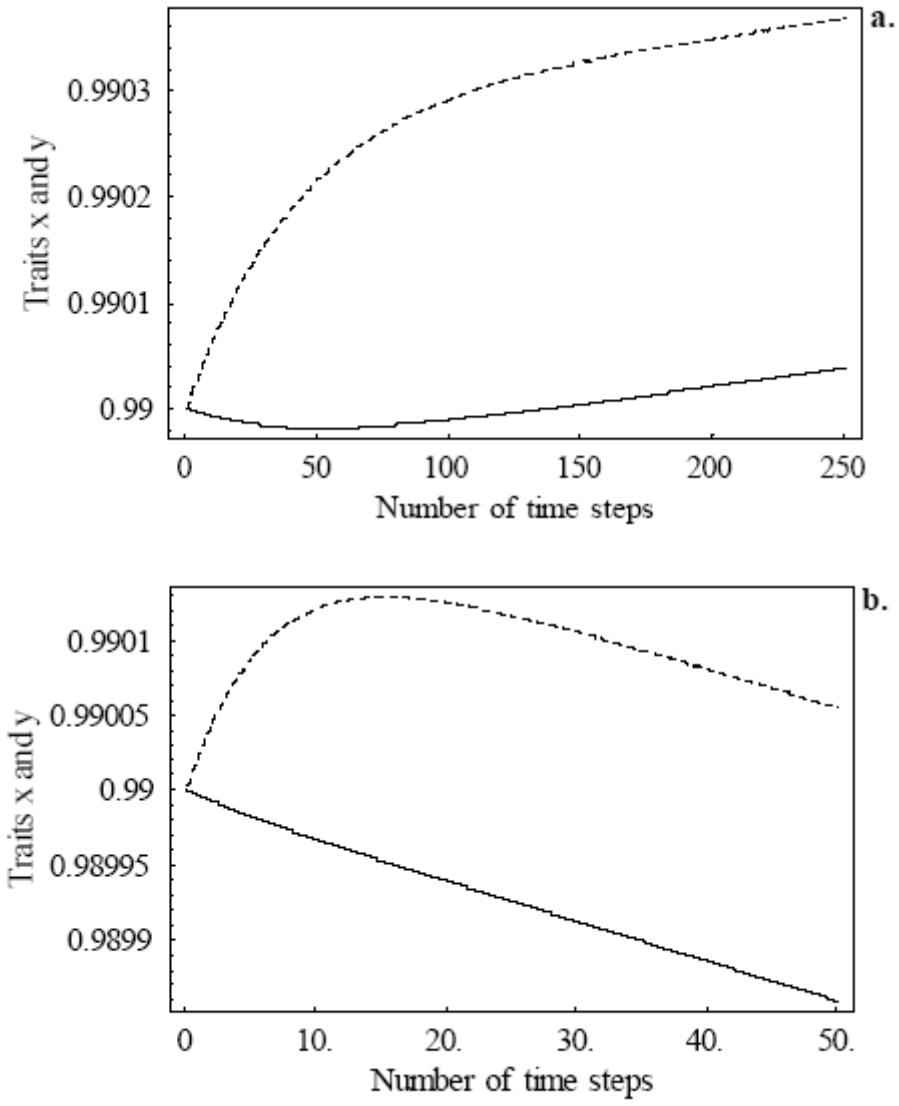

Figure 1.

Examples of short-term coevolutionary dynamics when traits first evolve in opposite directions until one trait changes its course. Full line: *Chiastocheta* preference *y*; dashed line: *Trollius* morphology *x*. *a* corresponds to the short-term dynamics of figure 2.c with $h_1 = 0.8$ and *b* to figure 2.a with $h_1 = 1$.

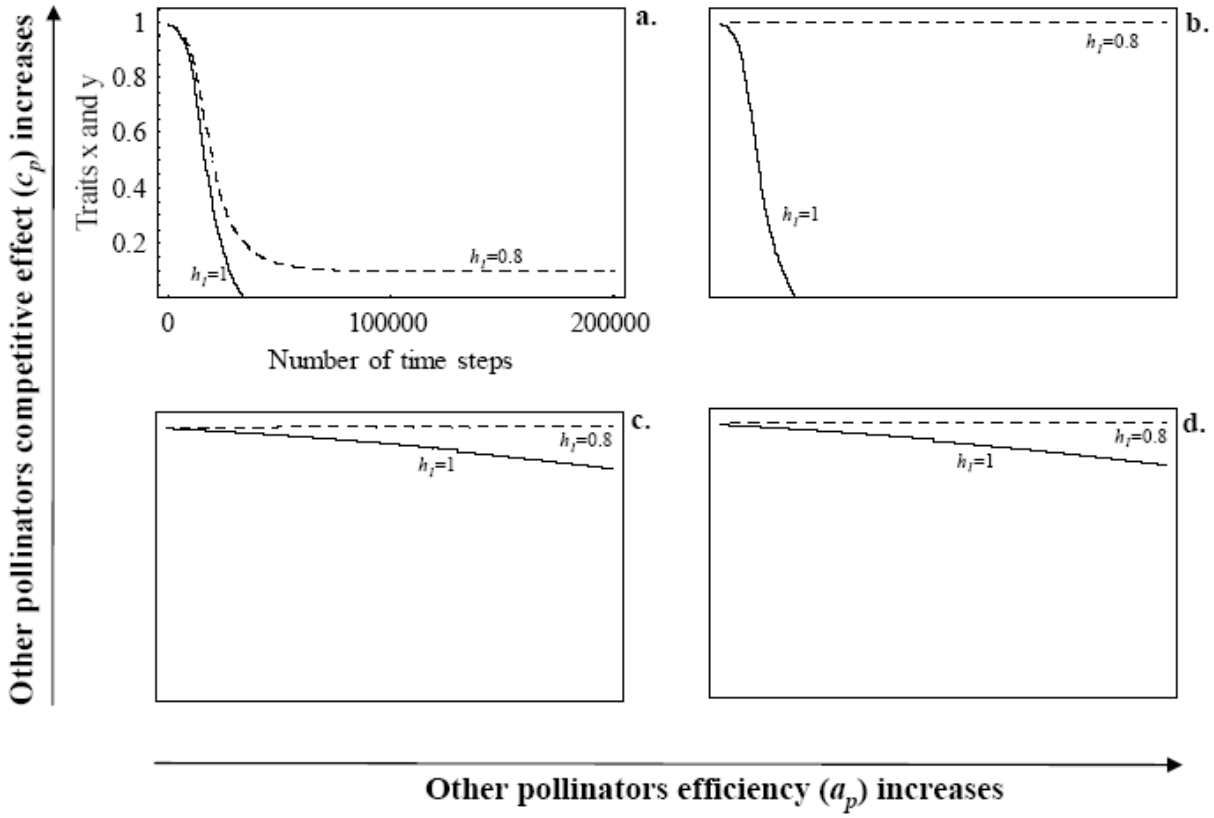

Figure 2.

Representative gallery of the coevolutionary dynamics encountered in the model for different parameter combinations of other pollinators' effect on *Chiastocheta* pollination (vertical, c_p ranging from 0.3 to 0.7) and pollination efficiency (horizontal, a_p ranging from 0.3 to 1), and of the intensity of *C/T* ratio-dependence for pollination (full lines, $h_1 = 1$; dashed lines, $h_1 = 0.8$). Each line represents dynamics of both traits *x* and *y* as the difference between traits never exceeded 0.001 (see Appendix 2). Dynamics can lead to generalisation (b., c. and d. with $h_1 = 0.8$) or specialisation. In the case of specialisation, coevolution can be fast (a.) or slow (c. and d. with $h_1 = 1$) and reach the phenotypic boundary 0 (a. with $h_1 = 1$) or stabilize between 0 and 1 (a. with $h_1 = 0.8$). Other parameter values are as in Table 1, with $a_{c1} = 8$, $a_{c2} = 5$, $\sigma_{c1} = 0.2$, $\sigma_{c2} = 0.8$, $\sigma_p = 0.5$, $h_2 = 2$.

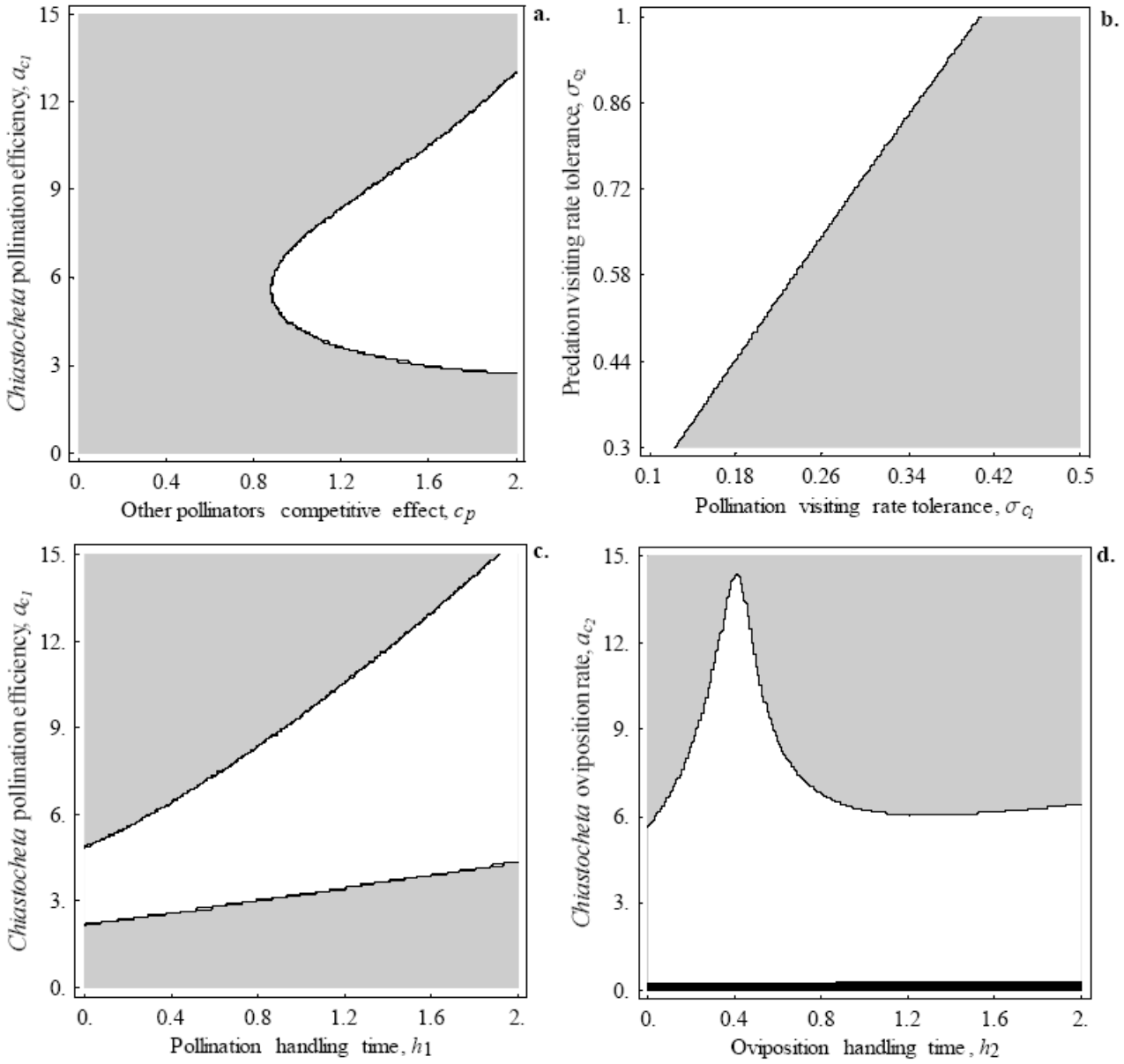

Figure 3.

Conditions for ecological viability of the *Trollius – Chiastocheta spp.* system (in black, unviable parameter values) and for the coevolution of specialisation (white) vs. generalisation (gray) derived from short-term coevolutionary dynamics. The effects of parameters c_p and a_{c1} (2.a), σ_{c1} and σ_{c2} (2.b), h_1 and a_{c1} (2.c), h_2 and a_{c2} (2.d) become visible. Other parameter values are as in Table 1, with $a_{c1} = 8$, $a_{c2} = 5$, $\sigma_{c1} = 0.2$, $\sigma_{c2} = 0.8$, $\sigma_p = 0.5$, $h_1 = 1$, $h_2 = 2$, $a_p = 0.3$, $c_p = 0.7$, except when those parameters are concerned by the sensitivity analysis.

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3. Les principaux résultats

- **Pour que le trolle se spécialise sur les chiastochètes, il faut que les autres pollinisateurs potentiels interfèrent avec ces dernières.**

- **Le trolle ne se spécialise pas lorsque la pollinisation par les chiastochètes est peu efficace ou lorsque leur taux d'oviposition est trop élevé.**

- **Lorsque la sélectivité des chiastochètes quant à la morphologie florale est plus forte pour les femelles ovipositrices que pour les adultes à la recherche de nectar ou de partenaire, la spécialisation est favorisée.**

- **Lorsque les femelles chiastochètes ovipositrices sont plus sélectives sur la morphologie florale que les adultes à la recherche de nectar ou de partenaire, la spécialisation est favorisée.**

CHAPITRE 3 : Rôle des composés organiques volatils émis par le trolle dans l'attraction des chiastochètes.

1. Introduction

Les signaux floraux impliqués dans la reconnaissance d'une plante par un pollinisateur sont visuels et olfactifs. Après avoir montré l'importance décisive de la morphologie globulaire, nous nous sommes intéressés au rôle des composés volatils organiques émis par la fleur au niveau des sépales et des étamines. Je me suis appuyé pour cela sur des résultats préliminaires que Laurence Després avait obtenu en collaboration avec Marie-Charlotte Anstett en 2003 et 2004, résultats repris dans le manuscrit ci-après. Les effluves de fleurs de plusieurs populations ont été collectées sur le terrain en conditions naturelles à l'aide d'un sachet en plastique inerte et de deux pompes (technique de « headspace ») et les principaux composés volatils émis ont été déterminés (les résultats sont inclus dans le manuscrit ci-après). A partir de ces données de départ, j'ai essayé de répondre à la question suivante : peut-on mettre en évidence le rôle des composés volatils dans l'attraction des chiastochètes, si oui lesquels, et quelle est leur importance en comparaison avec la morphologie globulaire et la couleur jaune du globe ? Pour cela, nous avons collaboré avec Sylvie Baudino et Jean-Claude Caissard, tous les deux membres du Laboratoire de Biotechnologies Végétales appliquées aux Plantes Aromatiques et Médicinales de l'Univerité Jean Monet à Saint Etienne ainsi qu'avec Stefan Dötterl, du Department of Plant Systematics de l'Université de Bayreuth (Allemagne). Stefan a déterminé quels étaient les composés volatils qui déclenchaient une réponse physiologique chez les chiastochètes. Les composés volatils émis par la fleur sont séparés dans un spectromètre de masse couplé à un électro-antennogramme. L'activité électrique de l'antenne de chiastochète en réponse à chaque composé présent dans le bouquet floral est ainsi mesurée. Sylvie et Jean-Claude nous ont aidé à concevoir et réaliser une expérience de terrain dans laquelle nous déterminions la composition du bouquet floral de plusieurs dizaines de fleurs dans la population, observions le nombre de visites reçues et mettions en relation visites et bouquet floral. Les détails de cette expérience sont présentés dans le manuscrit ci-dessous.

Enfin, dans une expérience complémentaire, nous nous sommes intéressé au rôle spécifique des volatils émis par les étamines.

2. Manuscrit (en préparation pour New Phytologist)

The role of volatile organic compounds emitted by globeflowers in the attraction of their specific pollinating flies.

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Running title: globeflower scent attractivity to specific pollinators

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Abstract

- Floral scents play a key role in plant insect interactions. Headspace collection in four populations in the field and GC-MS (gas chromatography-mass spectrometry) were used to identify and quantify the volatile compounds emitted by the globeflower *Trollius europaeus* involved in a highly specific mutualism with *Chiastocheta* flies. The blend is made of common fatty acid derivatives, benzenoids, mono- and sesquiterpenoids. Geographical variations among populations, compared to variations among individuals within populations, exists but are low.
- Electrophysiological analyses of the floral scent sample performed with a GC-EAD (gas chromatography-electroantennographic detection) system on *Chiastocheta* flies revealed that several compounds are electrophysiologically active: methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, and linalool.
- In two natural patches, we recorded the *Chiastocheta* visits to flowers, measured morphological and pigmentation traits and analysed the volatile blend of the observed flowers using hexane extracts. In one patch, the variability of the electroantennographic active compounds extracted explained half of the variability of the flowers visits, while in the other patch it explained only a negligible fraction. The variability of the other floral traits measured (e.g. morphology, pigments) explained one third of the variability of the visits.

Introduction

Species interactions are crucial to the functioning of ecosystems and drive the evolution of most plants and animals. Free-living organisms can, up to a certain point, choose to interact or not with other organisms. In host-parasite interactions, the hosts are selected to hide, and cues used by parasites to locate and reach their hosts are likely to be quite subtle. Conversely, organisms involved in mutualistic interactions, where both partners are selected to enhance the encountering rate, are expected to show clear attraction signals and efficient recognition mechanisms. For example, plants are selected to advertise their flowers, and pollinators are selected to locate the flowers where they will find resources (usually pollen or nectar). The main cues involved in pollinator attraction are visual and olfactory. Flower colour and shape have been thoroughly studied from genetics to ecology (Clegg & Durbin, 2003). Flower

scents have been less well-researched but recent technical developments in the collection and analysis of volatile compounds (Tholl & Röse, 2006) have rendered them easier to study. Floral scents are particularly effective over long distances and their variability in terms of chemical structure and blend composition seems considerable (Raguso, 2008). A comprehensive understanding of biosynthesis pathways of plant volatiles is just starting to form, with evidence of a molecular basis allowing much variability in the molecules produced (reviewed in Pichersky et al., 2006).

Until now, few generalities were known about flower scent functions: each compound can have zero (e.g. waste or by-product of the synthesis of other molecules), one, or several functions (e.g. pollinator attractant, insect repellent, or antibacterial activity; Dudareva & Pichersky, 2006), and may interact, or not, in synergy with other compounds. Floral scents of unrelated plant species may show some similarities according to the identity of their pollinators. For example, butterfly-pollinated plants often produce benzenoids in Europe, and linalool and its derivative in America (Andersson et al., 2002), bat-pollinated plants often produce sulphur compounds (Knudsen & Tollsten, 1995), hummingbird-pollinated taxa produce no or trace levels of volatile compounds (Knudsen et al., 2004), while bee-pollinated flowers are usually sweet-scented (Dobson, 2006), and fly-pollinated plants often smell foul, like carrion, faeces or rotten fruit (Jurgens et al., 2006). The scent of related species can vary widely according to their pollinators (Goldblatt & Manning, 2006). Despite these few broad generalities, the precise chemical language between plants and pollinators remains relatively unknown.

Species-specific pollination mutualisms, for which a single species of pollinator is the only pollinator of a plant species, could represent a Rosetta stone which allows the plant's pollinator language to be elucidated. Nursery pollination mutualisms, in which the specific pollinator's only egg laying site is located on its host plant reproductive structures, represents one such simple example (Dufay & Anstett, 2003). Among the nursery pollination mutualisms reported so far, odours produced by the host-plants were studied for figs (*Ficus* sp., Moraceae, Gibernau et al., 1998, Gibernau et al., 1997, Grison-Pige et al., 2002), dwarf palms (*Chamaerops humilis*, Arecaceae, for which odours attracting the pollinators are produced by the leaves and not by the flowers Dufay et al., 2003, Caissard et al., 2004), *Macrozamia* cycads (*Macrozamia sp.* Zamiaceae, Terry et al., 2004), Silenes (*Silene* sp., Caryophyllaceae, Dotterl & Jurgens, 2005), Yuccas (*Yucca* sp., Agavaceae, Svensson et al., 2005), and *Glochidion* trees (Okamoto et al., 2007). The volatiles identified in these highly specific systems were mostly ordinary compounds, frequently found in the floral bouquet of

many plants with unspecialised pollination systems. In three cases, the behaviour of the pollinator was studied. *Glochidion* floral scents were capable to attract the mutualistic *Epicephala* moths, which also discriminated host from non-host plants by floral scents (Okamoto et al., 2007). The nursery pollinator of *Silene latifolia,* i.e. *Hadena bicruris* (Lepidoptera, Noctuidae, Hadeninae), mainly reacts to lilac aldehydes (Dotterl et al., 2006). Also *Cycadothrips chadwicki* thrips are attracted by scent to their cycad host plant, *Macrozamia lucida*, and ocimene isomers as well as ß-myrcene were responsible for their attraction (Terry et al., 2007). No behavioural assays were conducted in the *Yucca-Tegeticula* pollination system, however, the pollinator of *Yucca filamentosa, Tegeticula cassandra* (Lepidoptera, Prodoxidae), strongly responded in electroantennographic analyses to an uncommon and so far undescribed dioxygenated compound ($C_{11}H_{18}O_2$), which might be responsible for attracting the moths (Svensson et al., 2005). Therefore, plants involved in nursery pollination may produce either specific molecules to attract the nursery pollinator as suggested for *Yucca,* or rely on a blend of less specific compounds.

The globeflower *Trollius europaeus* L. (Ranunculaceae) entirely depends for its pollination on specific Anthomyiidae flies of the genus *Chiastocheta* Pokorny. The adults spend most of their lifetime inside the globes where they mate, feed on nectar and where the females lay eggs. They are almost never found on other flowers. The flies' ability to detect globeflowers is therefore essential as soon as the adult emerge. The globe-shape of the flower is important in the attraction, artificially open flowers attract less flies than closed flowers (Ibanez et al., 2009). Further, when other yellow bowl-shaped flowers of the genus *Ranunculus* are present, *Chiastocheta* very rarely visit them compared to artificially opened *Trollius* flowers (S. Ibanez, pers. obs.), which suggests that olfactory stimuli might be implied in the specific attraction.

Here, we characterize the floral scent emitted by different globeflower populations, and investigate the role of these volatile organic compounds in the attraction of their specific pollinating and seed-eating flies. We also compare the importance of floral scents in comparison to other floral traits for attraction of flies to the flowers. Specifically, we address the following questions: 1) which compounds are emitted by globeflowers?, 2) among these compounds, which can be detected by the flies?, 3) can the electrophysiologically active compounds influence the behaviour of flies?, 4) how important are the VOCs in the attraction

of flies in comparison to other floral traits, such as globe morphology and pigment concentration?

Material and methods.

The Trollius europaeus - Chiastocheta *spp. system.*

The European globeflower, *Trollius europaeus* L. (Ranunculaceae), is a hermaphroditic, homogamous, arctic-alpine perennial species growing in moist meadows. In the Alps, natural populations range from 700 to 2500 meters altitude. The flowering is synchronised within populations and typically lasts 2-3 weeks. In the Alps, the plant is passively pollinated by six species of *Chiastocheta* flies (Anthomyiidae), but in this work we will consider them only at the genus level. *Chiastocheta* larvae feed only on *T. europaeus* seeds, they are obligate associates of globeflowers. Both male and female flies visit the globe-shaped flower where they eat nectar and pollen, and mate. Females deposit one to several eggs on or between the carpels, after hatching each larva eats several seeds and falls into the soil to pupate and overwinter.

Headspace volatile collection and scent analyses

Four study sites were chosen in two mountain massifs in the French Alps: 1) Banchet (1206 m a.s.l., 5°47'E 45°18'N), 2) Col de Portes (1326 m a.s.l., 5°47'E 45°17'N), 3) Lautaret (2100 m a.s.l., 6°24'E 45°03'N) and 4) Pré Gelé in the north side of col du Galibier (2300 m a.s.l. 6°24'E 45°04N), with pairwise distances among sites ranging between 1.5 and 100 km. We analysed the blend of 9-13 first day flowers in each of the four sites in 2003, resulting in a total of 47 flowers analysed, to determine the scent compounds emitted by *T. europaeus*, and to learn more about the variation in scent within and among populations. Floral odours were collected by dynamic headspace, a non-destructive method. First day flowers are easily recognised by their outer stamens just starting to dehisce. Each flower was enclosed in a nalophan® bag (Kalle Nalto, Wursthüllen, Germany), negative controls were made with empty bags. Air cleaned through a charcoal filter was blown into the bags at 400 mL min^{-1} and pulled out at 300 mL min^{-1} through an adsorption tube containing 30 mg of Alltech SuperQ (ARS Inc., Gainesville, Florida, USA). Each odour collection lasted 2 hours (increasing the duration of volatile collection did not lead to the detection of additional compounds). Back in the lab, the adsorption tubes were eluted with 150 µL CH_2Cl_2. We added 4 µg of each of two internal standards (nonane and dodecane) to each sample for

quantification. The eluents were analysed qualitatively with a Varian CP3800 gas chromatograph coupled with a Varian Saturn 2000 ion mass spectrometer (Varian Inc., California, USA). We injected 1 µL into a 1079 injector (200°C, splitless mode) and used a CP sil 8 CB column (30 m, 0.25 mm inner diameter, 0.25 µm film thickness) with helium as the carrier gas (constant flow rate: 1 ml min^{-1}). The oven temperature was kept at 50°C for the first 3 min, then programmed to increase 3°C min^{-1} to 100°C, 2.7°C min^{-1} to 140°C, 2.4°C min^{-1} to 180°C, and then 6°C min^{-1} to 250°C. To identify the compounds, we compared mass spectra of the samples with those of Adams (Adams, 1995), MassFinder, Wiley 6 and NIST98 libraries and with spectra of authentic compounds. We also compared the retention indices with those known for authentic compounds. For quantification, extracts were injected into a CP3800 gas chromatograph (FID detector, column EC-1, length 30m, internal diameter 0.25 mm, film thickness 0.25 mm, carrier gas: helium, oven temperature as for GC-MS analysis). The quantities of the compounds were estimated using as a scale the peak areas of the two internal standards (mean peak area of standards was used). Compounds found in the same quantity in sample and control were removed from the analysis.

Floral scent was additionally collected in the laboratory to get a sample for electroantennographic measurements (see below). Therefore, 40 flowers including the stem of *T. europaeus* were cut on 11 June 2008 at Col de Portes, sent via express mail to Bayreuth (Germany), where scent was collected on 12 June 2008 for 6.5 hrs in the afternoon. The flowers were enclosed in an oven bag (Topitts) and emitted volatiles were trapped in an adsorbent tube filled with 10 mg of of Tenax-TA 60-80 and 10 mg of Carbotrap 20-40. The air was sucked from the bag over the adsorbents (150 ml/min) by a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Volatiles were eluted with 80 µL of acetone (SupraSolv, Merck KgaA, Germany) to get the sample for the electrophysiological (and chemical) analyses (see below). By comparing this scent sample with scent samples collected in 2003 by dynamic headspace in the field we found all compounds with the exception of Z-3-hexen-1-ol and perrilene. Scent quality was therefore (almost) not influenced during sending the flowers from France to Germany.

GC-EAD (gas chromatography coupled to electroantennographic detection)

Electrophysiological analyses of the floral scent sample were performed with a GC-EAD system in Bayreuth. 14 different *Chiastocheta* individuals were tested. The flies were caught on 11 June 2008 at Col de Portes, where they were inside the collected flowers and also sent

via express mail to Bayreuth. The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID), and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Hilversum, Netherlands). 0.1 to 1 µL of the odor sample was injected splitless (injector temperature: 250°C) at 60°C oven temperature, followed by opening the split vent after 1 min and heating the oven with a rate of 10°C/min to 200°C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diameter 0.32 mm, film thickness 0.25 µm, Phenomenex). The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the FID and to the EAD setup. Makeup gas (He, 16 ml/min) was introduced through the fourth arm of the splitter. For measurements, the head was excised from the thorax and the postoccipital region was subsequently placed in a glass capillary electrode containing insect ringer (8.0 g/l NaCL, 0.4 g/l KCL, 0.4 g/l $CaCl_2$). The tip of one antenna was placed in another glass capillary electrode (recording electrode) containing also insect ringer. The electrodes were connected to silver wires.

To identify the EAD active compounds 0.1 µL of the scent samples was analyzed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector (Varian Inc., Palo Alto, USA). Column and settings were as described in Dötterl et al. (2005). Component identification was carried out using the NIST 02 mass spectral data base, or MassFinder 3, and confirmed by comparison with retention times and mass spectra of authentic standards.

Observation of flies visiting natural globeflowers and measurement of flower traits

To test, whether flowers visits by flies can be explained by the scent of these flowers or by other flower traits, the number of fly visits to specific flowers was correlated with their content of VOCs, their morphology, and their concentration of pigments.

Fly observations. In two patches A and B (distance: 200 m), located at Pré Gelé in the north side of col du Galibier (2300 m a.s.l. 6°24'E 45°04N, same location as population n°4 mentioned above, but different patches) we bagged 50 immature flowers each in order to prevent visits from flies and pollination before the experiment. On the 20 and 28 June of 2007, under sunny conditions with no wind, we removed the bags from the globeflowers, and five observers recorded the number of *Chiastocheta* flies that entered these flowers. When the flies are moving fast, it can be difficult to discriminate *Chiastocheta* and other flies, but

Chiastocheta are the only flies able to move freely between the sepals and enter the globeflowers. If a single *Chiastocheta* entered a flower, went out, and entered again, it was counted twice. Each globeflower was observed during 3 periods of 30 minutes (90 min in total), the observers and observation periods being randomised.

Morphological traits. We measured several morphological traits in situ: the distance of the globe to the ground, the globe outer diameter (defined as the diameter of the last circle of sepals), the globe inner diameter (defined as the diameter of the first circle of sepals), the globe height and the number of sepals.

Pigments. After the end of the observations we cut the flowers and brought them to the laboratory, where we immediately dissected sepals and stamens. A quarter of the sepals was kept frozen at –20°C and adonivernith (a phenolic pigment of *Trollius*) concentration was later on determined (see Gallet et al., 2007 for a detailed description of the protocol). Another quarter was weighed and immerged in a 70% acetone – 30% H2O solution during 15 minutes. The solution was then filtered and the absorbance at 470 nm was measured, from which the concentration of carotenoids was determined (Lichtenhalter, 1987).

VOCs. It was not possible to collect the floral scent from all the flowers observed using the time-consuming dynamic headspace technique described before; however, we determined the VOC content of observed flowers by extracting them in a solvent. As all flowers were in a similar developmental stage (unpollinated at the start of observation period), we assumed that a flower which contains e.g. a higher total content of VOCs than another flower, also emits a higher amount of scent.

In detail, the remaining half of the sepals and all the stamens of the flowers were weighed and immerged in twice their mass of hexane containing 40 mg L^{-1} camphor as an internal standard, during 24 hrs in separate tubes (two tubes per flower, one including sepals and one stamens), and then stored at –20°C until analyses. GC-FID analyses were carried on a gas chromatograph (Agilent 6850) equipped with a flame ionisation detector (FID). Nitrogen was used as carrier gas at a flow rate of 1 mL min^{-1}. A glass HP-Innowax 1909N-133E capillary column (30 m x 25 mm inner diameter, 0.25 µm film thickness) was used under the following conditions: 3 min at 40°C then 2°C min^{-1} up to 160°C and 12°C min^{-1} to 240°C with 2 min hold time. 1 µL of the extract was injected in split mode (10:1 ratio). Quantitative peak estimation was achieved by comparison with the internal standard and a molar response of one was assumed for all components as described by (Picone et al., 2004). After solvent extraction and analysis (see below), the quantity of each volatile compound contained in a

flower was determined using the following formula : 2*amount in the sepals + amount in the stamens.

To identify the volatile compounds of the solvent extract, parallel analyses were done on an Agilent 6890 gas chromatograph/mass spectrometer (using the databases CNRS, Wiley 275 and Nist 98 mass spectrum databases) with helium as carrier gas, but otherwise with the same GC column and oven program used to analyse these extracts by GC-FID. The temperature of the injector and detector was 250°C. Analysis parameters were as follows: ionising voltage in ei-mode 70eV; mass scan rate 2.94/s and mass scan range 50-550 m/z. All experiments were performed at least three times.

The solvent extracts also contained long chain alkanes and alkenes, which are known to be important in other pollination systems (e.g. sexual deceptive orchids, see Schiestl et al., 1999). *Chiastocheta* flies however never responded to such compounds in preliminary electroantennographic measurements, instead responded to compounds also found in the headspace samples. We therefore included only compounds with a Kovats retention index of less than 2000 (all typical compounds found in headspace floral scent samples have less than 2000) in the chemical analyses of these solvent extracts, and further did not use these samples for more detailed GC-EAD analyses.

Data analysis

To test for differences in scent composition (headspace volatile collections) among the different populations, we performed Permutational Multivariate Analysis of Variance using distance matrices (thereafter called PERMANOVA), based on Bray-Curtis indices. This method is available in the package "vegan" of the free software R 2.7.2 under the function name "adonis". Adonis is a multivariate procedure directly analogous to MANOVA (Anderson, 2001, McArdle & Anderson, 2001), and commonly used in community ecology. Instead of using the quantitative data, we used the percentage amount (relative amount) of compounds as we found high variation in the total amount of scent even within populations (see Results). To determine the compounds being responsible for differences in the percentage amount of scent emitted among population, we used a *SIMPER* analysis in Primer (Clarke & Gorley, 2006). All following statistical analyses were done with the software R 2.7.2.

The morphological and biochemical traits of observed flowers were highly correlated (out of the 91 pairwise correlation coefficients between traits, 35 were significantly different from zero in patch A and 27 in B), so we did not use classical multiple regression. Instead, we

modelled *Chiastocheta* visits to natural flowers against the traits measured using generalised Partial Least Squares (gPLS) regression. The number of *Chiastocheta* that entered the globes was modelled using "Poisson" family (function "glm", package "stats" in R). Following Bastien (2005), we built an algorithm which estimated the coefficient of each trait in the regression and a 95% confidence interval (1000 bootstraps), and the coefficient of determination (R^2) of the model. For each model, six PLS components were included in order to compare R^2 among models. All the traits measured were included in each gPLS component in order to avoid arbitrary trait selection based on p-values.

Results.

Floral scent of T. europaeus and how it varies among populations.

A single flower emitted in the median 342 ng of scent per 2hrs, and there were no significant differences among the populations (KW-ANOVA: $H(3, N = 47) = 4.60$, $p = 0.20$).

Sixteen floral scent compounds were detected in the 47 samples collected by dynamic headspace (Table 1), among them eight sesquiterpenoids, three monoterpenoids, three fatty acid derivatives, and two benzenoids. The scent was highly variable, and the compounds occurred in the mean only in 49%±8% (mean±SE) samples. Not any compound was detected in all of the samples, and only four compounds occurred in more than 80% of the samples: Z-3-hexen-1-ol (43 samples), methyl salicylate (41), β-caryophyllene (41), and linalool (39). Most of the compounds were emitted in small amounts, and only three compounds (β–caryophyllene 34%, linalool 18%, E,E-α-farnesene 7%) contributed a median of at least 5% to the total scent blend.

Eleven of the compounds occurred in all of the four populations analysed, among them E-β-ocimene, linalool, β-caryophyllene, E,E-α-farnesene, and β–caryophyllene oxide, which contributed a median of more than 5 % to the total amount of scent emitted in at least one population. The other five compounds occurred in three of the populations each, and these compounds were, if present, mostly emitted in small relative amounts. In two populations the scent samples were dominated by β-caryophyllene (49%, 41%), in the others similar high amounts of linalool (26%, 22%) and β-caryophyllene (27%, 24%) were emitted (all other 14 compound contributed with a median of less than 10% to the scent of the different populations), and this difference (as indicated by a SIMPER analysis) is mainly responsible for the differences in scent pattern found among the populations in a multivariate approach (PERMANOVA: $R^2 = 0.08$, $p = 0.0039$). However, though the differences are clearly

significant, the low R^2 value indicates that there was big overlap in scent samples among the populations. One explanation for this finding is that variation in scent was also high within the populations, and this is true for the main (β-caryophyllene, linalool) as well as for minor compounds (Table 1).

Electrohysiologically active compounds

The sample, which was used for the electroantennographic measurements, was collected from 40 cut flowers, and it contained all compounds found in samples collected in situ and listed in Table 1, except Z-3-hexen-1-ol and perillene. The measurements revealed the compounds being detected by the antennae of the flies. Though the baseline was quite unstable, several compounds consitently elicited antennal responses (Figure 1). Flies especially responded to the sesquiterpenes ß-caryophyllene, germacrene D, and E,E-α-farnesene, which were dominating the scent sample used for these measurements. Antennae of flies further responded to methyl salicylate and Z-jasmone, and in some runs also to linalool.

Observation of flies visiting natural globeflowers

Thirty three and 41 flowers were observed in patches A and B respectively. We recorded 502 (resp. 317) events of flies entering the flowers in patch A (resp. B), which represents15±0.27 (resp. 7.7±0.14) (mean±SE) visits per flower.

In the solvent extracts of these flowers (Table 3) we found most of the compounds (among them all EAD-active ones), which were also detected in the headspace analyses of corresponding population (Pré Gelé), but not perillene, ß-copaene, Z,E-α-farnesene, and caryophyllen oxide, which occurred only in some headspace samples and mostly in small amounts. In comparison to the headspace samples, the solvent extract samples were dominated by green leaf volatiles, such as hexanal and E-2-hexenal, which were not at all found in the headspace samples.

Two electrophysiologically active compounds were only present in a fraction of the solvent extract samples: germacrene D and methyl salicylate. Their presence was associated with a higher visitation rate (Table 4, in patch A the effect of germacrene D was only marginally significant) in both patches, which was generally not true for the inactive compounds which were present in a fraction of the flowers. The presence of inactive compounds was generally not associated with a higher visitation rate whatever the patch (except ß-bourbonene in patch B). The presence of Z-3-hexenyl acetate influenced the visitation rate negatively in patch A.

The variability of the visits could be partly described by the variability of the total amount of electrophysiologically active VOCs in patch B (Table 5, R^2=0.46) but not in patch A (R^2=0.01). This is to be compared with the variability of the visits explained by morphological and pigment traits (0.28 in B ; 0.10 in A). All over, the R^2 of the full model was 0.13 in patch A and 0.56 in B.

In patch B where the variability of the traits measured explained a large part of the variability of the visits, several traits seem to be important besides the total amount of EAD active VOCs. An increase in the globe diameter, the number of sepals, and the amount of germacrene D was linked to an increase of the visits; and an increase of the concentration of carotenoids in the sepals was linked to a decrease in the visits (Figure 2).

Discussion.

Though *Trollius europaeus* is involved in a highly specialized nursery pollination mutualism, the flowers did not emit uncommon compounds, instead, emitted fatty acid derivatives, benzenoids, and terpenoids, which are widespread and known as floral scent also from many other plants (Knudsen et al., 2006). The two compounds, linalool and ß-caryophyllene, which were the most abundant compounds in the headspace samples are even among the most common floral scents at all. The emission of common floral scent compounds by a plant, that is involved in a specialized pollination system is not exceptional, but was found also in other plants interacting with nursery pollinators (e.g. Terry et al., 2004, Okamoto et al., 2007, Dufay et al., 2003). In some of these systems, however, several compounds emitted by the plants are still unidentified, and among these ones, there may be uncommon compounds (e.g. Dufay et al., 2003). Several compounds identified in the headspace samples of this study, such as linalool and E,E-α-farnesene, were also found in the study of Jürgens & Dötterl (Jurgens & Dotterl, 2004). They thermally desorbed anthers of *T. europaeus* in a modified injector of a gas chromatograph, which was coupled to a mass spectrometer. Interestingly, ß-caryophyllene, the main compound in present work was not listed in that study. However, their unknown sesquiterpenoid with the retention index 1451 could in the meanwhile be identified as ß-caryophyllene (Dötterl, unpublished data), which contributed 3.7% to the anther volatiles. Compounds found in high relative amounts by Jürgens & Dötterl (Jurgens & Dotterl, 2004), and in low relative amount in present work may mainly originate from the stamens (e.g. E,E-α-farnesene), while compounds, which occurred in high relative amounts in present study and are absent or occurred in low amounts in the anther study may be emitted

exclusively/mainly by the sepals (e.g. ß-caryophyllene). However, different methods were used in both studies making a direct comparison difficult, and more studies are needed to determine qualitatively and quantitatively the compounds emitted by the different floral parts.

Also variations in scent within and between populations, as found in present work, make a comparison of studies, where plants of different populations were used (Jurgens & Dotterl -2004- used plants of unknown origin and cultivated in a botanical garden) difficult. We found high variation not only in the relative amount of compounds emitted within and between populations, but also in the scent quality. Not any compound present in the headspace samples (for a comparison of the headspace and solvent extract samples see below) was found in all samples analysed and most of the compounds even did not occur in all replicate samples within populations. Such a high variation is unusual for a plant involved in a highly specific nursery pollination system. In *Yucca* species for example the scent variability is much lower, and a strong conservatism even among species was found (Svensson et al., 2005, Svensson et al., 2006). In *S. latifolia*, which is the host of the nursery pollinator *H. bicruris*, variation in scent within and between populations was also high, nevertheless, at least lilac aldehyde, which dominated most of the samples (Dotterl et al., 2005), and which is most attractive to *Hadena* (Dotterl et al., 2006), was found in all samples analysed.

Instead of focusing on all compounds emitted by the flowers, it is more powerful to include only compounds, which play a role in the *Trollius-Chiastocheta* pollination system. Such compounds could be treated as *signals*, whereas compounds not important in that interaction are just *noise* as suggested by Raguso (Raguso, 2003). Our electrophysiological tests revealed that flies have antennal receptors for six compounds emitted (methyl salicylate, Z-jasmone, ß-caryophyllene, germacrene D, E,E-α-farnesene, linalool). We also found a positive correlation between the amount of EAD active compounds in the solvent extracts of flowers and the visitation rate (patch B, Figure 2). Flowers with a high total amount of these compounds were visited more often by *Chiastocheta* than flowers with a low amount of these compounds. When treating these six active compounds therefore as *signal*, and the others as *noise,* the variability of the signalling compounds is (tendentially) smaller than variability of the others in the headspace samples (t-test: $t_{df=14} = -2.11$; $p = 0.052$). The active compounds occurred in 68%±9% (mean±SE) of collected samples, and the inactive only in 38%±9% indicating stabilizing selection on the presence of compounds responsible for pollinator attraction (see also Ayasse et al., 2000). When testing for differences in the relative amount of

compounds among populations separately for EAD active and inactive compounds, we in both cases still find differences among populations (PERMANOVA: $p<0.05$ each) and again high variability also within populations. The presence of the EAD active compounds may therefore be more important for attraction of *Chiastocheta* than their relative amounts. Flowers containing germacrene D obtained more fly visits in the field than flowers that did not contain this compounds in one of the two patches observed, and we further found a positive correlation between the amount of this compound in the solvent extract of flowers and the visitation rate of flies in that patch. These results point towards a function of this compound in attracting flies and it possibly works in synergy with other compounds. Similarly, the presence of one compound in the solvent extracts, methyl salicylate, which was found in many headspace samples, but only in few solvent extract samples, resulted in an increase in the visitation rate of *Chiasocheta*. The six identified EAD active compounds are not only detected by *Chiastocheta*, but are known to be perceived by or to attract also other insects, among them dipterans (Jhumur et al., 2007, Zhu et al., 2003, Siderhurst & Jang, 2006, James, 2005, Bengtsson et al., 2001).

Most of the compounds found in the headspace samples of population # 4 also were found in the solvent extract samples of flowers, which were observed for fly visits in the field (patch A and B in population # 4) before extraction. In contrast to this similarity between headspace and solvent extract samples two main differences are evident. First, the solvent extract samples contained several C6 compounds (e.g. E-2-hexenal, hexanal) in all samples, which are known as typical green leaf volatiles (GLVs), and which were not at all found in the headspace samples. Such GLVs are known to be produced and released especially after injuring plant tissue (Matsui, 2006), and in our case it is likely that they appeared after cutting the stamens and the sepals before extracting them in hexane. Second, three of the EAD active compounds (linalool, ß-caryophyllene, E,E-α-farnesene) were found in all solvent extracts, but not in a few (1-3) of the headspace samples. We especially did not find these compounds in very weak headspace samples, and it maybe that these compounds were emitted as minor compounds by these flowers but in an amount too low for detection.

Additionally to floral scent, visual floral cues play a role for attraction of *Chiatocheta* to *Trollius* flowers. We for example could attract flies to scentless fake flowers, and besides their shape especially their yellow colour may have been responsible for fly attraction (indeed, blue, red or green fake flowers do not attract *Chiastocheta* flies and attract very few

other flies). Many fly species are known to respond to yellow colours (Lunau & Maier, 1995), and this is also true for *Chiastocheta*. Pellmyr (Pellmyr, 1992) could attract *Chiastocheta* flies to yellow scentless traps, and he further found that small changes in the colour of the traps strongly influenced the number of *Chiastocheta* flies trapped. We found high variation in the concentration of carotenoid pigments in patch B, and if we assume that carotenoids influence the colour of the flowers, our results even indicate that flies respond to colour differences naturally occurring within *Trollius* populations. The fly visitation rate was negatively correlated with the carotenoid concentration, indicating that flies preferred flowers with low concentrations over flowers with high concentrations. Adonivernith is close to the yellow pigment luteolin (it has been identified as luteolin 8-β-D-glucopyranosyl-2″-*O*- D-xylopyranoside, Gallet et al., 2007), but there was no correlation between its concentration in the sepals and the flies visitation rate. When located in the carpel walls, adonivernith is also involved in the plant's reaction to the presence of *Chiastocheta* larvae (Gallet et al., 2007), so it is not clear in which way flies should respond to it.

Altogether, our results revealed the compounds emitted by *Trollius* flowers and demonstrate that both floral scents as well as visual flower cues play a role in the *Chiastocheta-Trollius* interaction. The relative importance of visual versus olfactory cues may however vary and depend on several factors. On the day we observed the flies, VOCs variability explained a negligible fraction of visits variability in patch A, but nearly half of it in patch B. The other traits measured played a noticeable role, higher than VOCs in A, but lower in B. In patch B, *Chiastocheta* flies seemed to prefer larger flowers emitting large total amounts of electroantennographic active VOCs, among them especially germacrene D, with low concentrations of carotenoids. As flowers, which experience high visitation rates are likely to be better pollinated and, more importantly, will export more pollen (Ibanez et al., 2009) and have a higher male fitness, we expect that traits involved in the interaction are under *Chiastocheta*'s selection pressure. The selective pressures on floral traits "working" over a whole flowering period cannot be predicted from our single-day observations, which suggest that they are likely variable in time and space. However, there seems to be directional selection pressure on the presence of the EAD active in comparison to the inactive compounds, and also some other traits like the number of sepals and the amount of EAD active VOCs could be under *Chiastocheta*'s selection pressure. Recently there was further an ongoing strong directional selection pressure stabilizing the closed phenotype (Ibanez et al., 2009) demonstrated. Interestingly, in present work variability of the visits could be explained

by the measured flowers traits when the visitation rate was low but not when it was high. A more solid conclusion would have been possible if the number of observed patches would have been higher than two, but a more intense selection when the ecological conditions are harsh has been observed elsewhere (Wilson et al., 2006). Indeed, when *Chiastocheta* flies are more abundant and when flowers are crowded, the flies are likely to be less choosy. All over, selection on globeflowers floral traits might be more intense in populations where *Chiastocheta* flies are at low densities, and years when climatic conditions prevent the flies from being very active. Further, the traits used by *Chiastocheta* for host-plant finding may even change during the life of the flies. Flies visiting *Trollius* flower for their first time may use other floral cues than flies visiting flowers repeatedly. Flies are capable of learning (Papaj & Prokopy, 1989) and may learn specific visual and/or olfactory floral cues during flower visits, which are not important for flies in locating *Trollius* for their first time. In present work experiments were conducted with free-flying flies with unknown flower visiting history, we therefore may especially have measured the response of flower-experienced flies to *Trollius* floral traits, and the traits used by flower-inexperienced flies remains to being studied.

Table 1. Occurrence (in brackets the number of samples collected in each population), median and range (relative amount in %) of the scents emitted by first-day flowers (head-space collection) of four different *T. europaeus* populations (Banchet, Col de Portes, Lautaret and Pré Gelé).

	1) Banchet			2) Col de Portes			3) Lautaret			4) Pré Gelé		
	Occurrence (13)	Median	Range	Occurrence (12)	Median	Range	Occurrence (9)	Median	Range	Occurrence (13)	Median	Range
Total amount (ng/2h*flower)		342	16-2290		222	72-603		548	119-2420		370	23-1536
Fatty acid derivatives												
Z-3-hexen-1-ol*	10	4	0-99	11	5	0-38	9	4	*01-38	13	5	01-49
nonanal*	3	0	0-10	7	5	0-25	8	3	0-25	13	4	01-51
Z-Jasmone*	9	tr[1]	0-3	6	tr	0-3	5	tr	0-3	4	0	0-tr
Benzenoids												
methyl benzoate*	4	0	0-32	4	0	0-30	8	4	0-30	7	2	0-10
methyl salicylate*	12	1	0-5	9	tr	0-1	9	tr	tr-1	11	tr	0-1
Monoterpenoids												
E-ß-ocimene*	7	4	0-32	4	0	0-21	7	5	0-35	9	7	0-31
linalool*	9	9	0-30	9	15	0-32	9	26	15-41	12	22	0-47
Perillene	0	0	0-0	5	0	0-32	5	1	0-32	4	0	0-7
Sesquiterpenoids												
β-bourbonene	1	0	0-6	0	0	0-0	3	0	0-3	1	0	0-9
β-caryophyllene*	12	49	0-86	9	41	0-65	9	27	14-65	11	24	0-48
β-copaene	1	0	0-1	0	0	0-0	1	0	0-0	1	0	0-2
α-humulene*	1	0	0-1	2	0	0-9	1	0	0-9	0	0	0-0
germacrene D*	3	0	0-38	3	0	0-12	4	0	0-16	6	0	0-48
Z-E-α-farnesene*	1	0	0-2	0	0	0-0	2	0	0-4	1	0	0-2
E-E-α-farnesene*	9	8	0-23	6	4	0-19	6	2	0-29	10	8	0-35
caryophyllene oxide*	7	1	0-12	3	0	0-7	9	6	01-18	7	2	0-6

* identification is based on comparison of retention time and mass spectrum with an authentic standard. [1] tr: relative amount was less than 0.5%

Table 2. Mean and standard deviations of the traits measured in two patches of Pré Gelé, number of flowers (into brackets the number of flowers sampled in each patch) containing the compound (hexane extraction) in the case of VOCs.

	Patch A			Patch B		
	Occurrence (33)	Median	Range	Occurrence (41)	Median	Range
VOCs (mg per flower)						
Physiologically active compounds						
linalool	33	0.793	0.16-2.73	41	1.06	0.21-7.22
β-caryophyllene	33	3.474	0.10-21.61	41	1.337	0.23-4.77
germacrene D	28	1.863	0-15.52	31	2.836	0-15.51
E,E-α-farnesene	33	2.019	0.13-10.54	41	2.318	0.48-6.57
methyl salicylate	13	0	0-0.53	5	0	0-0.42
Z-jasmone	33	0.824	0.45-1.94	41	1.003	0.18-2.42
Physiologically inactive compounds						
hexanal	33	11.52	0.42-35.21	41	3.979	0.34-17.09
E-2-hexenal	33	15.96	4.17-68.77	41	11.75	1.73-31.7
Z-β-ocimene	31	0.108	0-0.40	41	0.217	0.01-0.83
Z-3-hexenyl acetate	26	0.09	0-0.38	39	0.221	0-1.57
1-hexanol	33	1.291	0.09-4.11	41	1.391	0.20-5.74
Z-3-hexenol	33	4.868	1.50-14	41	4.692	1.51-14.76
nonanal	31	0.236	0-0.83	41	0.952	0.04-3
β-bourbonene	10	0	0-0.38	23	0.122	0-0.64
methyl benzoate	15	0	0-0.10	24	0.013	0-0.35
hexanoic acid	15	0	0-0.40	40	0.272	0-1.39
nerolidol	26	0.631	0-7.61	34	0.168	0-4.19
Globe morphology (cm)						
Outer diameter		2.8	2.1-3.1		3.3	2.7-3.8
Inner diameter		2.3	1.8-2.7		2.6	2-3.1
Distance to ground		22.5	16-27.5		27	21-34
Globe height		1.5	1.2-1.9		1.6	1.3-1.8
Number of sepals		12	Okt 19		12	Okt 16
Sepal pigments (mg/g fresh mass)						
Adonivernith		8.47	3.2-16.72		6.32	2.47-16
Carotenoids		0.21	0.11-0.33		0.2	0.08-0.46

Table 3. Generalized linear models of the number of visits to natural flowers in two patches in Pré Gelé, depending on the presence of single compounds (only compounds, which were absent at least in three flowers in any patch were included in analysis). "na" means that the compound was absent in only one or two flowers, in which case the test is poorly robust. The first two compounds (germacrene D and methyl salicylate) are electrophysiologically active and the remaining inactive.

	Patch A				Patch B			
	estimate	SE	z	P	estimate	SE	z	P
germacrene D	0.185	0.104	1.786	0.074	0.220	0.098	2.255	0.024
methyl salicylate	0.183	0.070	2.615	0.009	0.224	0.111	2.011	0.044
Z-3-hexenyl acetate	-0.220	0.080	-2.746	0.006	na	na	na	na
β-bourbonene	-0.011	0.076	-0.144	0.885	0.185	0.081	2.291	0.022
methyl benzoate	0.008	0.070	0.114	0.909	0.072	0.081	0.897	0.370
hexanoic acid	-0.002	0.070	-0.025	0.980	na	na	na	na
nerolidol	-0.045	0.084	-0.541	0.589	0.158	0.111	1.426	0.154

Table 4. Coefficient of determination of each gPLS model explaining visitation rates to natural flowers in both patches of Pré Gelé, in function of VOCs contents, flower morphology and sepal pigmentation. Each model includes 3 PLS components. Mean number of visits per flower per hour±sd.

	All traits	Only VOCs	All except VOCs	Visits
Patch A	0.129	0.014	0.104	15.21±9.08
Patch B	0.561	0.460	0.286	7.73±5.69

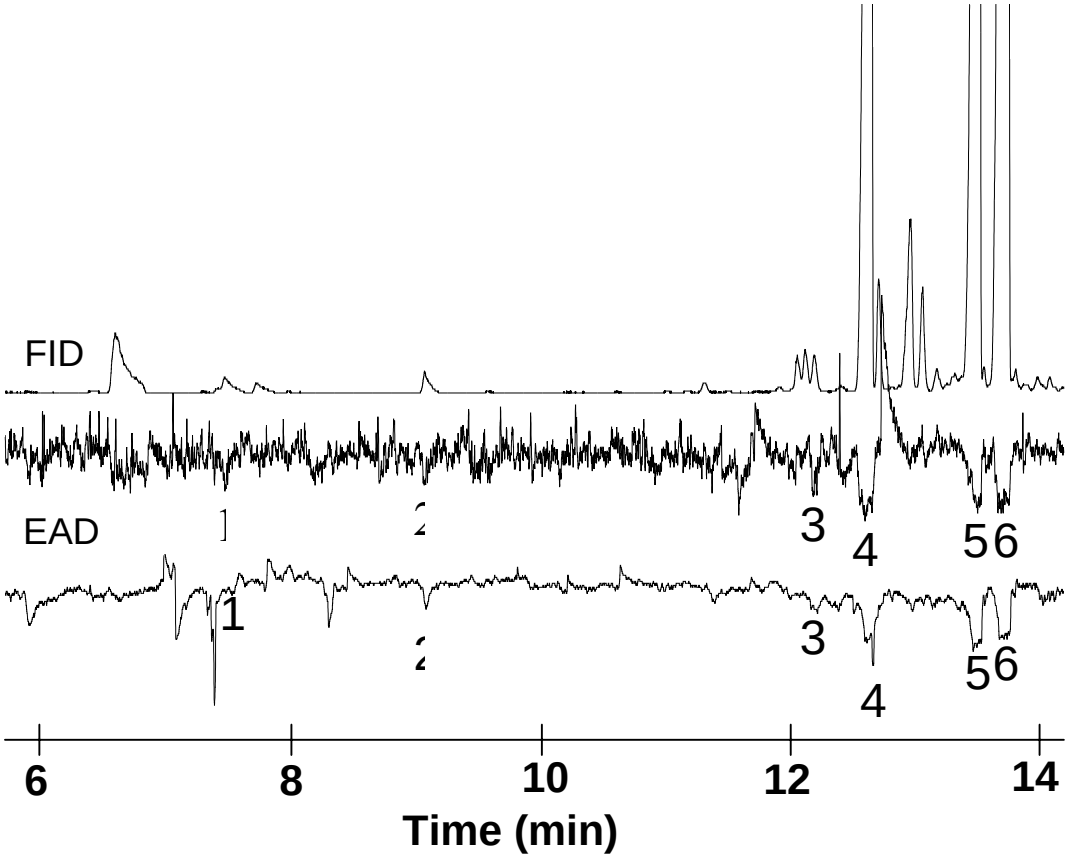

Figure 1. Coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *T. europaeus* flower scent sample tested on two different *Chiastocheta*. 1: linalool, 2: methyl salicylate, 3: Z-jasmone, 4: ß-caryophyllene, 5: germacrene D, 6: E,E-α-farnesene.

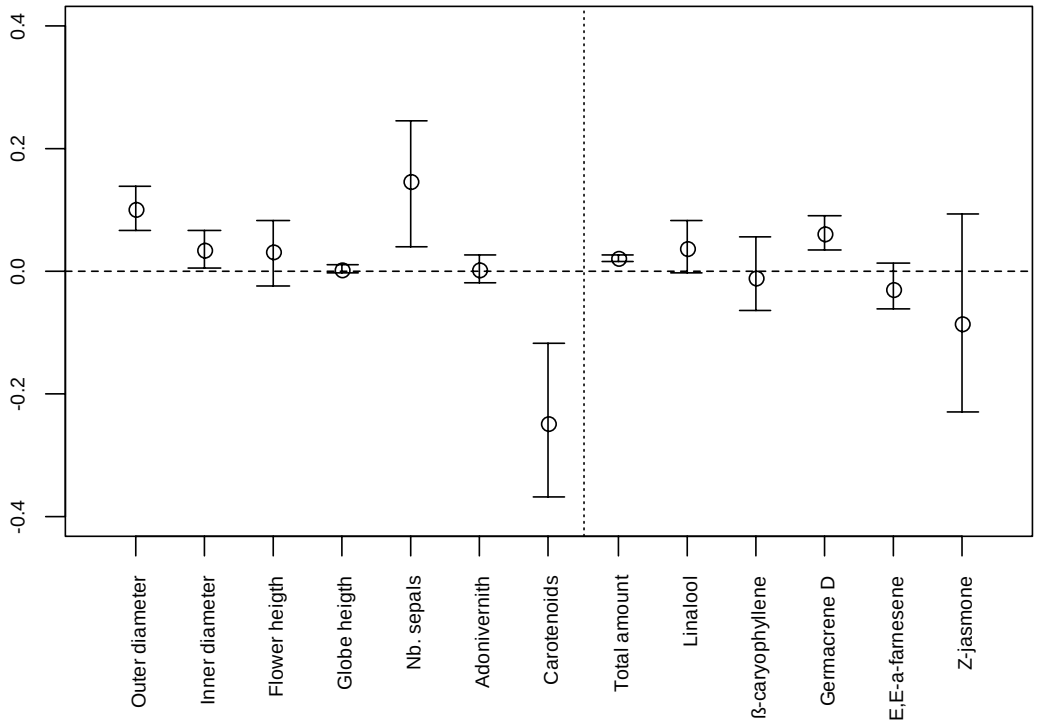

Figure 2:

95% bootstrap confidence intervals and estimated coefficients of the PLS regression for patch B. The results for patch A are not shown because the PLS regression has a very poor explicative power in this patch (see Table 5). The confidence interval and coefficient for methyl salicylate is not shown because this compound is present in only 12% of the individuals of pop. B, which makes the confidence interval very large.

3. Rôle des composés volatils des étamines dans l'attraction des chiastochètes.

Le bouquet de volatils physiologiquement actifs contenus dans les étamines est dominé par le E,E-α-farnesene et le linalol, tandis que le bouquet contenu dans les sépales est dominé par le germacrene D et le β-caryophyllene (Figure 14).

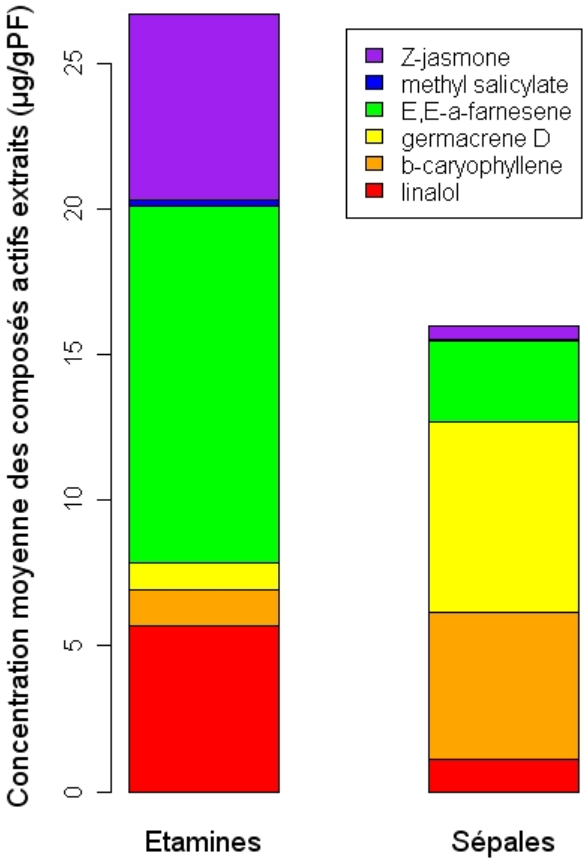

Figure 14. Concentration moyenne des composés physiologiquement actifs extraits dans l'hexane à partir des étamines (N=76) et des sépales (N=76), en µg/g de poids frais. Pour une description de la méthode, voir le manuscrit ci-dessus.

Afin de montrer le rôle du bouquet émis par les étamines dans l'attraction des chiastochètes, 10 amas ('patchs') de 10 fleurs encore en bouton ont été sélectionnés dans la population de Ruillas (proche du col du Lautaret). Toutes les fleurs ont été émasculées. Une semaine plus tard, alors que les fleurs étaient épanouies, des étamines fraîchement cueillies provenant de fleurs voisines ont été rajoutées dans 5 fleurs de chaque amas choisies au hasard. Une série d'observation a ensuite été conduite sur ces fleurs. 5 observateurs ont été répartis

aléatoirement sur les 10 amas pendant 4 sessions de 30 minutes. Chaque amas a alors été observé 2 fois 30 minutes, par deux observateurs différents. Les résultats obtenus sont représentés par la figure 15 ci-dessous.

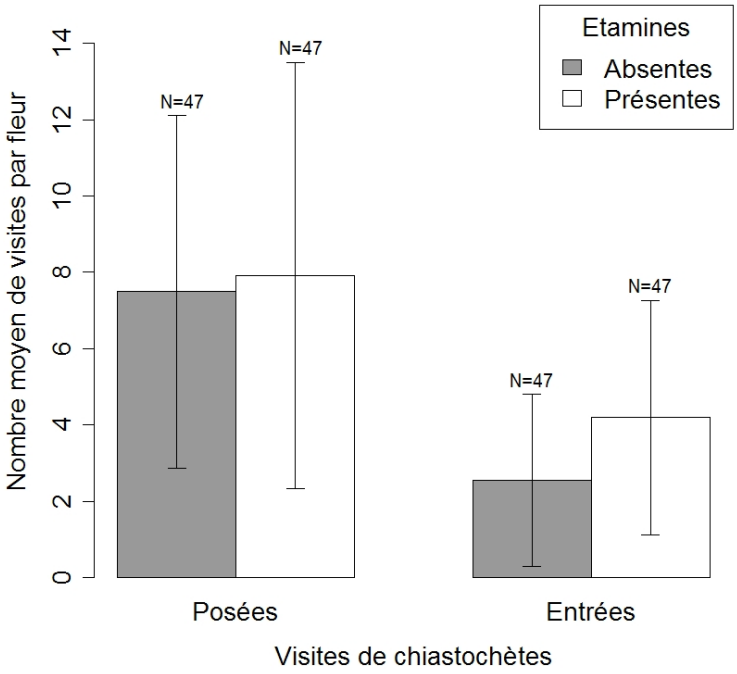

Figure 15. Effet de la présence d'étamines sur les visites de chiastochètes.

Le nombre de visites reçues suivant une loi de Poisson, un modèle linéaire généralisé a été appliqué pour ces données, en tenant compte d'un effet aléatoire de l'amas de fleurs. La fonction utilisée avec le logiciel R (package MASS) est « glmmPQL », c'est à dire « Generalized Linear Mixed Model, Penalized Quasi-Likelihood ». Ce modèle a été appliqué pour le nombre chiastochètes posées d'une part et entrées d'autre part, l'effet fixe restant la présence/absence d'étamines. Le tableau 4 ci-dessous présente les résultats pour les deux modèles.

Tableau 4. Effet de la présence d'étamines sur les visites de chiastochètes.

Modèle	Estimation	Erreur standard	ddl	t-value	p-value
Posées~Etamines ajoutées	0,055	0,110	1 ;83	0,502	0,617
Entrées~Etamines ajoutées	0,496	0,149	1 ;83	3,321	0,001

La présence d'étamines dans la fleur n'a pas d'effet significatif sur le nombre de chiastochètes qui viennent se poser sur la fleur, mais elle augmente significativement le nombre de chiastochètes qui pénètrent le globe et participent ensuite à la pollinisation. Etant donné que l'insecte ne peut voir les étamines lorsqu'il est posé sur la fleur, seuls les composés volatils émis par les étamines peuvent être responsables de leur plus forte attraction. Ces données confirment le rôle des composés volatils dans l'attraction des chiastochètes et montrent que ceux émis par les étamines jouent un rôle spécifique.

4. Les principaux résultats

- **Plusieurs composés volatils émis par la fleur de trolle déclenchent une réponse physiologique dans les antennes des chiastochètes : methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, linalool.**

- **Dans une population, la variabilité naturelle des composés volatils présents dans une fleur explique presque 50% de la variabilité naturelle des visites de chiastochètes, dans une autre elle en explique une part négligeable. La variabilité de la morphologie florale explique entre 10 et 30% de la variabilité des visites.**

- **Les composés volatils émis par les étamines jouent un rôle dans l'attraction à l'intérieur du globe des chiastochètes qui se posent sur la corolle.**

CHAPITRE 4 : Défense chimique contre les larves.

1. Introduction

Jusqu'à présent, nous nous sommes focalisés sur les traits floraux impliqués dans l'attraction des chiastochètes adultes, ce qui correspond à la partie mutualiste de l'interaction. L'existence d'intérêts contradictoires entre le trolle et les larves de chiastochètes à propos de la consommation de graines nous conduit à supposer l'existence chez le trolle d'un trait antagoniste capable de limiter la prédation. La déhiscence des carpelles permet de disperser les graines encore intactes et d'en soustraire une partie à l'appétit des larves (Jaeger et al., 2001). Mais quel mécanisme de défense pourrait être impliqué lorsque les graines encore immatures sont en développement à l'intérieur des carpelles ? Etant donné que l'utilisation de défenses chimiques est un mécanisme couramment utilisé par les plantes face à leurs ravageurs, notamment face aux prédateurs de graines, cette piste a été explorée. Avant mon arrivée au laboratoire, Christiane Gallet, Laurence Després et Lucie Zinger ont montré que la concentration en un composé secondaire de nature polyphénolique présent dans la paroi des carpelles était positivement corrélée au nombre de larves présentes dans le fruit ((Gallet et al., 2007), article en annexe n°2). Il s'agit de l'adonivernith, un glycoside de la lutéoline d'abord décrit chez *Adonis vernalis* (*Adonis* est le genre frère de *Trollius* , (Despres et al., 2003). Lors de mon stage de Master 2, j'ai montré qu'une cohorte de flavonoïdes était également corrélée au nombre de larves et que des dommages purement mécaniques ne suffisaient pas à induire ces composés : la réponse du trolle est donc probablement spécifique (Gallet et al., 2007). L'induction de flavonoïdes par les larves constitue-t-elle une défense chimique, les larves y sont-elles sensibles ? Quel est le mécanisme d'induction de l'adonivernith ? Existe-t-il une interaction entre le rayonnement UV et les larves sur l'induction ? C'est à ces questions que tente de répondre ce chapitre.

2. Manuscrit (soumis à BMC Evolutionary Biology)

Plant chemical defence: a partner control mechanism stabilising plant - seed-eating pollinator mutualisms.

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Abstract

Background

Mutualisms are inherently conflictual as one partner always benefits from reducing the costs imposed by the other. Despite the widespread recognition that mutualisms are essentially reciprocal exploitation, there are few documented examples of traits that limit the costs of mutualism. In plant/seed-eating pollinator interactions the only mechanisms reported so far are those specific to one particular system, such as the selective abortion of over-exploited fruits.

Results

This study shows that plant chemical defence against developing larvae constitutes another partner sanction mechanism in nursery mutualisms. It documents the chemical defence used by globeflower *Trollius europaeus* L. (Ranunculaceae) against the seed-eating larvae of six pollinating species of the genus *Chiastocheta* Pokorny (Anthomyiidae). The correlative field study carried out shows that the severity of damage caused by *Chiastocheta* larvae to globeflower fruit carpels is linked to the accumulation in the carpel walls of a C-glycosyl-flavone, adonivernith, which reduces the larval seed predation ability per damaged carpel. The different *Chiastocheta* species do not exploit the fruit in the same way and their interaction with the plant chemical defence is variable, both in terms of induction intensity and larval sensitivity to adonivernith.

Conclusions

Adonivernith accumulation and larval predation intensity appear to be both the reciprocal cause and effect. Adonivernith not only constitutes an effective chemical means of partner control, but may also play a role in the sympatric diversification of the *Chiastocheta* genus.

Background

Conflicts of interest are frequent in interspecific mutualisms (Herre et al., 1999, Bluthgen et al., 2007). In the case of plant/ seed-eating pollinator interactions, the conflict lies in the number of seeds eaten by the pollinator's larvae that therefore cannot contribute to the plant's fitness (Jaeger et al., 2001, Herre & West, 1997, Dufay & Anstett, 2003, Anstett et al., 1997). As a consequence, evolutionary theory has it that plants evolve traits that limit the costs imposed by the insect partners. Pellmyr & Huth (Pellmyr & Huth, 1994) showed that the selective abortion of fruits in the Yucca – Yucca moth interaction was an effective defence against the developing larvae, but this mechanism was found in only one of the three Yucca – Yucca moth systems studied by Adicott & Bao (Adicott & Bao, 1999). Selective abortion may not provide a general explanation for the stability of this mutualism (Shapiro & Adicott, 2004). Instead, density-dependent mortality in oviposition-induced 'damage zones', a characteristic specific to this system, may be a more important mechanism in terms of the regulation of the interaction (Shapiro & Adicott, 2003). Selective fruit abortion is also part of the Silene latifolia-Hadena bicruris interaction (Jolivet & Bernasconi, 2006, Burkhardt et al., 2009). Holland's investigation of the Senita cactus - Senita moth system (Holland et al., 2004) found no evidence of selective abortion but suggested that excess flower production followed by massive fruit abortion might actually increase a plant's male fitness, rather than serving to control seed predation by pollinator larvae. In the fig-fig wasp system it is theoretically possible that several mechanisms for reducing the plant's costs coexist (Yu et al., 2004). The geometry of the fig seems to play a crucial role in limiting the intensity of the damage inflicted by wasp larvae. Indeed, fig wasps preferentially oviposit in the inner ovules and avoid the outer ovules (Jousselin et al., 2001) presumably because the wasp larvae which develop in the outer ovules are more exposed to parasitoids that oviposit from outside the syconia than the larvae developing in the inner ovules (Dunn et al., 2008). The use by plants of chemicals to kill non-mutualistic pests or limit the damage they cause is a very common phenomenon (Fraenkel, 1959, Berenbaum & Zangerl, 2008) which may also play a role in mutualistic interactions. So far however, the importance of induced plant chemical defence in partner control has not been explored. Here we use the *Trollius europaeus* (L.) – *Chiastocheta* spp. (Pokorny) system and test whether plants can limit seed predation through chemical defence. The globeflower cannot respond to over-exploitation by *Chiastocheta* with the selective abortion of parasitized fruits, as it only produces one to three flowers per blooming, whereas yucca and senita cactus produce hundreds of flowers. Nor is the selective abortion of parasitized carpels an option because developing larvae move freely from one carpel to

another. In the Trollius – *Chiastocheta* interaction, it has recently been shown that the concentration of adonivernith, a luteolin based flavonoid (luteolin 8-β-D-glucopyranosyl-2"-O-D-xylopyranoside), in the carpel walls positively correlates to the number of developing larvae in the fruit (Gallet et al., 2007). As the protected carpel walls (with no larvae) contain significantly lower amounts of adonivernith than the parasitized fruits, it has been hypothesised that this compound is induced by larvae infestation and will act as a defence compound. Unfortunately, as *Chiastocheta* larvae cannot be reared on an artificial medium, this hypothesis could not be confirmed by means of in vitro toxicity experiments . However, other flavonoids have been identified as active inhibitors of larval growth on the larvae of the corn earworm (Heliothis zea, Elliger et al., 1980, the autumnal moth Epirrita autumnata, Ossipov et al., 2001, and the fall armyworm Spodoptera frugiperda Johnson et al., 2002, Urrea-Bulla et al., 2004). We hypothesise that adonivernith, the most abundant phenolic compound found in the carpel walls of *T. europaeus* , constitutes a chemical plant defence against *Chiastocheta* larvae by acting as a larval growth inhibitor. We predict that the accumulation of adonivernith in the carpel walls following larval damage will limit seed predation. Moreover, several species of *Chiastocheta* coexist in *T. europaeus* populations. They all feed on globeflower's seeds (which do not contain adonivernith, Gallet et al., 2007), but differ in terms of their exploitation pattern inside the fruit: it is possible that each species induces and reacts to adonivernith in a specific way.

To test these hypotheses, we carried out a field study on *T. europaeus* flowers in which we left only one egg of one of the different *Chiastocheta* species present (Figure 1). We dissected the fruit after full larval development and measured the mass of the larva, the number of damaged carpels, and the number of seeds eaten, we also estimated the fruit's seed/ovule ratio and the concentration of adonivernith in the carpel walls. During the analysis we tried to understand the interplay between adonivernith induction, adonivernith effect and seed predation by larvae.

Results

Fruit traits and the intensity of larval damage

The variation in adonivernith concentration between individual plants was wide enough to carry out the statistical analysis (range 0.12-1.01 mg/g, mean 0.48 mg/g, coefficient of variation 0.32). Adonivernith concentration positively correlated with the number of damaged

carpels when considering all species together (Linear Model LM, $t_{1,152}$=2.75, p=0.007, Table 1 & Figure 2). Although not significant due to the small sample size and high variability, the correlation was also positive when the species were analysed separately, except for *C. setifera/trollii* (Table 1). The seed/ovule ratio was not dependent on the number of damaged carpels (Generalised Linear Model GLM, binomial family, $t_{1,152}$=0.107, p=0.91).

Larval traits and adonivernith concentration

There was no link between larval mass and adonivernith concentration in the carpel walls when the species were pooled (ANOVA, $F_{1,137}$=0.0044, p=0.94) nor when they were analysed separately (not shown, p>0.12 in all cases). Similarly, there was no link between the total number of seeds eaten per larva and adonivernith concentration when the species were pooled (ANOVA, $F_{1,152}$=0.0018, p=0.96) nor when they were analysed separately (not shown, p>0.51 in all cases). However, when the number of seeds eaten per damaged carpel was considered, adonivernith had a negative effect (LM, $t_{1,151}$=-4.44, p<1E-4, Table 2 & Figure 3), and the seed/ovule ratio a positive effect on seed predation (LM, $t_{1,151}$=5.58, p<1E-6, Table 2) when the species were pooled. The R^2 of the corresponding multivariate linear model was 0.25 (when the species were treated separately, the R^2 were between 0.11 and 0.40, Table 2). Larval mass positively correlated with the number of seeds eaten per larva when all the species were pooled ($t_{1,137}$=6, p<1E-7, Table 3). When the species were analysed separately, the link was significant for *C. rotudinventris, C. inermella* and *C. dentifera* (Table 3).

Differences between Chiastocheta species

The adonivernith concentrations differed between fruits infested by different species. Fruits infested by *C. rotudiventris* and *C. macropyga* larvae had higher concentrations than those infected by *C. setifera/trollii* and *C. dentifera* larvae (ANOVA, Figure 4.a). The number of damaged carpels differed between species, *C. rotudiventris* damaged the most carpels, closely followed by *C. macropyga* and *C. setifera/trollii*. *C. inermell*a damaged around 3 carpels whereas *C. dentifera* damaged no more than two carpels (Figure 4.b). Larval mass differed between species; *C. dentifera* was the smallest species (Figure 4.c). The total number of seeds eaten per larva varied across species: *C. macropyga* and *C. setifera/trollii* ate more seeds than the others, followed by *C. rotudiventris* and *C. inermella*, and then *C. dentifera* (Figure 4.d). The seed/ovule ratio differed between species: *C. macropyga* and *C. setifera/trollii* had the highest ratio and *C. rotudiventris* the lowest (Figure 4.e). *C. dentifera* ate the most seeds per damaged carpel, and *C. rotudiventris* the least (Figure 4.f).

Discussion

Advantages and disadvantages of a correlative study

In a previous study, Gallet et al. (Gallet et al., 2007) showed that the amount of adonivernith in the carpel walls positively correlates to the number of larvae in the fruit. Here we only consider fruits infested by a single larva and show that 1) adonivernith concentration is dependent on the amount of damage caused by the larva and 2) the number of seeds eaten per damaged carpel decreases as adonivernith concentration increases. *Chiastocheta* larvae are specific to Trollius fruits, and cannot be reared on artificial medium under controlled laboratory conditions. Therefore we could not directly carry out bioassays to show that the cause of the reduced seed consumption is indeed adonivernith. Other correlated factors may be involved in the plant's response to larval damage and in its toxicity against larvae. For example, Gallet et al. (Gallet et al., 2007) showed that other undetermined phenolic compounds respond to increasing numbers of larvae, although the response is more marked with adonivernith. The chemical defence probably involves several compounds and possible synergistic effects: some may be precursors or the degraded compounds of others, and some may be more toxic to the larvae than others. Only bioassays performed in controlled conditions can link a cause (adonivernith concentration) to an effect (larval mass), but the correlative field study has the advantage of showing that the phenomenon is indeed at work in nature (Roush, 1995). The huge variability of flavonoids in the natural environment (Solar et al., 2005, Spitaler et al., 2008, Witzell et al., 2003), coupled with the wide range of factors that may influence their production and accumulation means an in vitro experiment would be entirely disconnected from nature and is unrealistic. Seed-eating pollinator mutualisms are complex systems to which observational studies or semi-experimental field studies are better adapted (Pellmyr & Huth, 1994, Jolivet & Bernasconi, 2006, Dunn et al., 2008).

Disentangling cause and effect.

Another advantage of the correlative approach is that it makes it possible to disentangle two processes which come into play simultaneously: the induction of plant defence (in response to carpel damage inflicted by larval predation) and the consequences of defence induction (on larval predation). The plant defence and larval predation are both the cause and effect (Levins & Lewontin, 1985). This explains why no direct link was found between adonivernith concentration and the total number of seeds eaten, nor between adonivernith concentration and larval mass: more seeds eaten means more carpel damage and therefore more

adonivernith induction, but at the same time more adonivernith induction means less seeds eaten. Instead, adonivernith induction can be explored by looking at the link between adonivernith concentration and the number of damaged carpels, and toxicity can be measured in terms of the link between the number of seeds eaten per damaged carpel and adonivernith concentration. Plant reactions to the damage vary between individuals, leading to variations in adonivernith concentrations in fruits with the same amount of damage. Thanks to this natural variability of plant defences, we were able to show that in the most reactive fruits, the larvae ate less seeds per damaged carpel. The variability of plant defences can have a genetic (e.g. Glynn et al., 2004) or an environmental (e.g. Miller & Woodrow, 2008) basis.

Origin of the chemical defence

Adonivernith is abundant in almost all parts of the globeflower, especially in the leaves and sepals. It is probably involved in the defence against herbivores and florivores, as well as in the resistance to ultra-violet radiation. Ultra-violet radiation has been shown to induce adonivernith production in globeflowers (S. Ibanez, unpublished results), and globeflower populations located at high altitudes contain higher concentrations of adonivernith in their carpel walls (Gallet et al., 2007). Adonivernith was first described in the genus Adonis (Harborne & Baxter, 1999), the sister genus of Trollius (Despres et al., 2003). It is also present in other Trollius species (Gallet et al., 2007), which suggests that it was already present in Trollius and Adonis's common ancestor. The chemical defence used by *T. europaeus* against *Chiastocheta* larvae is probably an exaptation. However, the accumulation of adonivernith in carpel walls is not induced by mechanical damage and appears to be specifically induced by *Chiastocheta* larvae (Gallet et al., 2007).

The ecological and evolutionary stability of the interaction

When several larvae are allowed to develop in a single fruit, each larva is exposed to increasing amounts of adonivernith as the number of larvae developing in the fruit increases (Gallet et al., 2007). The mechanism is therefore density-dependant: the higher the population density of *Chiastocheta*, the more it suffers from chemical defence. The density-dependant mechanism is also found in yuccas (Shapiro & Adicott, 2003). In two models exploring the evolutionary emergence of fruit abortion in yucca and senita cactus (Holland & DeAngelis, 2002, Holland et al., 2004), Holland et al. show that density-dependant mechanisms which limit seed predation by moths can maintain the costs of seed predation at a lower level than the benefits of pollination thereby stabilising the interaction. Although adonivernith induction

by larvae is an evolutionary response on the individual plant level, it can lead to a limitation of *Chiastocheta* density on the population level as smaller larvae produce less fecund adult flies, and maintain the benefits of mutualism at this level. This ensures the ecological stability of the interaction in the sense that globeflower populations are more likely to persist. The modelling carried out by Ferdy et al. (Ferdy et al., 2002) showed that if the closure of the globeflower corolla led to an increase in intraspecific contest competition due to an increase in egg survival, then females would evolve a reduced clutch size per flower thus stabilising the interaction, but unpublished field data (L. Després) does not support the model hypothesis (i.e. higher egg survival in closed corolla). However, the chemical defence mechanism described here may play exactly the same role as globe closure in Ferdy et al's model if it indirectly increases intraspecific competition between larvae. The chemical defence would then lead to an evolutionary stabilisation of the mutualism. Finally, the larvae are likely to evolve a resistance to adonivernith. Preliminary results suggest that the activity of the detoxifying enzyme cytochrome P450 (frequently involved in insect resistance to plant chemicals, Despres et al., 2007) in *Chiastocheta* larvae is greater when they are exposed to adonivernith (L. Després, unpublished results). In any case the results of this study suggest that adonivernith is more likely to act as a growth inhibitor or a feeding deterrent rather than a lethal compound.

Plant defence and sympatric speciation in the Chiastocheta genus

Phylogenetical and biogeographical data indicates that the diversification of the *Chiastocheta* genus mostly occurred in sympatry (Despres et al., 2002). The dominance of intra- over inter-specific competition could have driven the radiation (Despres & Cherif, 2004) through resource partitioning in space (exploitation pattern, Pompanon et al., 2006) and time (oviposition time, Despres & Cherif, 2004). Both processes are affected by plant defence: exposure to adonivernith will depend on the exploitation pattern, and the larvae of late-ovipositing species will be exposed to higher concentrations resulting from the damage inflicted by early-ovipositing species. The accumulation of adonivernith in the carpel walls will depend on the exploitation pattern (the number of damaged carpels) and on oviposition timing. Interestingly, the larva of the late-ovipositing species *C. dentifera* only mines through a single carpel, thereby avoiding contact with the carpel walls containing adonivernith. In the present study, it is the species which least induces a response, and the least sensitive to adonivernith. Intra- and inter-specific competition may be direct in the form of larval contests and the data presented here suggests that it may also be indirect by means of adonivernith

induction. Adonivernith may have played a key role in the sympatric speciation of the *Chiastocheta* genus through the following three mechanisms: 1) by increasing competition between larvae; 2) by provoking a behavioural avoidance strategy in *C. dentifera*; and 3) by mobilising different capacities to metabolize this chemical compound. We have already shown that larval foraging behaviour varies across species and we predict that the larval capacity for resistance also varies across species.

Conclusions

Adonivernith induction reduces the costs of mutualism for the plant, which has a stabilising effect on the plant's specialisation in *Chiastocheta* flies. The interaction between the larvae and adonivernith varies between the six *Chiastocheta* species, which may have played a role in the sympatric speciation of the genus. The two processes are interlinked: adonivernith induction by larvae and adonivernith toxicity on larvae. Adonivernith accumulation and larval predation are both the reciprocal cause and effect.

Methods

Study species

The European globeflower *Trollius europaeus* L. (Ranunculaceae) is a hermaphroditic, homogamous, outcrossing, arctic-alpine perennial species growing in moist meadows. Each yellow flower is composed of around 10 tightly-closed sepals which form a globose corolla that contains approximately 10 nectariferous staminodias, 30 multiovulate carpels, and numerous stamens that sequentially dehisce throughout flower longevity (typically 5-9 days, Despres & Jaeger, 1999, Jaeger et al., 2000). In the Alps, the plant is passively pollinated by six species of *Chiastocheta* flies (Anthomyiidae): *C. rotundiventris* Hennig, *C. dentifera* Hennig, *C. inermella* Zetterstedt, *C. macropyga* Hennig, *C. setifera* Hennig, and *C. trollii* Zetterstedt. *Chiastocheta* flies are the only pollinators of *T. europaeus* and *Chiastocheta* larvae feed only on *T. europaeus* seeds (Pellmyr, 1989, Jaeger & Despres, 1998). The female deposits one or several eggs on, or between the carpels, at various flower stages depending on the species (Pellmyr, 1989, Despres & Jaeger, 1999). Shift in oviposition time among species ranges from 2 days to one week (Pompanon et al., 2006). Egg morphology, colour and position on the fruit make it possible to assign them to a species (Pellmyr, 1992). The early ovipositing fly species *C. rotundiventris* visits young, unpollinated flowers, and typically deposits just one egg per flower. The late ovipositing species *C. dentifera* lays several eggs on pollinated, fading flowers. After hatching, larvae develop on seeds throughout fruit

maturation (about 4 weeks). Larvae from each species have a specific location in the globeflower complex fruit, composed of several follicles (hereafter referred to as carpels, Figure 1). The larvae of the early ovipositing species *C. rotundiventris* are found in the floral receptacle. Each larva enters several carpels in succession through their bases and eats one to several seeds in each carpel. The larvae of the late ovipositing species *C. dentifera* are found in one single carpel and consume most of its seeds. The larvae of the intermediate species forage their way through several carpels (Figure 1). At the end of their development, the larvae exit the fruit and drop into the soil to overwinter as pupae.

Adonivernith is present in most parts of the globeflowers excluding the seeds and flower receptacle (Gallet et al., 2007). The concentrations reach levels as high as 3.9 mg of luteolin eq. g-1 in the sepals. In the carpel walls levels range between 0.1 and 1 mg of luteolin eq. g-1 depending on the individuals and the populations (Gallet et al., 2007). *Chiastocheta* larvae feed on the seeds, but they have to mine through the carpel wall each time they enter or leave a carpel. We do not know whether the larvae consume the carpel wall or destroy it without swallowing it. Either way, they come into contact with adonivernith either by ingestion or diffusion through the cuticle.

Field study design

We conducted the field study around the "Station Alpine Joseph Fourier UMS 2925", col du Lautaret, France, in a single large population "Ruillas", 2025 m a.s.l in June -July 2007. A sample of 289 flowers was chosen randomly and left untouched until naturally pollinated. We then removed the eggs from each flower and waited one day for a new set of ovipositing females to lay their eggs. At the end of the day, we inspected the flowers and removed all the newly-laid eggs but one. The flower was then covered with a nylon bag to prevent further oviposition. If no eggs were found, we repeated the same procedure the following day. We recorded the day each flower was bagged (ranging from June 8th to 19th) and collected them 28 days later.

Back in the laboratory, the fruits were stored at 4°C for a maximum duration of 24h before dissection. For each fruit, all carpels were checked for damage, and the number of damaged carpels recorded. Five intact carpels were chosen at random and dissected, and the ratio of the number of developing seeds to the number of ovules (developing and degenerating) per carpel was determined. All damaged carpels were dissected in order to estimate the number of developing seeds that had been eaten (Jaeger et al., 2001). The larvae were located either in the damaged carpels, or in the flower receptacle (in the case of *C. rotundiventris*), and

weighed. The position and the trajectory of each larva inside the fruit (Figure 1) were used to assign it to one of the five following species: *C. rotundiventris, C. macropyga, C. inermella, C. setifera or C. trollii* (recorded as *C. setifera/trollii*, as these two species cannot be distinguished at the larval stage, Despres & Jaeger, 1999) and *C. dentifera*. If the fruit happened to contain no larvae, or more than one larva, it was excluded from the analysis. The carpel walls of five damaged carpels and the five intact carpels used for pollination analysis were pooled for the chemical analysis as preliminary results had shown that adonivernith concentration in intact carpels as opposed to damaged carpels was not significantly different ($F_{1,29}=2.039$, $p=0.164$). If the larva had damaged less than five carpels, the intact carpels were chosen at random and dissected so that all chemical analyses were carried out on ten carpel walls. Of the 289 flowers included in the first stage of the design, 154 were used for the statistical analysis. Missing samples were either lost in the field, contained no, or more than one larva (some hidden eggs might have been missed), or had been consumed by herbivores such as bush crickets (Tettigoniidae species) despite the protection offered by the nylon bag. *C. rotundiventri*s developed in 24 of the 154 fruits, *C. macropyga* in 25, *C. inermella* in 22, *C. setifera/trollii* in 34 and *C. dentifera* in 49.

Chemical analysis

All samples were individually stored at –18 ° C until analysis. This individual storage and the very small size of some of the samples meant dry weight could not be measured: all the results were given as fresh weight (FW). Each sample was weighed and extracted using 50 ml of an ethanol-water (50/50) mixture under reflux (Gallet et al., 2007). Aliquots (20 µl) of the ethanolic solution were used for HPLC analysis on a RP C18 µBondapak column, 4.6mm x 250 mm, monitored using a Waters 600 Controller. Spectra were recorded on a Waters 996 PDA. Solvent A was acetic acid 0.5 % in distilled water and solvent B acetic acid 0.5 % in acetonitrile. Adonivernith was separated with an isocratic flow (1.5 ml min-1) of 20 % of B in A and its area was recorded at 354 nm. Concentration was expressed in luteolin equivalent, based on a calibration curve established with pure luteolin (obtained from Extrasynthese, Lyon, France).

Data analysis

All statistical analyses were carried out using the software R 2.6.0 (R Development Core Team 2007). We carried out ANOVAs, univariate and bivariate linear regressions using the R function "lm" in the "stats" package. We produced generalised linear models (binomial

family) using the R function "glm" in the "MASS" package. The datasets corresponding to the five taxonomical subdivisions of the *Chiastocheta* genus we used were either analysed all together in order to draw conclusions at the genus level; or analysed separately in order to explore the differences between species.

Authors' contributions

SI, CG and LD participated in the conception and design of the study. SI and FD carried out the field study and the statistical analysis. CG and SI carried out the chemical analysis. SI, CG and LD drafted the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

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Figures

Figure 1 - Exploitation patterns

Fruit architecture and the exploitation pattern of a single larva for each species studied.

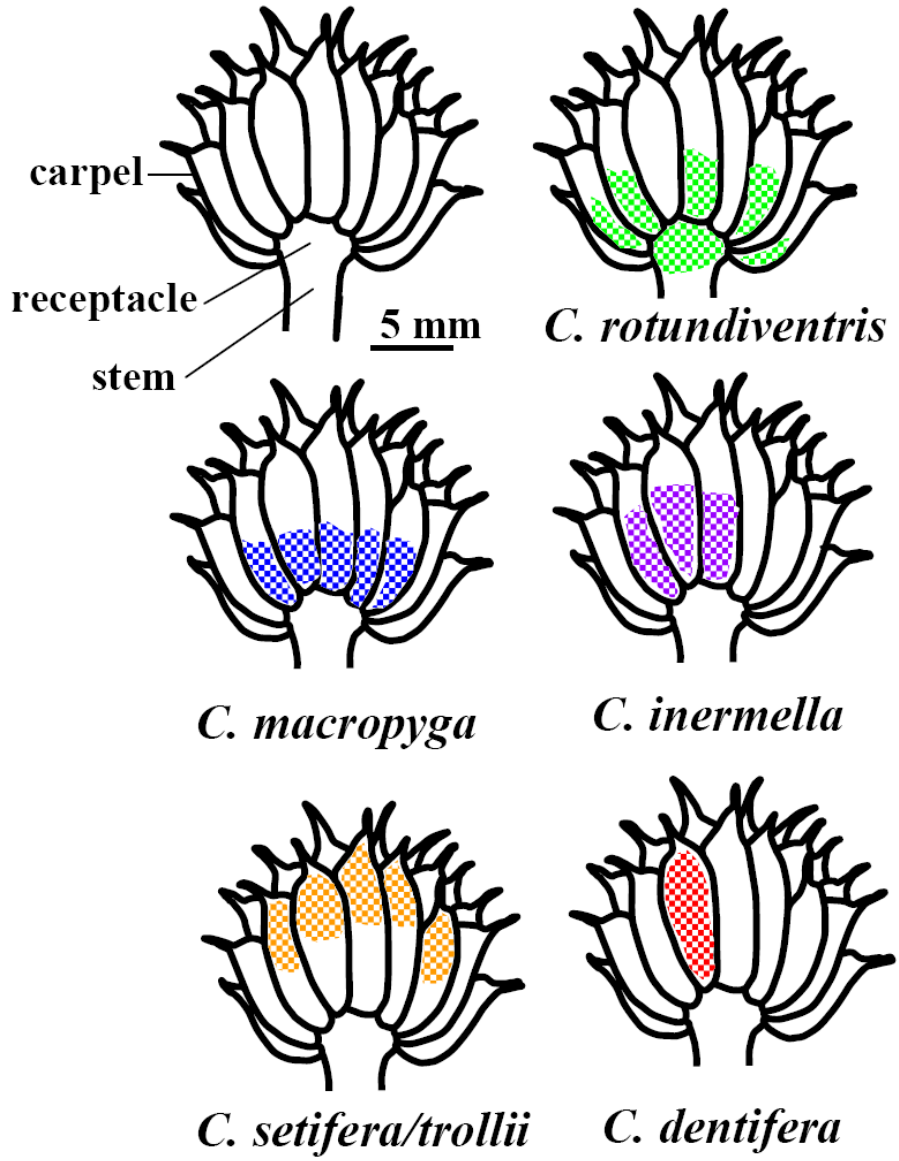


Figure 2 - Adonivernith induction

Adonivernith concentration in the carpel walls according to the number of damaged carpels. Green: *C. rotundiventris*, blue: *C. macropyga*, purple: *C. inermella*, orange: *C. setifera/trollii*, red: *C. dentifera*. See Table 2 for the statistical significance of the relationship.

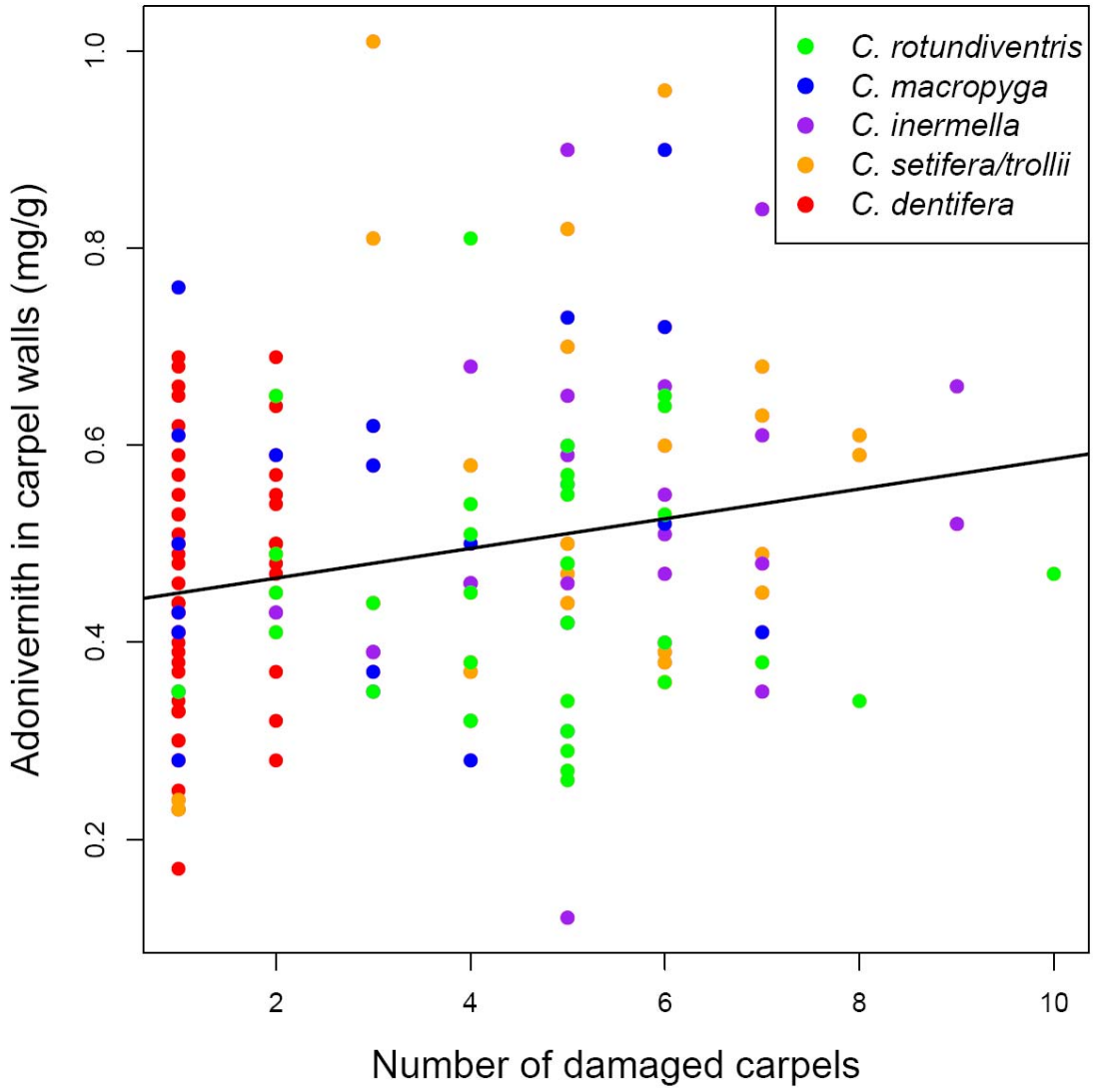


Figure 3 - Adonivernith effect

Number of seeds eaten per damaged carpel according to the adonivernith concentration in the carpel walls. Green: *C. rotundiventris*, blue: *C. macropyga*, purple: *C. inermella*, orange: *C. setifera/trollii*, red: *C. dentifera*. See Table 3 for the statistical significance of the relationship.

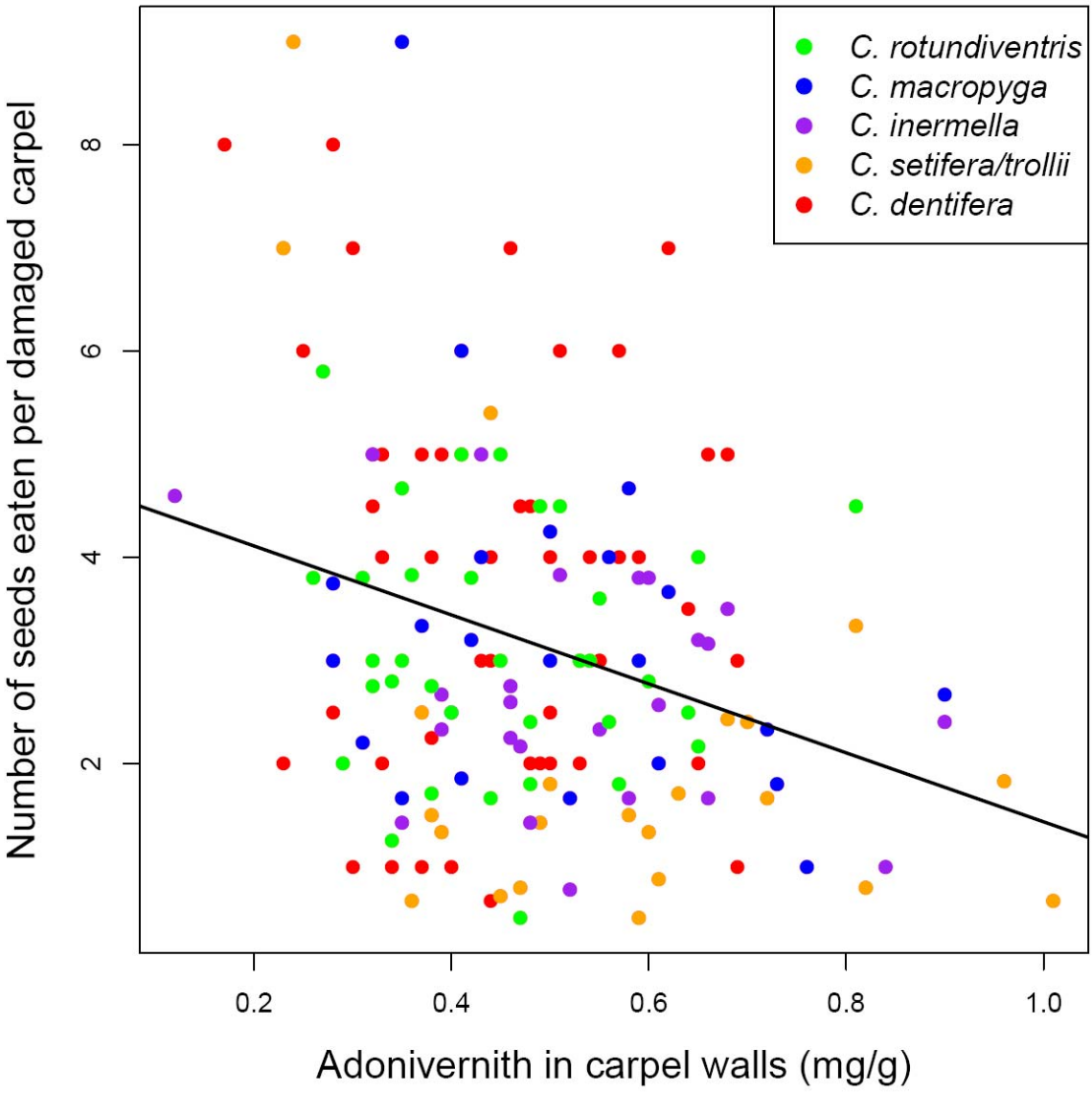
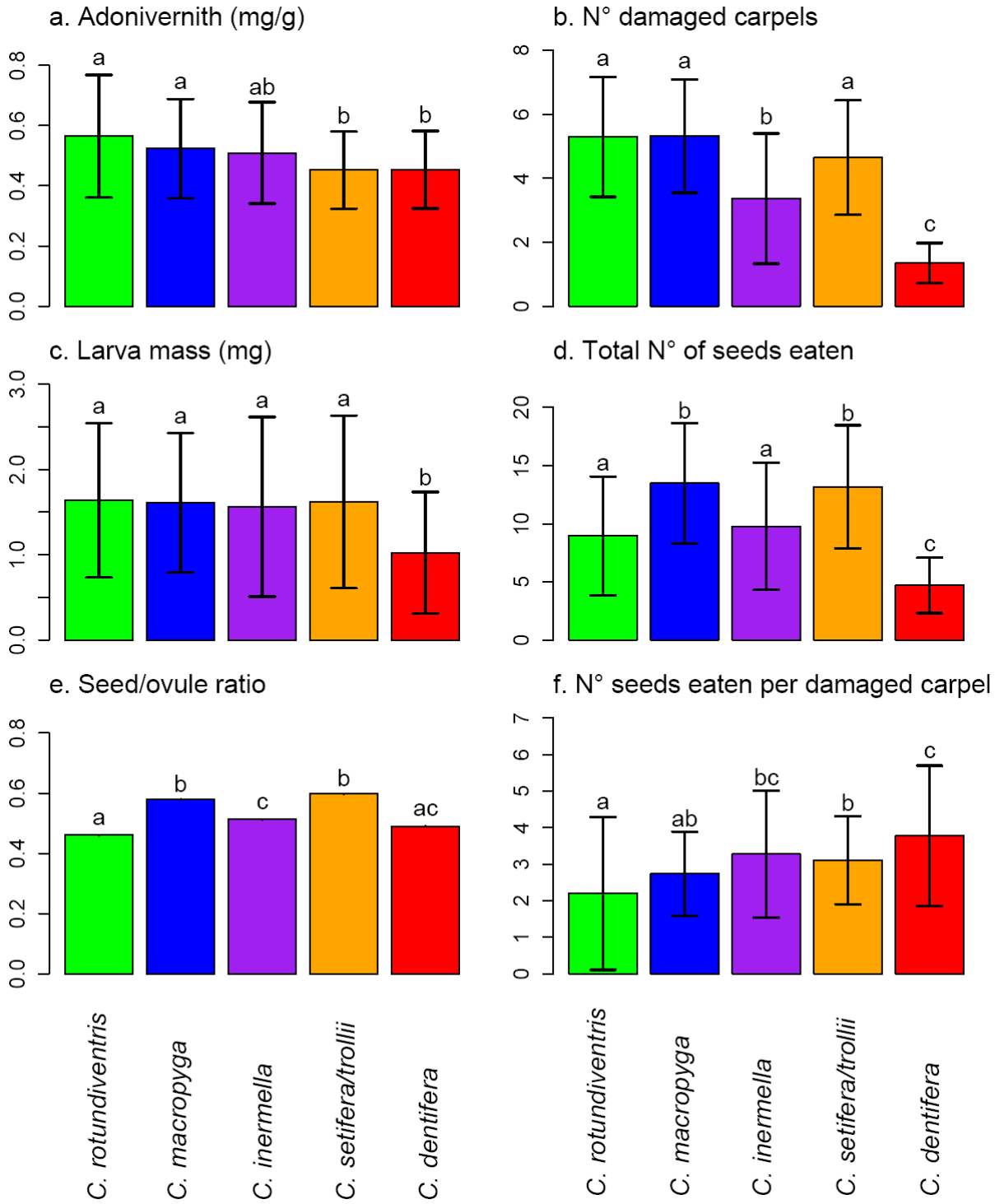


Figure 4 - Differences between fruits infested by different Chiastocheta species

Mean (bar) and standard deviation (bracket) for each group of fruits infested by the different *Chiastocheta* species of: a. adonivernith concentration in carpel walls in mg/g, b. number of damaged carpels by a single larva, c. larval mass in mg, d. total number of seeds eaten per larva, e. seed/ovule ratio and f. number of seeds eaten per damaged carpel.



Tables

Table 1 - Adonivernith induction

Adonivernith concentration in carpel walls in relation to the number of damaged carpels (univariate linear model).

	Regression coefficient	t	p-value	Residuals d.f.	R^2
All species	0.02	2.75	0.007	152	0.05
C. rotudiventris	0.02	0.87	0.39	22	0.03
C. macropyga	0.02	1.24	0.23	23	0.06
C. inermella	0.02	0.98	0.34	20	0.05
C. setifera/trollii	-0.002	-0.13	0.90	32	0.001
C. dentifera	0.01	0.40	0.69	47	0.003

Table 2 - Seed predation

Number of seeds eaten per damaged carpel in relation to adonivernith concentration in the carpel walls and the developing seed/ovule ratio (multivariate linear model).

	Adonivernith effect			Seed/ovule ratio effect			Model Residuals	
	Regression coefficient	t	p-value	Regression coefficient	T	p-value	d.f.	R^2
All species	-3.46	-4.44	<1E-4	3.51	5.58	<1E-6	151	0.25
C. rotudiventris	-4.27	-2.22	0.04	2.73	1.43	0.17	21	0.27
C. macropyga	-2.42	-1.69	0.10	0.52	0.40	0.69	22	0.12
C. inermella	-4.66	-2.47	0.02	5.51	3.02	0.01	19	0.40
C. setifera/trollii	-0.71	-0.44	0.66	2.57	1.92	0.06	31	0.11
C. dentifera	-2.05	-1.20	0.24	5.41	5.40	<1E-5	46	0.40

Table 3 - Larval growth

Larval mass in relation to the number of seeds eaten (univariate linear model).

	Regression coefficient	t	p-value	Residuals d.f.	R^2
All species	0.07	6.00	<1E-7	137	0.21
C. rotudiventris	0.12	4.28	<1E-3	22	0.45
C. macropyga	-0.001	-0.03	0.98	20	<1E-4
C. inermella	0.12	3.43	0.003	19	0.38
C. setifera/trollii	0.05	1.26	0.22	28	0.05
C. dentifera	0.19	4.44	<1E-4	40	0.33

3. Effet du rayonnement Ultra-Violet sur l'accumulation d'adonivernith dans la paroi des carpelles.

Dans beaucoup de cas, une augmentation du rayonnement UV conduit à une plus forte accumulation de composés secondaires dans les organes aériens des plantes, en particulier des flavonoïdes (Reinfenrath, 2007) qui protègeraient la plante du rayonnement UV (Harborne & Williams, 2000). L'influence des conditions environnementales sur les composés secondaires des plantes peut modifier leurs capacités de défense (Roberts & Paul, 2006). Si le rayonnement UV favorise l'accumulation d'adonivernith dans la paroi des carpelles, il est alors possible que le rayonnement UV interagisse avec la présence de larves dans le fruit sur l'accumulation d'adonivernith. La figure 16 ci-dessous est tirée de l'article en annexe 2 :

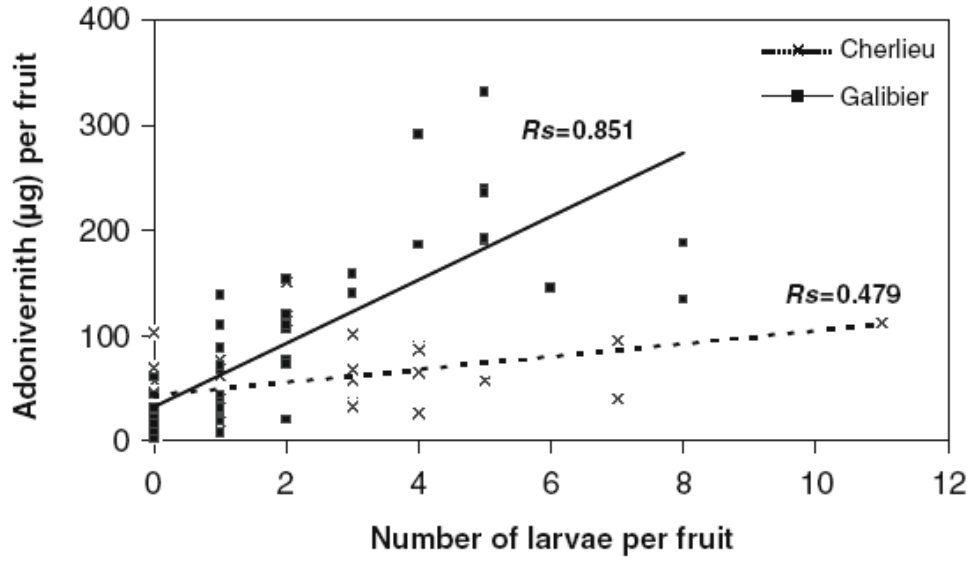


Figure 16. Corrélation entre le nombre de larves par fruit et la concentration en adonivernith dans le fruit.

La concentration en adonivernith dans le fruit est positivement corrélée au nombre de larves ($P<0.001$). L'intensité de l'induction varie également entre les populations de Cherlieu (Chartreuse, 980m.) et du Galibier (2400m.) : l'interaction entre les deux facteurs est significative ($P<0.001$, résultats présentés dans l'article en annexe 2). J'ai émis l'hypothèse que cette interaction est due au rayonnement UV : à plus forte altitude, les trolles sont dans un état de stress physiologique dû aux UV qui accentue leur réponse à la présence de larves.

Pour tester cette hypothèse, j'ai mis en place l'expérience suivante. Des fleurs de trolles provenant d'individus voisins groupés en petits amas étaient sélectionnées au début de leur floraison. Pour la moitié des fleurs de chaque amas, les œufs déjà pondus étaient retirés ; et les fleurs étaient ensachées pour éviter les ovipositions ultérieures. Des plaques de PVC qui absorbent dans le rayonnement UV étaient alors placées au-dessus de la moitié des amas sélectionnés (voir la photo ci-dessous). Les deux facteurs « œuf » et « UV » étaient donc croisés, avec un effet aléatoire de l'amas (split-splot design). Ce dispositif expérimental (figure 17) a été mis en place dans deux populations du versant nord du Col du Galibier, à Pré Gelé (2374m.) et dans une tourbière sous le col du Galibier nommée « Galibier » (2459m.).

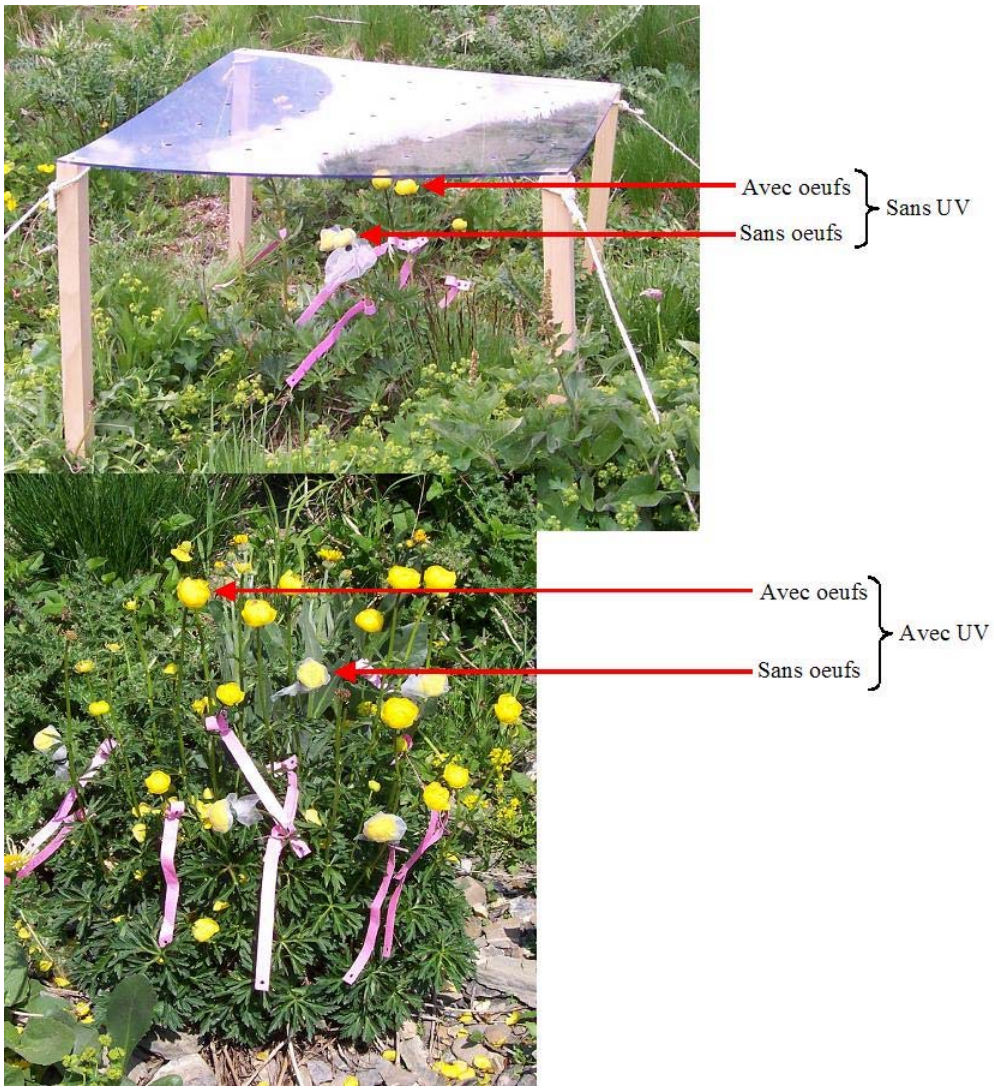


Figure 17. Photos du dispositif expérimental.

Une fois les fleurs fanées, les sachets de nylon été retirés, ainsi que les sépales fanés. Les fruits étaient récoltés après deux semaines de maturation et de développement des larves, et la concentration en adonivernith dans la paroi des carpelles était dosée par HPLC avec

la même méthode que celle décrite plus haut. Les résultats obtenus sont présentés dans la figure 18 ci-dessous.

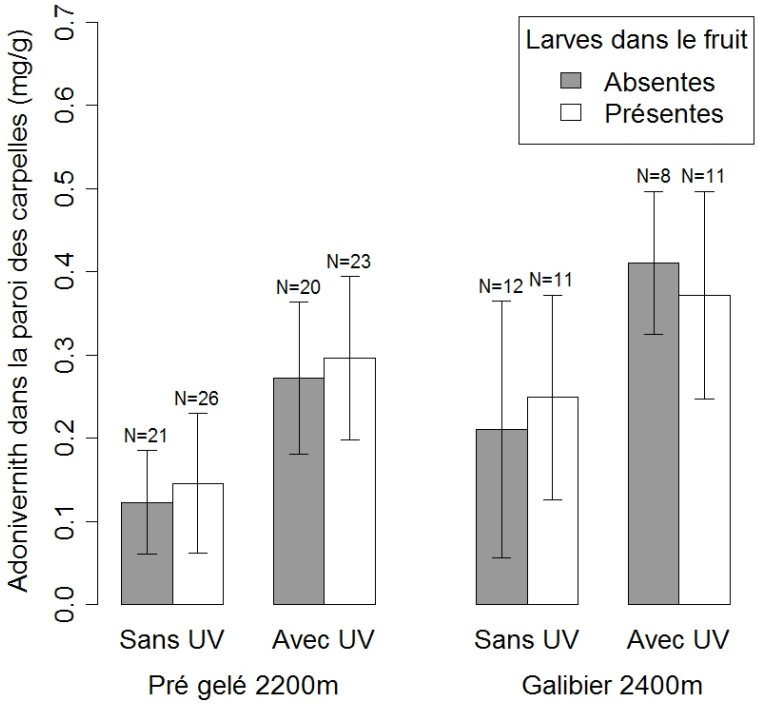


Figure 18. Concentration en adonivernith dans la paroi des carpelles en fonction du rayonnement UV, de la population et de la présence de larves.

Le tableau 5 ci-dessous présente les résultats statistiques d'un modèle mixte (fonction « lme » du package « nlme » du logiciel R) qui intègre dans les effets fixes le rayonnement UV, la population, la présence de larves et l'interaction UV*larves sur la concentration en adonivernith, et en effet aléatoire les amas de fleurs.

Tableau 5. Effet du rayonnement UV, de la population, de la présence de larves et de l'interaction entre les UV et les larves sur l'induction d'adonivernith.

	Estimation	Erreur standard	ddl	t-value	p-value
Rayonnement UV (absence de filtre)	0,166	0,033	1;12	4,995	0,0003
Population (Galibier)	0,097	0,030	1;12	3,200	0,008
Présence de larves (pas d'ensachage)	0,024	0,022	1;115	1,104	0,272
Interaction UV*larves	-0,018	0,032	1;115	-0,578	0,564

Il existe un net effet du rayonnement UV et de la population. La présence de larves n'a pas d'effet significatif, de même que l'interaction UV*larves. L'effet positif du rayonnement UV sur l'accumulation d'adonivernith confirme les nombreuses données à ce sujet dans la littérature. Les concentrations en adonivernith dans la population du Galibier reflètent peut être l'effet de l'altitude et du rayonnement UV plus intense. Sachant la différence d'altitude entre les deux sites est d'environ 200 mètres, d'autres facteurs peuvent être en jeu (effet du sol, différentiation génétique). La présence de larves dans le fruit n'a pas d'effet sur la concentration en adonivernith, ce qui est en totale contradiction avec l'article de l'annexe 2 ainsi qu'avec le manuscrit présenté plus haut. Nous n'avons jusqu'à présent pas d'explication. Par conséquent, l'interaction UV*larves est également non-significative, ce qui invalide l'hypothèse à l'origine de cette expérience. Ces données n'ont pas fait l'objet d'une publication.

4. Mécanisme de l'induction de l'adonivernith par les larves.

L'article présenté en annexe 2 (Gallet et al., 2007) s'intéresse également au mécanisme d'induction. Dans la population de Cherlieu, de légères blessures ont été infligées à l'aide d'une aiguille (10 trous) soit aux carpelles (figure 19, en noir), soit à la tige (en gris). Aucune différence significative n'existe entre les traitements. Au Galibier, des blessures plus sévères ont été infligées avec un scalpel, sans non plus conduire à des différences significatives.

Fig. 4 Amounts (mean + SE) of adonivernith in carpel walls of *T. europaeus* mechanically damaged. (**a**) In Cherlieu, wounds (10 holes) were inflicted with a needle (= low wound) on the carpel (■ black, *N*=19) or on the stem (■ gray, *N*=21) and compared to control plants (□ white, *N*=19). (**b**) In Galibier, wounds (five incisions) were inflicted with a scalpel (= severe wound) on the carpel (■ black, *N*=21) and compared to control plants (□ white, *N*=25). Adonivernith amounts were expressed in micrograms of luteolin equivalent. No statistically significant differences were observed

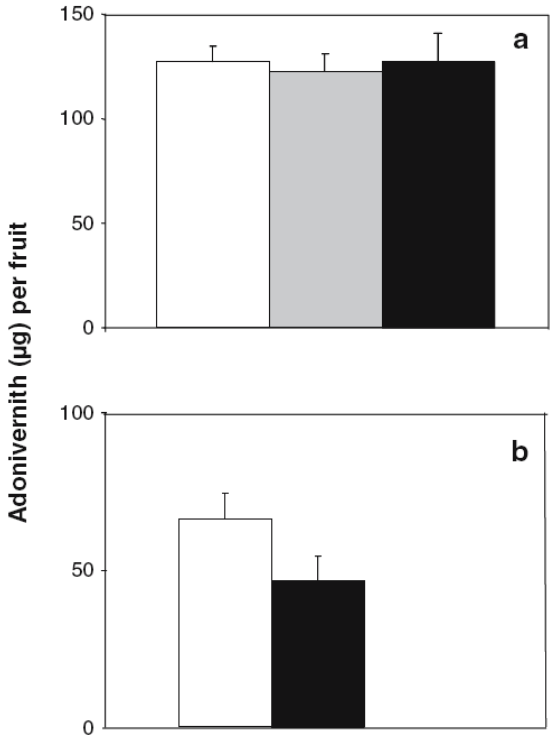


Figure 19. Quantité d'adonivernith dans le fruit en fonction des blessures.

Nous avons alors pensé que la salive des larves de chiastochètes induisait spécifiquement l'accumulation d'adonivernith dans la paroi des carpelles. Pour tester cette hypothèse, une expérience similaire à celle présentée dans le panneau b de la figure 18 a été conduite.
Un traitement supplémentaire a été imposé à une partie des fruits manipulés, il consiste à blesser les carpelles à l'aide d'un scalpel puis d'appliquer sur les blessures un broyat de larves à l'aide d'un pinceau. Des larves entières ont été utilisées pour préparer le broyat car leur petite taille rend très difficile la dissection des glandes salivaires. La figure 20 ci-dessous présente les résultats.

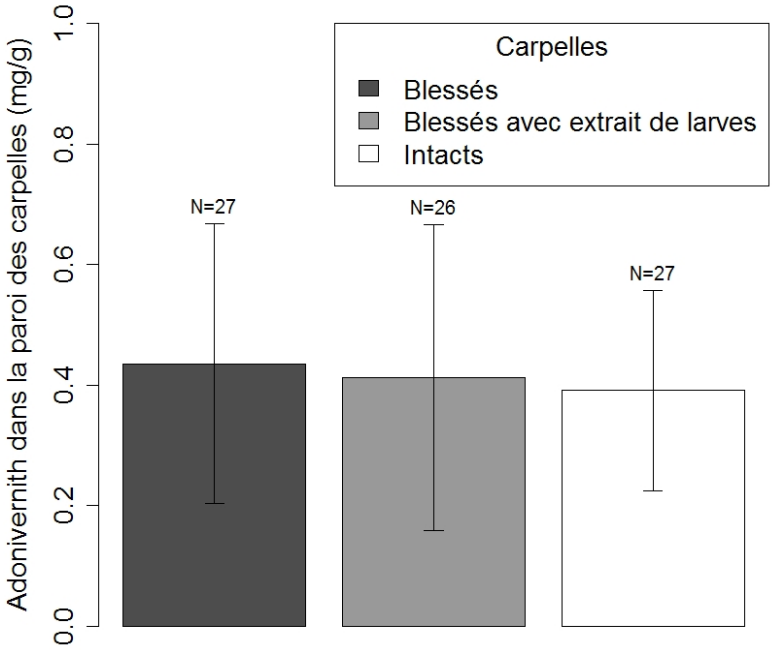


Figure 20. Concentration en adonivernith dans la paroi des carpelles en fonction du type de blessure.

Il n'existe aucune différence significative entre les traitements ($F_{2,77}$=0.277, P=0.758), l'hypothèse à l'origine de cette expérience ne peut donc être validée. Il est cependant possible que la salive larvaire soit effectivement capable d'induire l'accumulation d'adonivernith, mais que le dispositif expérimental conduit ici ne permette pas de reproduire cet effet.

5. Les principaux résultats

- **La concentration en adonivernith dans la paroi des carpelles est positivement corrélée au nombre de carpelles endommagés par la larve qui se développe dans le fruit.**

- **Pour un nombre de carpelles endommagés donné, lorsqu'il y a plus d'adonivernith dans la paroi des carpelles, la larve consomme moins de graines, ce qui diminue son poids.**

- **Les 6 espèces de chiastochètes induisent et sont affectées de différentes manières par l'adonivernith.**

- **Le rayonnement ultraviolet induit également l'accumulation d'adonivernith dans la paroi des carpelles.**

- **Des blessures mécaniques ne suffisent pas à induire l'adonivernith.**

CONCLUSION : Contradictions des relations interspécifiques.

Dans la conclusion de ce travail de thèse, je vais revenir sur deux questions soulevées dans l'introduction :

- la question du « bénéfice » mutualiste et du niveau d'organisation, population ou individu, adéquat pour le mesurer.
- la question des intérêts opposés qui peuvent exister entre partenaires mutualistes.

Il en découlera ensuite une discussion sur la nature contradictoire des relations interspécifiques et sur le rôle moteur que peuvent jouer les contradictions dans la coévolution.

1. Bénéfice mutualiste et niveaux d'organisation.

Définir le bénéfice mutualiste à l'échelle de l'individu revient à comparer la fitness d'individus qui interagissent avec le partenaire mutualiste à celle d'individus seuls. Dans le chapitre 1, nous avons choisi cette approche. Les trolles ouverts interagissent peu avec les chiastochètes, tandis que l'interaction est forte pour les trolles fermés. Nous avons alors comparé les fitness femelle et mâle de ces deux phénotypes.
Définir le mutualisme à l'échelle de la population revient à étudier l'effet du partenaire mutualiste sur le taux d'accroissement de la population. Cette échelle est pertinente lorsque l'on étudie la dynamique d'une communauté. Par exemple, dans un réseau d'interaction entre une communauté végétale et ses partenaires mycorhiziens, le bénéfice mutualiste reçu à l'échelle de la population par une espèce de plante est un paramètre qui entre en jeu dans la prédiction de son statut de dominance. Dans une logique de conservation, le niveau populationnel est également le plus pertinent. Finalement, quel niveau choisir pour mieux comprendre les interactions mutualistes, individu ou population ? Les résultats du chapitre 1 permettent d'y répondre.

Le résultat le plus intéressant de ce chapitre, à mon avis, est que la pression de sélection qui s'exerce contre le phénotype ouvert via la fitness femelle est très faible par rapport à celle qui

s'exerce via la fitness mâle. La fitness mâle est souvent négligée dans les travaux sur les interactions entre une plante et un pollinisateur prédateur de graines, par exemple lorsque (Holland, 2002) mesure les coûts et bénéfices du cactus senita (*Lophocereus schottii*) en interaction avec *Upiga virescens* (Pyralidae). Ce biais est peut être dû au fait que les coûts imposés par les insectes concernent seulement la fitness femelle, le bénéfice sur lequel on a alors tendance à se focaliser est celui qui vient contrebalancer la prédation, c'est-à-dire le pollen que reçoit (et non qu'exporte) la plante.

J'ai le sentiment que l'idée véhiculée dans la littérature sur les interactions mutualistes est que chaque partenaire fournit à l'autre un service vital, faute de quoi la survie de la population des partenaires est menacée. Cette idée se retrouve par exemple dans les modèles de dynamique des populations des systèmes mutualistes (voir par exemple ceux de (Morris et al., 2003), (Wilson et al., 2003), (Bronstein et al., 2003). En suivant cette idée, la spécialisation sur un partenaire donné est alors vue comme le choix du « meilleur » partenaire, celui qui va permettre la survie de l'espèce. Les résultats présentés ici montrent que chez le trolle d'Europe la spécialisation est avant tout une affaire de compétition intraspécifique entre gamètes mâles pour l'accès aux ovules : c'est le niveau individuel qui est le plus pertinent.

2. Intérêts opposés entre partenaires mutualistes.

Le dispositif expérimental du chapitre 1 a l'avantage de synthétiser les coûts et bénéfices de l'interaction pour le trolle d'Europe en ce qui concerne la fitness femelle : le résultat des processus de pollinisation et de prédation se retrouve condensé dans la production de graines. La conclusion est qu'il est avantageux pour un individu de trolle d'interagir avec les chiastochètes, en ce qui concerne la fitness femelle (et, on l'a vu, encore plus en ce qui concerne la fitness mâle). Il est donc utile de procéder à une telle « synthèse » : cela permet de montrer que la spécialisation du trolle sur les chiastochètes est stable et adaptative.

Cependant, une telle synthèse passe sous silence les intérêts opposés qui peuvent exister entre partenaires mutualistes. Dans le cas des systèmes impliquant une plante et un pollinisateur parasite de graines, cette divergence d'intérêts se manifeste à propos des graines : la consommation de graines est vitale pour l'insecte (et souvent, plus une larve en consomme, plus sa fitness est élevée) tandis que la plante a intérêt à en soustraire le plus possible à son appétit (et si possible, à empêcher toute prédation).

Le chapitre 4 s'est penché sur la traduction évolutive de ce conflit d'intérêt. Nous avons montré qu'il existe une interaction de type antagoniste entre le trolle et les larves : une défense chimique, comparable aux défenses produites par presque toutes les plantes contre leurs ravageurs.

3. L'interaction *Trollius-Chiastocheta* : une mosaïque de traits mutualistes et antagonistes.

Le tableau 6 ci-dessous récapitule les traits impliqués dans la spécialisation de l'interaction *Trollius-Chiastocheta*.

Traits du trolle d'Europe	Traits des chiastochètes	Interaction
Morphologie florale globulaire	Préférence pour cette morphologie	Mutualiste
Composés volatiles organiques émis par la fleur	Réponse spécifique à ces composés	Mutualiste
Adonivernith dans la paroi des carpelles	Résistance et/ou localisation dans le fruit	Antagoniste

A l'intérieur des couples de traits mutualistes, il peut y avoir des tensions. Comme nous l'avons vu dans le chapitre 1, la morphologie globulaire entraîne une augmentation de la prédation. De la même manière, une augmentation de l'attractivité de la fleur *via* les composés volatils peut conduire à une augmentation du nombre d'œufs pondus.

Le tableau 6 fait apparaître l'interaction *Trollius-Chiastocheta* comme la juxtaposition de deux types d'interactions : une interaction plante-pollinisateur et une interaction plante-insecte ravageur. Au sens populationnel présenté en introduction, l'interaction *Trollius-Chiastocheta* est bel et bien mutualiste : le bénéfice de la pollinisation est supérieur au coût de la prédation. En se plaçant à l'échelle de l'individu, qui est le niveau le plus pertinent en biologie évolutive, l'interaction n'est ni mutualiste, ni antagoniste. Elle est une mosaïque de traits antagonistes et mutualistes, une « liaison dangereuse » selon l'expression de van Baalen et Jansen (van Baalen & Jansen, 2001).

4. Contradictions des relations interspécifiques.

La coexistence de traits antagonistes et mutualistes n'est pas l'apanage des interactions mutualistes et a été établie dans les interactions d'exploitation et de compétition. Voici quelques exemples ci-dessous.

Exploitation

Les herbivores participent parfois au recyclage des nutriments, notamment de l'azote. Lorsque l'herbivorie est modérée, la production primaire peut être plus élevée, comme cela a été décrit pour des nématodes consommateurs de racines (Bardgett et al., 1999), des criquets (Belovsky & Slade, 2000), pour le couple zoo- et phyto-plancton (Covich et al., 1999), et dans plusieurs modèles théoriques (voir par exemple (de Mazancourt & Loreau, 1998)). L'interaction est en général étudiée à l'échelle de la population, et même de la communauté puisqu'il est question des cycles biogéochimiques. En fonction de l'intensité de l'herbivorie, les herbivores peuvent alors avoir un impact positif ou négatif sur la production primaire. A l'échelle individuelle, une mosaïque de traits est à l'œuvre.

De manière similaire, le poison développé par une proie et la résistance élaborée par le prédateur sont des traits antagonistes, mais la livrée aposématique de la proie et la capacité visuelle du prédateur à la reconnaître sont des traits mutualistes.

Compétition.

Les papillons du genre *Heliconius* se nourrissent de pollen sur des cucurbitacées du genre *Gurania* et *Anguria* (les chenilles se développent sur les passifloracées). Les adultes des différentes espèces sont en compétition pour l'accès au pollen : les traits qui leur permettent de détecter les fleurs et de les utiliser pour se nourrir ont évolué en réponse à cette compétition. Par contre, ces espèces étant toutes toxiques, leur intérêt commun est d'exprimer un signal aposématique pour avertir les prédateurs : le mimétisme Müllérien est l'expression du côté mutualiste de l'interaction (Templeton & Gilbert, 1988).

Drosophila melanogaster et *Drosophila simulans* sont en compétition pour l'accès aux ressources. Pour leur développement, elles ont besoin des stérols des levures qu'elles ingèrent et métabolisent pour en faire leur « propres » stérols ; les stérols de levures métabolisables par les drosophiles varient d'une espèce à l'autre. Sur certaines souches de levures, *D. simulans* survit difficilement seule, et *D. melanogaster* pas du tout. En revanche, lorsque les deux

espèces sont élevées ensemble, leur développement est mutuellement facilité, probablement grâce au fait que chaque espèce fournit à l'autre des stérols qu'elle ne peut acquérir pour elle-même (Bos et al., 1977).

Dernier exemple, lorsque plusieurs espèces d'arbres produisent leurs graines en même temps (« masting »), leurs prédateurs sont saturés, mais les graines dont la survie a ainsi été favorisée donnent des plantules qui se retrouvent en compétition pour l'accès aux ressources.

5. Conséquences sur la dynamique coévolutive.

Dans les trois principaux types de relations interspécifiques, on peut trouver des exemples de systèmes dans lesquels coexistent des traits antagonistes et des traits mutualistes. Au-delà des classifications, cela souligne la profonde unité des relations interspécifiques.

En quoi est-ce utile de critiquer les concepts de mutualisme, compétition et exploitation au niveau individuel ? Pourquoi mettre en avant la coexistence de traits contradictoires dans les relations interspécifiques ? Est-ce simplement une question de vocabulaire, ou de convention ? Je pense que non, car la coexistence de traits contradictoires est susceptible de modifier les dynamiques évolutives (et d'être simultanément affectée par de telles dynamiques). Plusieurs mécanismes font de la coexistence de traits antagonistes et mutualistes un moteur d'évolution, j'en cite ci-dessous quelques uns.

Corrélations entre traits.

Des contraintes génétiques, développementales ou physiologiques peuvent conduire à une corrélation entre traits, ce qui modifie alors la dynamique évolutive de l'interaction.
Chez le trolle par exemple, l'adonivernith est impliqué à la fois dans la défense chimique et dans la coloration UV des sépales, dans un trait antagoniste et dans un trait mutualiste qui est peut être impliqué dans l'attraction des chiastochètes. L'évolution de l'un peut donc contraindre celle de l'autre. Chez les deux espèces de drosophiles citées ci-dessus, il peut exister des corrélations entre l'aptitude compétitive et la complémentarité des ressources : les gènes qui régulent l'expression des enzymes métaboliques conduisent souvent à un réseau métabolique interdépendant. Chez les plantes soumises à l'herbivorie, il existe un trade-off

entre les défenses anti-herbivores qu'elles produisent et leur capacité à récupérer les nutriments recyclés par les herbivores (Herms & Mattson, 1992). Ce trade-off est à la base d'un modèle de dynamique adaptative qui suggère que l'évolution des défenses anti-herbivores peut conduire à une interaction mutualiste entre plantes et herbivores (de Mazancourt et al., 2001).

Interaction entre les mécanismes lorsque les espèces interagissent.

Le paragraphe précédent s'intéressait aux contraintes qui peuvent exister « à l'intérieur » des espèces en interaction. Il existe également des contraintes au niveau de l'interaction elle-même : une variation d'un trait chez un individu peut modifier l'environnement sélectif de l'autre trait. Chez le trolle par exemple, une fleur capable d'attirer particulièrement bien les chiastochètes va produire un peu plus de graines et exporter beaucoup plus de pollen que les autres. Simultanément, plus de larves vont se développer dans son fruit : la pression de sélection conduisant à l'induction d'adonivernith par les larves sera alors plus forte. On voit ici que l'évolution du trait mutualiste renforce celle du trait antagoniste.

Le raisonnement réciproque est également possible. Le modèle présenté au chapitre 2 montre que lorsque le taux d'oviposition est faible, l'évolution de la spécialisation morphologique du trolle est favorisée. En fait, tout ce qui conduit à une augmentation de la prédation est défavorable à l'évolution de la spécialisation, notamment lorsque le nombre de graines mangées par larve augmente. L'induction d'adonivernith permet de limiter la prédation, et renforce donc l'évolution de la spécialisation. L'évolution des deux traits antagonistes et mutualistes se renforcent mutuellement.

Dans le cas de l'aposématisme décrit plus haut, une livrée qui permet à la proie d'être bien reconnue et donc évitée par les prédateurs diminue la pression de sélection que les prédateurs exercent sur la toxicité. Dans ce cas, l'évolution du trait mutualiste affaiblit celle du trait antagoniste. Des données sur des grenouilles de la famille des Dendrobatides établissent une corrélation négative entre toxicité et coloration, ce qui supporte cette hypothèse (Darst et al., 2006).

Bouclage entre la dynamique des populations et la dynamique évolutive.

Chez le trolle, l'évolution de défenses chimiques plus intenses peu provoquer une baisse de la densité en chiastochètes, ce qui peut conduire à l'évolution de traits floraux plus attractifs. Réciproquement, l'évolution des traits floraux plus attractifs peu provoquer une augmentation de la densité en chiastochètes, ce qui peut conduire à l'évolution de défenses chimiques plus

intenses. Une dynamique coévolutive s'enclenche alors, menant simultanément à plus de défense et plus d'attraction. Ce type de mécanisme peut être proposé pour d'autres interactions. En fonction de la forme des réponses fonctionnelles qui lient les espèces, des variations de la densité des populations peuvent conduire à une intensification ou à une modération de la coévolution. En tous cas, la coexistence de traits contradictoires ne signifie pas nécessairement le développement de l'un au détriment de l'autre !

Le tableau 7 ci-dessous récapitule les différents niveaux d'organisations discutés pendant ce travail.

Niveau d'organisation	Mesure du bénéfice mutualiste	Variabilité du résultat de l'interaction	Chez le trolle
Population	Taux de croissance de la population	Entre populations d'environnements différents	Peu de variations (chap. 1)
Individu	Fitness de l'individu	Entre individus d'une même population	Peu de variations (annexe 1)
Infra-individuel	Plusieurs mesures potentiellement contradictoires	Entre fonctions mâle et femelle d'un même individu	Mesures dans le même sens, d'intensité très différentes (chap. 1)
		Entre traits d'un même individu	Traits mutualistes (chap. 1,2 & 3) et antagonistes (chap. 4)

OUVERTURE : Ecologie évolutive et matérialisme dialectique.

Il y a 150 ans, trois semaines après la publication de « L'origine des espèces » de Darwin (Darwin, 1859), Friedrich Engels écrit à Karl Marx le 11 ou 12 Décembre 1859 : « Darwin, que je lis justement en ce moment, est excellent. Dans ce domaine la téléologie n'était pas encore détruite, maintenant c'est chose faite. En outre, il n'y a pas eu jusqu'ici tentative aussi grandiose de démontrer le développement historique dans la nature, et cela avec un pareil succès » (Marx & Engels, 1973). Les deux philosophes suivaient attentivement le développement des sciences naturelles et des mathématiques de leur époque, l'œuvre fondatrice de la biologie évolutive a logiquement attiré leur attention. Ce qui est plus intéressant, c'est l'homologie soulignée par Engels : il y a un « développement historique dans la nature », de la même manière qu'il y a un développement historique dans la société humaine. Engels insiste sur ce point lors de son oraison funèbre prononcée en anglais devant la tombe de Marx au Highgate Cemetery à Londres, le 17 Mars 1883 : « Just as Darwin discovered the law of development of organic nature, so Marx discovered the law of development of human history ». Les théories darwiniennes et marxistes recherchent les causes matérielles de deux processus historiques : il y a bien une homologie fondamentale, et non une analogie vide de sens. Ce faisant, elles détruisent la téléologie, comme l'a fait remarquer Engels.

L'idée d'un développement historique apparaît également très tôt dans l'histoire de l'écologie. En étudiant les successions végétales dans les dunes du lac Michigan, Henry Chandler Cowles écrit : « Le développement d'une forêt à partir d'une bruyère est facile à comprendre et peut être observé à presque tous les niveaux. La bruyère est assez dense pour empêcher l'érosion éolienne mais pas assez pour empêcher les sauvageons de divers arbres de commencer à croître » (Cowles, 1899), cité par (Acot, 1988). L'homologie fondamentale révélée par Engels n'est pas formulée par Cowles, mais la démarche est là : il s'agit de chercher quelles sont les causes matérielles responsables du développement historique des dunes du Michigan.

Une fois l'homologie révélée, est-il possible d'aller plus loin ? Pour l'étude du développement historique des sociétés humaines, Marx et Engels ont proposé une méthode, inspirée entre

autres de Hegel et appelée ultérieurement matérialisme dialectique. Pour l'étude de la nature, Engels a jeté quelques bases de cette méthode dans un ouvrage resté malheureusement inachevé, la « Dialectique de la nature » (Engels, 1883). Cent ans plus tard, Richard Levins et Richard Lewontin (Levins & Lewontin, 1985) ont continué à creuser dans cette direction dans « The dialectical biologist ». Ici l'objectif n'est pas d'exposer les fondements du matérialisme dialectique, simplement de citer trois de ses piliers en les illustrant brièvement par des exemples issus de ce travail de thèse.

La pénétration réciproque des contraires.

« Identité et différence – nécessité et contingence – cause et effet – tels sont les principaux contraires qui, considérés isolément, se convertissent l'un en l'autre.» (Engels, 1883), page 218. Dans le chapitre 4, nous avons vu que l'accumulation d'adonivernith dans la paroi des carpelles est à la fois cause et conséquence de l'intensité de la prédation des larves (et réciproquement).

La forme en spirale du développement.

La conversion de la cause en conséquence conduit à un développement dans le temps en forme spirale. L'induction de la défense chimique conduit à une modification de la prédation, qui conduit à une modification de l'induction de la défense chimique, etc.

Le formalisme mathématique utilisé dans le modèle du chapitre 2 se base sur ce même principe. La dynamique écologique du modèle constitue le matériel de base sur lequel vient se greffer la dynamique évolutive : la fitness d'un mutant est définie comme son taux d'accroissement lorsqu'il est initialement rare dans une population résidente monomorphe. Lorsque la dynamique évolutive modifie les valeurs des phénotypes, la dynamique écologique est modifiée, ce qui change ensuite les conditions d'invasion de nouveaux mutants. S'enclenche alors un développement éco-évolutif circulaire.

Le développement par contradiction.

Dans la conclusion, j'ai développé dans le détail la manière dont la coexistence de couples de traits antagonistes et mutualistes au sein d'une relation interspécifique est un moteur de la dynamique coévolutive. A l'intérieur d'un système en développement, il existe des pôles contradictoires. Loin de conduire à un anéantissement mutuel, la coexistence de pôles contradictoires est un moteur du développement historique, quel qu'il soit.

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ANNEXES

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PLANT ANIMAL INTERACTIONS

Geographic and within-population variation in the globeflower–globeflower fly interaction: the costs and benefits of rearing pollinators' larvae

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Abstract Interspecific interactions can vary within and among populations and geographic locations, and this variation can influence the nature of the interaction (e.g. mutualistic vs. antagonistic) and its evolutionary stability. Globeflowers are exclusively pollinated by flies, whose larvae feed only on their seeds. Here we document geographic variability in costs and benefits in globeflowers in sustaining their pollinating flies throughout the range of this arctic-alpine European plant over several years. A total of 1,710 flower heads from 38 populations were analysed for their carpel, egg and seed contents. Individual and population analyses control for the confounding influences of variation in both: (1) population traits, such as fly density and egg distribution among flower heads; and (2) individuals traits, such as carpel and egg numbers per flower head. Despite considerable variation in ecological conditions and pollinator densities across populations, large proportions (range 33–58%) of seeds were released after predation, with a benefit-to-cost ratio of 3, indicating that the mutualism is stable over the whole globeflower geographical range. The stability of the mutualistic interaction relies on density-dependent competition among larvae co-developing in a flower head. This competition is revealed by a sharp decrease in the number of seeds eaten per larva with increasing larval number, and is intensified by non-uniform egg distribution among globeflowers within a population. Carpel number is highly variable across globeflowers (range 10–69), and flies lay more eggs in large flowers. Most plants within a population contribute to the rearing of pollinators, but the costs are greater for some than for others. Large globeflowers lose more seed to pollinator larvae, but also release more seed than smaller plants. The apparent alignment of interests between fly and plants (positive relationship between numbers of seed released and destroyed) is shown to hide a conflict of interest found when flower size is controlled for.

Keywords Pollination mutualism · Seed predators · Egg aggregation · Density-dependent competition · Flower size

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Introduction

Some plants are highly specialized for their pollination by insects whose larvae feed on developing seeds. In these systems, there is potential for a conflict of interest between interacting species, as an increase in insect fitness (i.e. more eggs laid and more seeds destroyed) is costly for plant seed production, and a negative correlation between viable and destroyed seeds is expected. The classic and best studied examples of extreme obligate mutualism between a plant and a pollinating seed

predator include the fig–fig wasp, the yucca–yucca moth, the senita–senita cactus, and the globeflower–globeflower fly interactions (Addicott 1986; Pellmyr 1989; Anstett et al. 1997; Jaeger and Després 1998; Holland and Fleming 1999). Despite apparent similarity in these mutualisms, the nature of the interaction differs between fig–fig wasps and other systems. A fig-wasp's life is entirely devoted to the transportation of pollen from its natal fig to another fig, the laying of its eggs, and its death in the fig. The only way for a fig to export its pollen is to rear its pollinator's progeny. Male fig success, therefore, entirely depends on pollinator progeny developmental success, and the resource allocated by a fig to the rearing of its pollinator larvae is approximately half its seeds, as expected by sex allocation theory (Charnov et al. 1976). By contrast, in other systems, pollinating insects are free to move from an individual plant to another, and transport pollen from several individuals throughout their lifespan. There is no direct individual benefit for a plant to rear pollinator larvae in terms of pollen export, as pollinators reared by other plants may also transport its pollen. One may expect these systems to be highly susceptible to invasion by cheaters (Axelrod and Hamilton 1981; Herre et al. 1999; Yu 2001), plants that prevent oviposition and/or kill developing larvae (Bao and Addicott 1998). The European globeflower *Trollius europaeus* L. (Ranunculaceae) is a perennial arctic-alpine herb pollinated by *Chiastocheta* flies, whose larvae are specific seed predators of globeflowers (Pellmyr 1989; Jaeger and Després 1998). Each individual plant typically produces, every second year, a single flower composed of several carpels, each containing about 12 ovules. Both male and female flies contribute to passive pollination when visiting flowers to feed on nectar and search for sexual partners (Després 2003), so flowers with many eggs are not necessarily better pollinated than flowers with few or no eggs (Jaeger et al. 2000). Egg hatching success is close to one, and larval mortality is low, so that the number of eggs laid on a flower head is a good indication of the number of larvae co-developing in the flower (Jaeger et al. 2001; Pompanon et al. 2006). Larvae can freely move from one carpel to another, and there is usually only one flower per plant, so selective fruit abortion is not an option for the globeflower (Bull and Rice 1991; Pellmyr and Huth 1994). After larval development, the last instar falls to the soil and overwinters, to emerge as a short-lived adult pollinator the following spring. As a globeflower typically flowers only every second year (Å. M. Hemborg and L. Després, unpublished data), an individual plant never benefits from pollination services by the individual fly it reared as a larva. The difference between gross benefits (the number of ovules fertilized by flies) and costs (the number of seeds eaten by larvae) determines the net benefit of the interaction for the globeflower. The gross benefit is likely to vary with fly density in the population, because when fly density is high, pollination efficiency is high, while the cost is likely to vary with number of eggs laid on a particular globeflower and the size of this flower (carpel number). The magnitude of variation between benefits and costs is therefore likely to vary with fly density across populations, egg distribution across globeflowers within a population, and individual flower size and egg content.

In this paper, we evaluate costs and benefits of rearing pollinator flies for the European globeflower throughout its ecological range. We answer the following questions:

1. How much does it cost a globeflower population to rear pollinator larvae, and how does this cost vary across populations with variable fly densities and egg distribution among globeflowers?
2. How variable are costs and benefits among individuals within a population, and what is the effect of flower size on the individual cost/benefit outcome?

Materials and methods

A total of 26 globeflower populations were studied at various elevations, six in Swedish Lapland (range 400–670 m a.s.l.) and 20 in the French Alps (range 800–2,500 m a.s.l.) including six populations sampled for 3 consecutive years (1995, 1996, 1997). Study sites represent a wide range of ecological conditions (Jaeger and Després 1998; Hemborg and Després 1999). This resulted in a total of 38 records, each representing a group of globeflowers sharing their pollinators, hereafter called a "population". An average of 45 flower heads (range 9–119) were sampled per population, resulting in a total of 1,710 globeflowers analysed. For each flower, we counted the number of eggs and the number of carpels. Egg distribution among flowers within a population was estimated as the coefficient of dispersion (CD) = V/M, where M is the mean number of eggs per flower and V its variance. If CD = 1, eggs are randomly distributed in the population (Poisson distribution) and if CD > 1, eggs are aggregated, i.e. a few flowers have more eggs than others. We estimated the proportion of fertilized ovules (gross seed production) by counting the number of undeveloped ovules and developing seeds per carpel in five undamaged carpels (Jaeger and Després 1998). Undeveloped ovules were counted on only 1,524 flowers in 36 populations, because some flowers were too damaged and no intact

carpels were available. In each studied population, relative *Chiastocheta* density was estimated as the mean number of eggs per flower.

To estimate seed loss due to a pollinator's larval predation, we selected a total of 308 other flower heads with a number of *Chiastocheta* eggs ranging from 1 to thirty-five, 2 weeks after the end of flowering in three populations (Jaeger et al 2001). The number of carpels was counted, and flowers were bagged to prevent seed release. Flower heads were collected after completed larval development (4–5 weeks after the end of flowering), and the number of seed destroyed by larvae was estimated by the difference between the number of seeds remaining intact after predation and the estimated number of seeds initiated. *Chiastocheta* larvae are the only predator of globeflower seeds. Although up to six *Chiastocheta* spp. co-exist in alpine globeflower populations (Després and Jaeger 1999), seed consumption per larva was shown to be similar for all species (Pompanon et al. 2006), so that we did not distinguish between *Chiastocheta* spp. in the present study. The proportion of seed eaten per flower head was plotted against individual egg density per carpel and the best fitting model was selected. We then applied this model to each of the 1,710 flowers sampled in the 38 populations to estimate individual and population costs. The proportion of seed released after predation (net seed production) equals the proportion of seed initiated multiplied by one minus the proportion of seed eaten.

Data analysis

Data were first checked for normality (Kolmogorov–Smirnov test of normality) and homogeneity of variances (Levene test). Seed proportions were arcsine square root transformed prior to analyses. Two-way ANOVAs (GLM procedure for unbalanced experimental design) were performed on carpel and egg numbers, and on the absolute number and on the proportion of seed initiated, eaten, and released after predation. All tested effects (population and year) were considered as fixed effects, and mean squares adjusted for unequal sample sizes were used in the ANOVAs. To examine whether fly density in a population had an effect on the slope between pollination efficiency and egg number, we performed an analysis of covariance (ANCOVA) testing for the effect of population, egg density, and interaction on the number of seeds initiated: a significant interaction indicates that the slopes are different within each population. To relate this difference to variation in fly densities, we performed a linear regression of the slopes against fly density across populations. Linear and non-linear regressions predicting the proportion of seed eaten against egg density per carpel were performed and the best fitting model [residual sum of squares (SS_R) minimum] was selected. Multiple linear regressions predicting the absolute number and proportion of seed initiated and of seed released after predation against egg density per carpel and carpel number were performed in each population, and across populations. We analysed distributions of costs among populations and among globeflowers within a population using Kolmogorov–Smirnov normality test. Spearman's correlations were performed for all pairwise combinations of variables.

Results

All study populations contained *Chiastocheta* flies, with densities ranging from 0.5 to 17 eggs per flower head (Table 1). There were significant differences in carpel and egg numbers per flower across populations (carpel $F_{25,1672} = 15.12$, $P < 0.001$, egg $F_{25,1672} = 23.28$, $P < 0.001$), years (carpel $F_{2,1672} = 22.2$, egg $F_{2,1672} = 77.18$, $P < 0.001$) and population by year interaction (carpel $F_{10,1672} = 5.11$, egg $F_{10,1672} = 21.53$, $P < 0.001$), reflecting highly variable ecological environments. Mean carpel number per flower varied across populations and years ($n = 38$ observations) from 25 to 42 (Table 1).

At the individual level, 92% of the 1,710 analysed flowers contained at least one egg (range 0–63) and carpel number was highly variable (range 10–69). Unparasitized flowers were significantly smaller than parasitized flowers (30.3 vs. 32.85; $F_{1,1708} = 8.94$, $P = 0.003$). The proportion of unparasitized flowers ranged from 0 to 75% across populations and this proportion decreased with increasing fly densities ($r_s = -0.876$, $P < 0.001$). Within each population, eggs were not randomly distributed among flowers as shown by a coefficient of dispersion higher than 1 in all populations (range 1.07–7.39, significantly higher than one in 35 out of 38 populations, Table 1). Large flowers tended to be more heavily infected than small flowers, as shown by a positive correlation between the number of eggs and the number of carpels per flower, significant in 24 out of 38 populations (Table 2). Although large flowers attracted more eggs than small flowers, they were not better pollinated (no effect of carpel number on gross seed production in 29 out of 36 populations; when significant, either positive of negative, see Table 2). Furthermore, carpel number was not correlated with egg density per carpel, i.e. large flowers do not concentrate more eggs than small flowers

Table 1 Characteristics of the 38 globeflower populations analysed. The number of sampled flower heads and mean contents per flower head are given. SDs are given *in parentheses* for directly measured data and for the total mean. *CD* Coefficient of deviation, % proportion, *n.s.* non-significant ($P > 0.05$)

Population name	Flower heads (*n*)	Carpel no.	Egg no.	CD	% Seeds initiated	No. seed initiated	No. seed eaten	No. seed released	% Ovule released	% Seeds eaten	% Seeds released	Benefit /cost[a]
Chables	40	38 (10)	6.00 (5.7)	5.42	0.86 (0.14)	408 (144)	157	250	0.54	0.38	0.62	2.6
Chamoss	39	28 (9)	4.77 (5.9)	7.39	0.69 (0.21)	206 (89)	74	132	0.44	0.35	0.65	2.8
Che95	45	33 (9)	4.29 (4.4)	4.50	0.89 (0.10)	297 (101)	105	193	0.58	0.34	0.66	2.8
Che96	50	40 (8)	4.08 (3.1)	2.37	0.76 (0.16)	365 (130)	122	243	0.5	0.33	0.67	3.0
Che97	49	29 (9)	4.06 (2.1)	1.07 n.s.	0.79 (0.11)	263 (94)	102	161	0.48	0.39	0.61	2.6
Cot95	38	32 (9)	2.97 (2.6)	2.30	0.84 (0.12)	269 (113)	90	179	0.56	0.33	0.67	3.0
Cot96	50	38 (9)	3.04 (2.2)	1.54	0.75 (0.14)	358 (104)	111	247	0.51	0.31	0.69	3.2
Cot97	49	32 (7)	2.63 (2.3)	1.96	0.74 (0.12)	271 (95)	87	184	0.51	0.31	0.69	3.1
Cro95	50	27 (7)	6.92 (4.4)	2.84	0.93 (0.05)	274 (95)	123	151	0.51	0.45	0.55	2.2
Cro96	49	28 (7)	5.20 (4.0)	3.09	0.74 (0.19)	248 (248)	100	148	0.45	0.39	0.61	2.5
Cro97	73	29 (9)	5.10 (4.3)	3.60	0.73 (0.16)	264 (109)	102	162	0.46	0.37	0.63	2.6
Etelley	46	34 (7)	3.20 (3.3)	3.53	0.67 (0.15)	272 (92)	89	183	0.45	0.32	0.68	3.1
Fardelay	78	42 (9)	5.27 (4.9)	4.52	0.84 (0.14)	386 (123)	133	253	0.53	0.35	0.65	2.9
Gal95	49	31 (7)	16.96 (9.1)	4.90	0.80 (0.12)	299 (79)	167	133	0.36	0.55	0.45	1.8
Gal96	50	29 (8)	4.74 (3.9)	3.24	0.61 (0.17)	183 (64)	66	117	0.39	0.37	0.63	2.8
Gal97	30	24 (8)	8.67 (6.5)	4.86	0.70 (0.16)	194 (96)	93	100	0.37	0.47	0.53	2.1
JouxPlan	92	37 (7)	12.83 (7.1)	3.99	0.89 (0.10)	411 (110)	202	209	0.46	0.49	0.51	2.0
Lac95	46	36 (10)	7.41 (5.9)	4.81	0.68 (0.17)	302 (116)	123	179	0.41	0.4	0.6	2.5
Lac96	49	40 (11)	4.00 (3.7)	3.55	0.56 (0.16)	260 (96)	89	172	0.37	0.33	0.67	2.9
Lac97	40	35 (10)	8.58 (4.9)	2.79	0.77 (0.16)	289 (146)	128	161	0.43	0.45	0.55	2.3
Lau95	39	29 (6)	7.13 (5.9)	4.87	0.86 (0.12)	262 (75)	115	147	0.49	0.43	0.57	2.3
Passy	119	35 (9)	3.82 (3.3)	2.91	0.73 (0.20)	276 (113)	93	183	0.49	0.32	0.68	3.0
Pelly	38	35 (7)	5.71 (4.6)	3.68	0.87 (0.12)	373 (105)	144	228	0.54	0.38	0.62	2.6
PrazFarou	76	36 (10)	5.12 (4.1)	3.33	0.79 (0.16)	328 (139)	124	204	0.5	0.37	0.63	2.7
Rhi1	12	29 (9)	2.25 (2.3)	2.35	0.57 (0.19)	190 (95)	49	141	0.43	0.26	0.74	3.9
Rhi2	20	32 (10)	2.70 (2.0)	1.52	0.53 (0.21)	209 (117)	65	144	0.37	0.3	0.7	3.2
Rhi3	9	24 (5)	2.33 (1.9)	1.50 n.s.	0.49 (0.13)	139 (54)	44	95	0.33	0.32	0.68	3.2
Sales	40	29 (7)	1.78 (1.9)	2.04	–	178 (83)	45	133	–	0.23	0.77	4.0
Salmoiry	38	30 (9)	3.42 (2.7)	2.10	0.79 (0.14)	255 (92)	92	163	0.5	0.35	0.65	2.8
Som95	60	25 (5)	16.0 (11.2)	7.80	0.94 (0.06)	266 (74)	151	115	0.4	0.56	0.44	1.8
Som96	49	28 (7)	4.41 (3.0)	2.09	0.75 (0.17)	259 (71)	101	158	0.45	0.39	0.61	2.6
Som97	50	27 (6)	5.36 (3.1)	1.76	0.80 (0.13)	262 (91)	110	152	0.46	0.42	0.58	2.4
Tete	40	28 (5)	0.50 (1.0)	2.05	0.53 (0.19)	162 (66)	15	147	0.49	0.08	0.92	11.0
Vagnys	37	34 (8)	4.89 (3.6)	2.60	0.82 (0.20)	331 (105)	126	206	0.51	0.37	0.63	2.6
Vallon	20	27 (5)	2.15 (2.3)	2.46	–	222 (68)	61	161	–	0.26	0.74	3.6
tre1	22	31 (8)	2.09 (1.84)	2.09	0.68 (0.16)	236 (83)	63	174	0.5	0.25	0.75	3.7
tre2	15	34 (8)	1.60 (1.8)	1.6	0.63 (0.08)	228 (67)	54	174	0.48	0.23	0.77	4.2
tre3	14	27 (6)	2.71 (1.9)	2.71	0.67 (0.12)	213 (61)	71	142	0.44	0.33	0.67	3.0
Total	1710	31.8 (4.6)	5.12 (3.6)	3.2 (1.5)	0.74 (0.11)	268.7 (66)	99.6 (37)	169.1 (39)	0.46 (0.06)	0.36 (0.09)	0.64 (0.09)	3 (1.4)

[a] Number of seeds initiated/number of seeds eaten

(Table 2). The proportion of seed initiated neither increased significantly with egg number in most populations, nor did the absolute number of seeds initiated when corrected for carpel number (Table 2), i.e. flowers with more eggs were generally not better pollinated than flowers with few eggs. However, the slopes of the relationship between the number of seed initiated and the number of eggs differed across populations (ANCOVA, population $F_{37,1709} = 7.13$, $P < 0.001$; egg density $F_{1,1709} = 14.23$, $P < 0.001$; population by egg density interaction $F_{37,1709} = 1.65$, $P = 0.008$). In low fly density populations, the slope was more positive than in high fly density populations (Fig. 1), indicating that globeflowers in low fly density populations benefited more in terms of pollination from visits by ovipositing females than in high fly density populations. Across populations, the mean proportion of seed initiated increased with mean egg number per flower head (Table 3): populations with high fly densities were better pollinated than populations with low fly densities.

Predation costs

In the predation study ($n = 308$) we found no effect of flower size on the proportion of seed eaten (linear regression $F_{1,306} = 0.08$, $P = 0.7$). Therefore, the proportion of seed eaten per flower is best predicted by egg density per carpel rather than by the absolute number of eggs in that flower (DeAngelis and Holland 2006). As *Chiastocheta* larvae are the only predators of globeflower seeds, seed destruction in the absence of larva is zero. A classic assumption in modelling the probability that a seed will be eaten is that larvae move at random

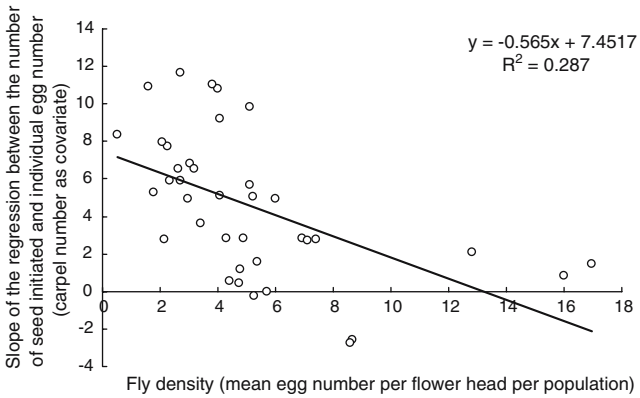

Fig. 1 Linear regression of the slopes of the relationship between the individual number of seeds initiated and individual egg number (with carpel number as covariate) within each population ($n = 38$) against fly density per population estimated as the mean egg number per flower. The negative slope is significant ($P = 0.001$, $r^2 = 0.27$): individual plants benefit more from pollination by ovipositing females in low fly density populations than in high fly density populations

Table 2 Summary of Spearman's correlation analyses within globeflower populations ($n = 36$ or 38) showing the number of populations for which the correlations were significantly positive (+), negative (−) ($P < 0.05$), or n.s.

	Carpel number			Egg number		
	+	n.s.	−	+	n.s.	−
Egg number	24	14	0	–	–	–
Egg density (egg no./carpel no.)	3	31	4	–	–	–
Proportion of seed initiated	2	29	5	5	31	0
Number of seeds initiated	38	0	0	22	16	0
Number of seeds eaten	31	7	0	38	0	0
Number of seeds released	37	1	0	0	36	2
Proportion of seed released	1	33	2	0	10	26

and eat any encountered seed; given Poisson-distributed visits with a mean of ax (where x is egg density per carpel, and a is a constant), the probability for a seed to be eaten is $1 - e^{-ax}$ (e.g. one minus the probability of no visit; Morris et al. 2003). We call this model the "random search model". The best fitting model predicting the proportion of seed eaten per flower as a function of egg density per carpel ($y = 0.66x^{0.26}$, $SS_R = 10.39$, Fig. 2a) is superior to the linear model ($y = 0.33 + 0.41x$, $SS_R = 12.79$), and much superior to the random search model ($y = 1 - e^{-2.43x}$, $SS_R = 14.89$). This model ("individual cost model") was then applied to each of the 1,710 flower heads sampled in the studied populations. Costs varied across years ($F_{2,1708} = 59.84$, $P < 0.001$) and populations ($F_{25,1684} = 25.84$, $P < 0.001$). The proportion of seed lost to pollinator larvae ranged from 0 to 87% among individuals within populations, and from 8 to 56% across populations, ca. an average of 36% of the initiated seeds destroyed (Table 1; Fig. 3). Ninety-five percent of all studied populations lost between 25 and 45% of initiated seeds to pollinator larvae, and these costs were normally distributed across populations (Kolmogorov–Smirnov test, $P > 0.05$); by contrast, costs were normally distributed among globeflowers within a population in only 13 populations among 38: in these populations, almost all individuals had at least one egg. In all the 25 remaining populations, where up to 75% of the individuals sampled had no eggs, costs were not normally distributed among individuals: some pay less than others. Mean predation costs per population were plotted against mean egg density per population ($n = 38$). The best fitting model ($y = 0.69x^{0.34}$, $SS_R = 0.02$) takes into account egg aggregation on flowers within each population (Després and Jaeger 1999) to predict the change in population costs with increasing fly density ("population cost model", Fig. 2b). The population data always lie below the individual cost model, which

Table 3 Simple and multiple regressions predicting the number of seeds initiated and released against egg number in 38 populations. *n* Number of flower heads sampled

Covariate		Seeds initiated						Seeds released					
		None			Carpel			None			Carpel		
Population	*n*	Slope	SE	*P*	Slope	SE	*P*	Slope	SE	*P*	Slope	SE	*P*
Chables	40	17.04	3.02	<0.0001	4.92	2.94	0.102	4.11	2.14	0.06	−4.44	2.08	0.040
Chamoss	39	2.96	2.41	0.229	1.16	1.93	0.552	−2.11	1.76	0.24	−3.36	1.46	0.027
Che95	45	11.22	3.08	0.001	2.83	1.90	0.145	0.58	2.20	0.80	−5.21	1.47	0.001
Che96	50	11.69	5.80	0.050	9.22	4.50	0.046	−3.25	4.34	0.46	−5.25	3.15	0.103
Che97	49	21.01	5.82	0.001	5.11	4.87	0.300	7.60	3.89	0.06	−3.88	2.98	0.199
Cot95	38	12.13	6.91	0.088	4.93	5.20	0.350	0.39	4.93	0.94	−5.29	3.30	0.118
Cot96	50	4.25	6.90	0.541	6.82	5.00	0.179	−11.31	5.20	0.03	−9.37	3.78	0.017
Cot97	49	15.89	5.63	0.007	6.54	4.52	0.155	1.86	3.98	0.64	−4.64	3.24	0.159
Cro95	50	10.44	2.71	<0.001	2.85	2.05	0.172	1.72	1.62	0.29	−3.20	1.08	0.005
Cro96	49	14.14	3.34	<0.001	5.05	3.07	0.107	3.20	2.06	0.13	−2.45	1.88	0.199
Cro97	73	14.13	2.53	<0.0001	5.66	1.94	0.005	2.48	1.76	0.16	−3.83	1.23	0.003
Etelley	46	8.38	3.92	0.038	6.52	3.32	0.056	−1.63	2.86	0.57	−3.08	2.33	0.194
Fardelay	78	−0.41	2.90	0.889	−0.23	2.06	0.913	−7.55	2.28	<0.01	−7.41	1.57	<0.0001
Gal95	49	3.99	1.13	0.001	1.47	0.88	0.103	−0.20	0.62	0.75	−1.80	0.39	<0.0001
Gal96	50	2.17	2.33	0.355	0.45	1.85	0.809	−3.98	1.78	0.03	−5.26	1.44	0.001
Gal97	30	6.41	2.51	0.017	−2.59	1.75	0.150	0.94	1.43	0.52	−4.45	0.86	<0.0001
JouxPlan	92	7.63	1.40	<0.0001	2.07	1.26	0.103	0.48	0.78	0.54	−2.95	0.64	<0.0001
Lac95	46	10.57	2.45	<0.0001	2.79	2.47	0.265	1.05	1.87	0.58	−5.44	1.76	0.004
Lac96	49	15.59	2.92	<0.0001	10.83	3.33	0.002	4.36	2.09	0.04	0.18	2.28	0.936
Lac97	40	8.36	4.65	0.080	−2.77	2.72	0.314	1.50	2.84	0.60	−5.46	1.55	0.001
Lau95	39	7.02	1.75	<0.001	2.72	1.76	0.131	0.18	1.13	0.88	−3.09	1.02	0.005
Passy	119	11.00	2.97	<0.001	11.04	2.55	<0.0001	−1.54	2.08	0.46	−1.51	1.73	0.385
Pelly	38	8.59	3.52	0.020	−0.02	2.44	0.994	−1.43	2.53	0.57	−7.53	1.80	<0.001
PrazFarou	76	15.43	3.49	<0.0001	9.84	2.49	<0.001	3.21	2.32	0.17	−0.47	1.67	0.779
Rhi1	12	14.63	2.17	0.257	7.72	7.51	0.331	−3.21	10.60	0.77	−9.59	5.66	0.124
Rhi2	20	17.11	2.98	0.204	5.92	8.19	0.480	1.33	9.72	0.89	−7.24	5.87	0.234
Rhi3	9	6.61	0.58	0.552	5.93	7.30	0.448	−4.10	8.50	0.64	−4.67	5.38	0.419
Sales	40	14.43	6.70	0.038	5.27	4.90	0.289	−1.57	5.38	0.77	−8.92	3.93	0.029
Salmoiry	38	9.14	5.51	0.106	3.62	3.75	0.341	0.27	3.77	0.94	−3.67	2.40	0.135
Som95	60	2.42	0.81	0.004	0.83	0.62	0.187	−1.11	0.43	0.01	−2.03	0.31	<0.0001
Som96	49	0.55	3.42	0.872	0.53	2.71	0.846	−5.39	2.30	0.02	−5.40	1.71	0.003
Som97	50	4.19	4.27	0.330	1.58	2.95	0.594	−2.52	2.78	0.37	−4.32	1.78	0.020
Tete	40	11.83	10.46	0.265	8.36	9.92	0.405	−12.34	9.86	0.22	−15.68	9.32	0.101
Vagnys	37	9.25	4.72	0.058	2.85	4.76	0.554	−0.34	3.21	0.92	−5.01	3.17	0.123
Vallon	20	12.25	6.37	0.070	2.80	4.72	0.561	−3.98	5.26	0.46	−11.52	4.08	0.012
tre1	22	18.40	9.14	0.058	7.96	9.38	0.407	−3.29	8.35	0.70	−12.50	8.65	0.165
tre2	15	15.98	9.24	0.108	10.95	7.85	0.188	−3.44	7.97	0.67	−7.34	7.15	0.324
tre3	14	11.51	8.51	0.201	11.65	4.25	0.019	−3.16	7.39	0.68	−3.05	4.30	0.493

means that egg aggregation among globeflowers results in decreasing global predation costs as compared to predicted costs if eggs were uniformly distributed (Fig. 2b).

Net benefit: female fitness

At the individual level, the proportion of seed released after predation decreased with increasing egg number in most populations, while carpel number had no effect on this proportion (Table 2). By contrast, the absolute numbers of seeds initiated, eaten, and released after predation increased with increasing carpel number (Table 2). This resulted in positive correlations between the number of seeds eaten and released (significant in 31 out of 38 populations). When carpel number was controlled for by using a multiple regression analysis, the slope of the number of seeds eaten versus seeds released was negative in all populations (significant in 24 out of 38 populations), and the number of seeds released decreased with increasing egg number (Table 3). At the population level, despite a considerable variation in fly densities, the proportion of fertilized ovules released after larval predation was strikingly similar across populations (mean ± SD = 0.46 ± 0.06, $n = 36$; Table 1), with no significant effect of egg number variation across populations on net seed production (Table 4). Populations with low fly

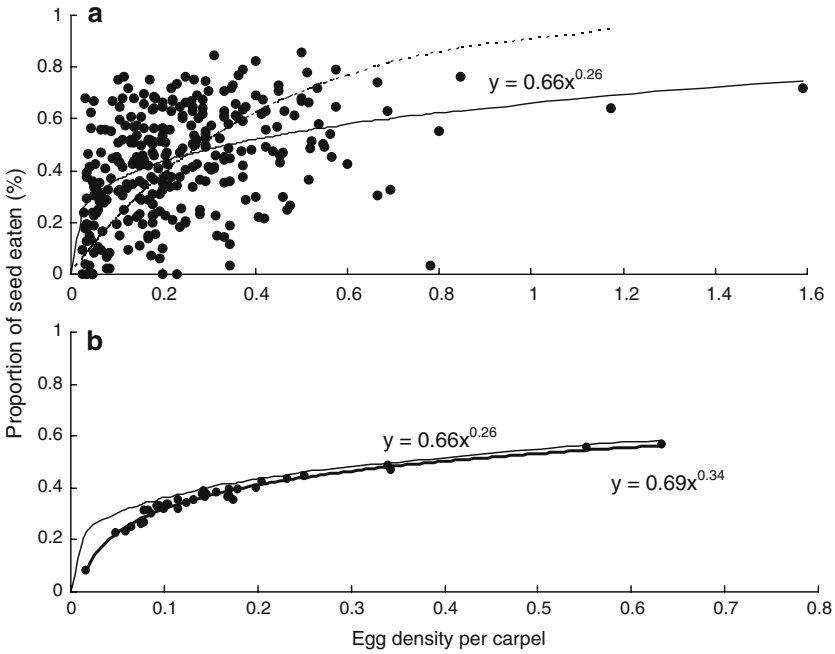

Fig. 2 **a** Proportion of seed eaten per flower head as a function of individual egg load ($n = 308$). The best fitting model: individual cost model ($y = 0.66x^{0.26}$, $SS_R = 10.39$; *solid line*) is superior to the linear model (not shown), and much superior to the random search model ($y = 1 - e^{-2.43x}$, $SS_R = 14.89$; *dashed line*). It shows strong competition among larvae at high densities. **b** Mean proportion of seed eaten per population as a function of mean egg density per carpel per population ($n = 38$). The best fitting model: population cost model ($y = 0.69x^{0.34}$, $SS_R = 0.02$; *thick line*) is below the individual cost model (*thin line*), which means that egg aggregation among globeflowers within populations results in decreasing predation costs as compared to costs in populations with uniformly distributed eggs

densities produced fewer seeds, because they were less efficiently pollinated, as shown by a significant positive effect of egg number on the proportion of seed initiated, but they were also less infected, resulting in a similar proportion and absolute number of seeds released after predation (Figs. 4, 5). The benefit-to-cost ratio (number of seeds initiated over number of seeds eaten) was about 3, ranging from 1.8 to 11 (Table 1).

Discussion

Pollination efficiency

Gross seed set increased with increasing fly densities across populations, i.e. individual plants benefit from being in a population with many flies; however, within populations, seed set generally did not increase with increasing individual egg load, or only marginally increased in low fly density populations. This indicates that pollination and oviposition are not as closely coupled as in other pollinating seed-eating mutualisms which have been described, such as the fig–fig wasp, the yucca–yucca moth, and the senita cactus–senita moth mutualisms where pollination takes place only during oviposition (Janzen 1979; Holland and Fleming 1999). Indeed, both male and female *Chiastocheta* were shown to efficiently transfer pollen from a globeflower to another, independently from egg-laying attempts (Després 2003). However, when pollen transfer is very limiting, in low fly density populations, every visit contributes to increase seed set, including visits by ovipositing females. Pollination ranged from 49 to 93% of seed initiated across populations, which is comparable to the pollination efficiency observed in several fig species (46–95%, Herre and West 1997), where pollination is active but performed only by ovipositing females.

Predation costs

Theoretical studies on the population dynamics of seed predators are usually based on the assumption that insects eat seeds randomly (Holland and De Angelis 2001; Morris et al. 2003). Our empirical data do not support a random search model as the best predictor of the proportion of seed eaten as a function of egg density. The best fitting model generated values superior to those of the random search model for low larval density and inferior to those for high larval density. This indicates that larvae do not move randomly from a seed to another but through specific pathways increasing search efficiency at low density (Pellmyr

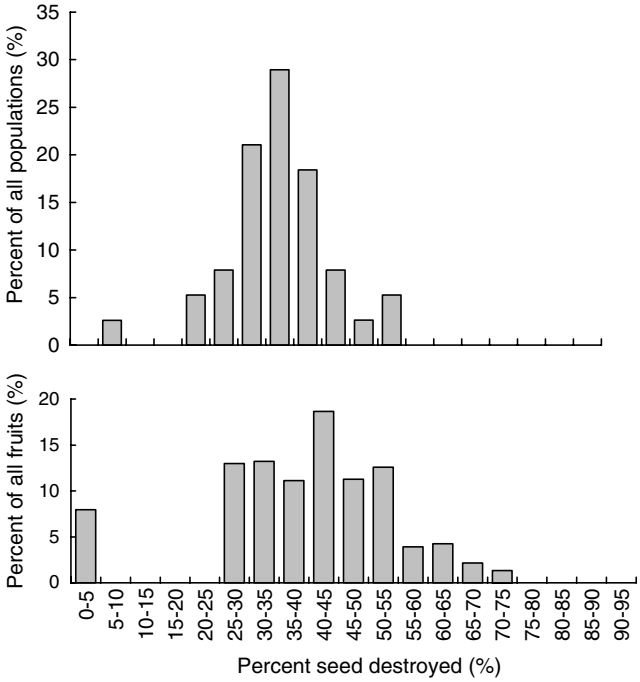

Fig. 3 Distribution of the proportion of seed lost to pollinator larvae for records of 38 globeflower populations (different populations and/or different years), and for all globeflower individuals analysed ($n = 1{,}710$) among the 38 study populations. Costs are grouped in intervals of 5%. Costs are normally distributed among populations, but not among individuals, because some individuals have no eggs and therefore no costs; such a departure from normality is verified in all populations with more than one unparasitized globeflower (Kolmogorov–Smirnoff tests)

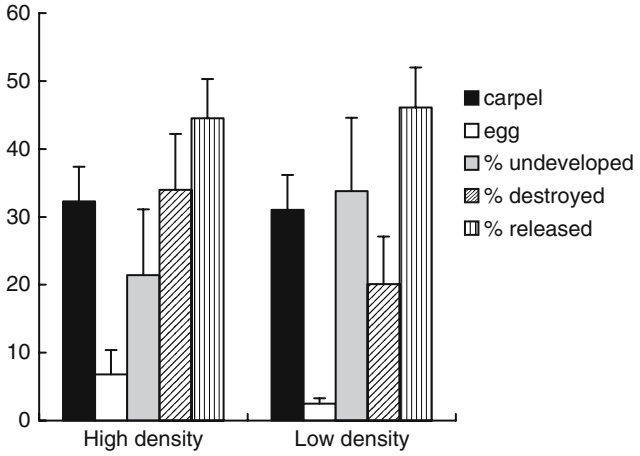

Fig. 4 Mean (±SE) carpel and egg numbers and proportion of ovules undeveloped, destroyed and released, in globeflower populations with high and low *Chiastocheta* densities. Fly density per population was estimated as the mean number of eggs per flower head [four or more eggs per flower head ($n = 23$) (*High density*); less than four eggs per flower head ($n = 15$) (*Low density*)]. Effect of *Chiastocheta* density on the proportion of: undeveloped ovules $H_{1,\ 36} = 9.9$, $P < 0.001$ (*% undeveloped*); destroyed ovules $H_{1,36} = 17.9$, $P < 0.001$ (*% destroyed*); released seeds $H_{1,36} = 0.55$, $P = 0.459$ (*% released*) (Kruskal–Wallis non-parametric tests)

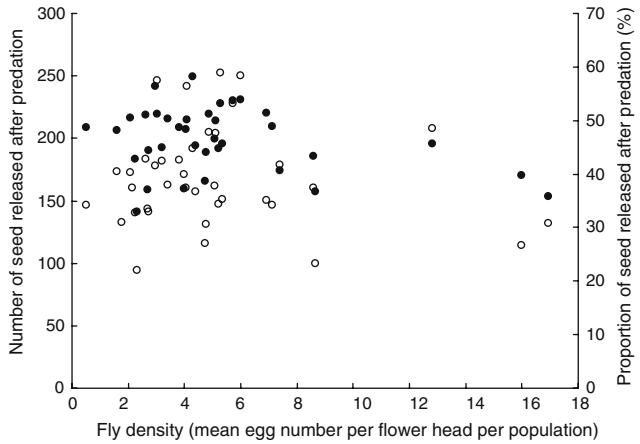

Fig. 5 Mean number (*white circles*) and proportion (*black circles*) of seed released per population ($n = 38$ and $n = 36$, respectively) as a function of fly density (mean number of eggs per flower head). The linear regressions are non-significant (not shown)

1989), but that at high larval density, larval competition prevents efficient seed predation (Jaeger et al. 2001). The population cost model generates values inferior to those of the individual cost model, i.e. egg aggregation on flowers within population decreases costs predicted by the individual cost model. *Chiastocheta* egg aggregation among globeflowers within populations occurs at various latitudes (Johannesen and Loeschcke 1996; Després and Jaeger 1999). Egg aggregation is species dependent, i.e. five out of six *Chiastocheta* sp. do aggregate their eggs, while the first species to lay (*C. rotundiventris*) uniformly distributes eggs, avoiding laying more than one egg per flower head (Després and Jaeger 1999); the differences in egg aggregation observed across populations may reflect different species composition of the community of pollinators. This study shows that non-uniform egg distribution among globeflowers benefits the whole population (lower global predation cost due to increased larval competition) but is costly for the few individual plants attracting higher egg densities. These individuals are not larger globeflowers as carpel number is not correlated with egg density per carpel: although larger plants attract more eggs, they are not disproportionately more infected than smaller plants. This suggests that ovipositing females have a precise evaluation of the size of the resource and of the number of eggs already laid. This evaluation can be visual, olfactory, or tactile, through pheromone deposition by previous ovipositing females (Huth and Pellmyr 1999) or through egg associated compounds (de Jong and Stadler 2001). Whether the female decides to oviposit or not, evaluation of flower size and egg content involves penetrating the closed

Table 4 Multiple regressions predicting absolute number and proportion of seed produced before (*Gross benefit*) and after predation (*Net benefit*) against carpel and egg numbers across populations. *n* Number of populations analysed

Gross benefit	Proportion of seed initiated ($n = 36$)				Number of seeds initiated ($n = 38$)			
	Parameter estimate	SE	*t*-value	*P*-value	Parameter estimate	SE	*t*-value	*P*-value
Intercept	0.4847	0.1205	4.02	0.0003	−124.09	41.70	−2.98	0.0053
Carpel	0.0050	0.0036	1.40	0.1722	11.154	1.2633	8.83	<0.0001
Egg	0.0181	0.0046	3.90	0.0004	7.4753	1.6220	4.61	<0.0001
Net benefit	Proportion of seed released ($n = 36$)				Number of seeds released ($n = 38$)			
	Parameter estimate	SE	*t*-value	*P*-value	Parameter estimate	SE	*t*-value	*P*-value
Intercept	0.3302	0.0669	4.93	<0.0001	−64.405	23.476	−2.74	0.009
Carpel	0.0049	0.0020	2.49	0.0178	7.4968	0.7110	10.54	<0.0001
Egg	−0.0048	0.0025	−1.87	0.0704	−0.9362	0.9130	−1.03	0.3122

corolla and coming into close contact with stigmas and pollen, thereby favouring pollen transfer. Therefore, variation in carpel number among globeflowers within a population may be a way to manipulate fly behaviour by forcing females to visit many flowers.

The mean proportion of seed eaten, 36% (range 8–56% across populations) is comparable to that observed in other pollinating seed parasite mutualisms (18–60% across several fig species, 19–29% in senita cactus and 1–45% in several *Yucca* sp., reviewed in Bronstein 2001). However, the distribution of costs among individuals differs in these various mutualistic interactions. Most globeflowers (92%) bred at least one larva, whereas most senita cactus and yucca fruits contain no pollinator larvae due to egg/larval mortality and/or failed oviposition (Holland and Flemming 1999). In dioecious *Ficus* sp., half the individuals (the female trees) contain no pollinator larvae, while in monoecious *Ficus* sp. all individuals contribute to the pollinator population. The average benefit-to-cost ratio for *T. europaeus* interacting with *Chiastocheta* was 3, comparable to that observed in yucca and senita cactus interacting with their specific pollinating seed predators (2–5, Fleming and Holland 1998). The population benefiting the most from the interaction was that with the lowest fly density, with a benefit-to-cost ratio of 11.

Is there a conflict of interest between the plant and the fly?

As both the plant and the fly rely on seeds for their reproduction, there is potential for a conflict of interest between the partners, each being selected to maximize its fitness by monopolizing the common resource. The expectation is therefore to observe a negative relationship between the number of seeds released by the plant and the number of seeds eaten by the larvae. However, simple correlations across the 38 populations showed a positive relationship between the number of seeds released (plant fitness) and the number of seeds eaten (fly fitness); such a positive relationship has also been observed across 17 *Ficus* sp. (Herre and West 1997). This suggests that the plant and the pollinator have aligned interests: plants that provide more food to pollinators are also those that produce more viable seeds. However, when the size of the available common resource pool was controlled for statistically, the negative trade-off between eaten and released seeds was ubiquitous (see also Herre and West 1997), as expected in a conflictual relationship.

How are costs distributed across globeflowers?

All individuals do not share equally the cost of rearing pollinator larvae: some, generally large flowers, attract more eggs than others. However, despite large flowers being more heavily infected, and losing a higher absolute number of seeds to larvae, they still released the same proportion of seed after predation as did small flower heads (no effect of carpel number on net proportion of seed produced). Interestingly, individuals with no eggs had significantly fewer carpels than average. These individuals which benefit from pollination while losing fewer seeds to predation can hardly be viewed as cheaters; first, their probability of escaping from oviposition is directly linked to fly density in the population; second, their low investment in carpels reflects more the quality of their microenvironment and/or past reproductive history than a genetically different strategy. Indeed, carpel number is largely environmentally determined, as showed by the significant effect of year on carpel number, and by experimental nutrient supply: transplanted plants given a

nutrient supply 8 times higher than that in natural populations increased flower size by 33% (Å. M. Hemborg and L. Després, unpublished data). Carpel number is thus a plastic character. As globeflowers are long-lived plants, their flower size is likely to vary across years depending on their age and past reproductive history, so that the global cost of rearing pollinators' larvae throughout a plant's lifetime may not differ strongly across individuals.

Mechanisms of stabilization of the interaction

Despite the large fluctuations in fly density observed over time and space, larval predation costs and net seed production remain strikingly constant across populations. The lack of substantial variation in costs and net seed set among populations has been observed in other obligate mutualisms, and supports theoretical predictions for specialized and obligate interactions that selection pressures should be stronger than in facultative mutualisms on traits controlling partner overexploitation. In yucca and senita cactus both larval/egg intrinsic low survival and high probability of fruit abortion were shown to be important factors limiting seed predation and controlling for seed predator population size (Addicott 1986; Pellmyr and Huth 1994; Holland and Fleming 1999). In the case of the globeflower–globeflower fly interaction, egg/larval survival is high and there is no fruit abortion. The stability of the interaction comes from fly population regulation by density-dependent competition, as revealed by negative correlations between fly fitness and fly density: larval mass decreases with increasing number of larvae co-developing in a flower head (Després and Cherif 2004). At low fly densities, there is virtually no competition among larvae, and insect reproductive success is high, leading to an increase in pollinator population size the following generation. At high fly densities, larvae compete intensively but never to the point of destroying all the developing seeds: flower heads release part of their seeds before full development of larvae (Jaeger et al. 2001), therefore controlling pollinator population size by starving the larvae. Variation in carpel number leading to non-uniform egg distribution among globeflowers could be another plant trait involved in the manipulation of a pollinator's behaviour, enhancing the efficiency of pollination and intensifying larval competition. Furthermore, preliminary experiments suggest another way for globeflowers to control overexploitation by larvae: infected globeflowers over-produce a *C*-glycosyl flavone, and the concentration of this induced chemical increases with increasing number of larvae co-developing in a flower head (unpublished results). *C*-glycosyl flavones were shown to be involved in larval growth inhibition in the corn earworm (Wiseman et al. 1993).

In conclusion, this study shows that the globeflower–globeflower fly association is strongly mutualistic over a wide geographic area, with on average 74% of seeds initiated (range 49–94%), from which 64% (range 44–92%) are devoted to plant reproduction. The benefit-to-cost ratio varies little across populations, as previously found in other obligate mutualisms involving a plant and a seed-eating pollinator. Moreover, most analysed plants (92%) were parasitized by at least one larva, i.e. most plants contribute to sustain the pollinator population. However, costs are not equally distributed across globeflowers: large plants host more larvae and lose a higher absolute number of seeds to predation than smaller plants, but the proportion of seed eaten is independent from flower size. Flower size reflects the level of resource a plant can allocate to its reproduction in a given year, which is likely to vary throughout an individual's life. Individual plants pay proportionally for their current resource status to sustain the pollinator population.

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Plant Chemical Defense Induced by a Seed-Eating Pollinator Mutualist

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Abstract Plant-seed parasite pollination mutualisms involve a specific pollinator whose larvae develop by consuming a fraction of the host plant seeds. These mutualisms are stable only if the plant can control seed destruction by the larvae. Here, we studied the chemical response of the European globeflower *Trollius europaeus* to infestation by an increasing number of *Chiastocheta* fly larvae. We used liquid chromatographic analysis to compare the content of phenolic compounds in unparasitized and parasitized fruits collected in two natural populations of the French Alps, and mass spectrometry and nuclear magnetic resonance to elucidate the structure of adonivernith, a C-glycosyl-flavone. This compound is present in many of the organs of *T. europaeus*, but not found in other *Trollius* species. Furthermore, it is overproduced in the carpel walls of parasitized fruits, and this induced response to infestation by fly larvae is density-dependent (increases with larval number), and site-dependent (more pronounced in the high-altitude site). Mechanical damage did not induce adonivernith production. This tissue-specific and density-dependent response of *T. europaeus* to infestation by *Chiastocheta* larvae might be an efficient regulation mechanism of seed-predator mutualist population growth if it decreases survival or growth of the larvae.

Keywords Mutualism · *Trollius europaeus* (globeflower) · Seed predators · Adonivernith · Chemical defense · Phenolic metabolism

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Introduction

Mutualisms are widespread, ranging from plant pollinators to microbes hosted by plant roots or animal guts. Their long-term persistence is striking given that selection should favor individuals that increase their fitness at the expense of the mutualist partner. To counter this evolutionary instability, each partner must be able to prevent overexploitation by the other. Identifying the mechanisms that prevent overexploitation of one species by its partner is fundamental to understanding the evolutionary stability of mutualisms.

Some plants are highly specialized in pollination and use insects whose larvae feed on the developing seeds of the host plant. An increase in insect fitness (i.e., more eggs laid and more seeds eaten) is costly for plant seed production, creating a conflict of interest between the interacting species. The strategies evolved by plants to prevent overexploitation by seed predators include morphological traits, such as long styles preventing oviposition in figs, or yucca fruit morphology preventing seed access to yucca moth larvae, and fruit abortion in yucca and senita cactus (Pellmyr and Huth 1994; Holland and DeAngelis 2006). Surprisingly, although the chemical response of plants to damage from phytophagous insects through constitutive (Wittstock and Gershenzon 2002) and induced (Bennett and Wallsgrove 1994; Roda and Baldwin 2003) secondary compounds is an acknowledged phenomenon, the role of plant chemical defenses in regulating plant-seed eating pollinator mutualisms has so far been underexplored.

The European globeflower *Trollius europaeus* is exclusively pollinated by six species of flies (genus *Chiastocheta*), whose larvae feed only on globeflower seeds. The female deposits one to several eggs, on or between the carpels at various flower stages, depending on the species (Pellmyr 1989; Després and Jaeger 1999). The larva from each species has a specific location in the globeflower complex fruit, which is composed of several follicles (hereafter referred to as carpels). After hatching, the larvae develop on seeds throughout fruit maturation. Previous experiments have shown that the number of seeds eaten by each larva sharply decreases when the number of larvae per fruit increases, despite the fact that seeds are still available (Jaeger et al. 2001; Després and Cherif 2004). Moreover, larval mass is negatively correlated with the number of larvae co-developing in an infrutescence. Such density-dependent larval growth inhibition might result from the induction of a chemical defense by the attacked plant.

Among the secondary metabolites, phenolic compounds have traditionally been considered to play an important role in plant–insect interactions (Harborne 1999; Simmonds 2003) and can act as attractors, feeding deterrents, or toxic compounds (Summers and Felton 1994; Takemura et al. 2002). They have been identified as active inhibitors of larval growth for the larvae of corn earworm (*Heliothis zea*) (Elliger et al. 1980), autumnal moth (*Epirrita autumnata*) (Ossipov et al. 2001), and fall armyworm (*Spodoptera frugiperd*a) (Johnson et al. 2002; Urrea-Bulla et al. 2004). To determine whether plant phenolics could be involved in the control of seed predation in the *Trollius europaeus–Chiastocheta* system, we compared the phenolic profiles of unparasitized fruits vs. fruits naturally parasitized by an increasing number of larvae in two alpine sites at different altitudes. We found that a flavonoid is overproduced in the carpel walls of parasitized fruits. We quantified the variation of this compound in several *Trollius* species, in various plant organs, and at different developmental stages. Mechanical damages were performed on unparasitized plants to test for the specificity of the plant response. We discuss the potential implication of this tissue-specific, density-dependent induced chemical compound in the evolutionary stability of this obligate plant-seed eating mutualism.

Material and Methods

Study Species The European globeflower *Trollius europaeus L.* (Ranunculaceae) is a hermaphroditic arctic-alpine perennial species growing in moist meadows (Després and Jaeger 1999; Jaeger et al. 2000). Two study sites were chosen in two mountain massifs in the French Alps (Chartreuse and Galibier), 100 km apart. These two sites (Cherlieu, 950 m a.s.l. 5°46′E 45°18′N and Galibier 2,300 m a.s.l. 6°24′E 45°04′N) below and above the tree line, respectively, were previously extensively studied (Jaeger and Després 1998; Hemborg and Després 1999; Jaeger et al. 2000, 2001; Després et al. 2007).

Phenolic Profiles of Parasitized vs. Unparasitized Fruits To obtain unparasitized fruits, flower buds were bagged with a nylon mash bag and hand pollinated with pollen from several donors. Parasitized and unparasitized fruits were sampled 3 wk after flowering in 2003, June 18th in Cherlieu (N=15 and N=30, respectively) and July 8th in Galibier (N=11 and N=36, respectively). Back in the laboratory, the fruits were dissected immediately, and the developing larvae were counted and discarded. From each fruit, three parts (the carpel walls, seeds, and floral receptacle) were carefully separated and individually stored at −18°C until they were analyzed. A first subset of 22 fruits from Cherlieu (eight without larva, seven with one larva, and seven with several larvae) was submitted for an analysis of total phenolic compounds, which was carried out on each of the three fruit components. Based on preliminary results (see below), the entire set of carpel walls was submitted for complementary analysis (phenolic acids and flavonoids). This individual procedure and the tiny size of some of the samples meant that dry weight could not be measured. Therefore, all results were expressed in fresh weight (FW). Five of the larvae were pooled, stored at − 18°C, and analyzed for phenolic content.

Each sample was weighed and separately extracted twice with 50 ml of an ethanol–water (50:50) mixture under reflux. After evaporation under vacuum, the residue was redissolved in a precise volume of distilled water, filtered (0.45 μm), and used for Folin-Ciocalteu colorimetric determination of total amounts of phenolic compounds (Marigo 1973).

Aliquots (20 μl) of the ethanolic solution were used for high performance liquid chromatography (HPLC) analysis on a RP C18 μBondapak column, 4.6×250 mm, and gradients were monitored by a Waters 600 Controller. Solvent A was acetic acid 0.5% in distilled water and solvent B acetic acid 0.5% in acetonitrile. Phenolic acids were determined by using a linear gradient from 0 to 20% of B in A in 45 min, with a 1.5 ml min^{-1} flow. The spectra were recorded with a Waters 996 PDA in the 200- to 400-nm range. Chromatographic peak areas were determined at 280 nm. Areas were compared after multiplying the area by the volume of extraction corresponding to each carpel. Standards of common phenolic acids and aldehydes were obtained from Sigma. Flavonoids (including adonivernith) were separated with an isocratic flow (1.5 ml min^{-1}) of 20% of B in A. The peak areas were recorded at 354 nm. Standards (luteolin, orientin, homorientin) were obtained from Extrasynthese (Lyon, France). The standards were prepared in methanol as stock solutions at 1 mg ml^{-1}.

Structural Elucidation of Flavonoid Liquid chromatography-mass spectrum (LC-MS) analysis: ten ethanolic extractions were pooled, concentrated, and used for mass determination with electrospray ionization MS (ESI-MS) on the positive mode [M+H]. Mass spectra were recorded from a ZQ Waters Mass detector (electrospray voltage 4.2 kV) coupled to a μBondapak RP C18.

Nuclear magnetic resonance (NMR) analysis: To obtain sufficient amounts of flavonoid, 75 g FW of frozen flowers were extracted with methanol (70:30) under reflux for 30 min. The methanolic extract was filtered and evaporated. The aqueous residue was successively extracted with petroleum ether and ethyl acetate. The ethyl acetate fraction was evaporated

to dryness, redissolved in MeOH, and fractionated by gel filtration using Sephadex LH-20 (Amersham, 3.5×50 cm, MeOH, 2 ml min^{-1}) to obtain 40 fractions. The fractions were monitored by HPLC, and fractions containing adonivernith were combined and dried.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 and a Varian Unity+ 500, respectively, in MeOH-d_4 as solvent at 303 K. Standard pulse sequences and parameters were used for the experiments (COSY, DEPT, HMQC, HMBC, TOCSY, and NOESY). Chemical shift references were given from the solvent resonances at 3.30 ppm for ^{1}H and 49 ppm for ^{13}C.

Adonivernith Occurrence in T. europaeus and in Other Trollius Species To assess the distribution of adonivernith within the whole *T. europaeus* plant, six flowering plants (without larvae) were collected in Cherlieu in 2004 and dissected into seeds, floral receptacle, leaves, staminodias, sepals, carpels, and stamens. The six samples were pooled and extracted by ethanolic mixture as described above.

The kinetics of the synthesis of adonivernith by the whole flower was followed over five developmental stages (N=3), from green bud to old fruits (21 d after flowering). The entire organ (flower or fruit) was analyzed for flavonoid content as previously described. Fruits of three other species of *Trollius of Asian Origins Trollius chinensis*, *T. ledebouri*, and *T. pumilus* were collected during summer 2005 in the Alpine Garden of the Joseph Fourier station (Col du Lautaret, 2,100 m a.s.l.) and stored frozen (−18°C) until analysis.

Mechanical Damage Mechanical damage was inflicted in two ways: first, low-intensity wounds were inflicted in Cherlieu by piercing the carpel walls or the stem (to measure a systemic response) with a needle; and second, high-intensity wounds were inflicted in Galibier by incising the carpel wall with a scalpel (five incisions). In Cherlieu, 59 young floral buds were bagged and hand-pollinated 1 wk later. Ten holes were pierced on the stems of 19 flowers and on the carpel walls of 21 other flowers; this treatment was repeated 1 wk later. Three-week-old fruits were collected, and stored at −18°C until they were analyzed. In Galibier, incisions were made on 25 flowers. The wounded flowers and 21 control flowers (at the same developmental stage) were bagged to prevent infestation. Fruits were collected 11 d later and kept frozen (−18°C) until they were analyzed.

Statistical Analysis Data were checked for normality of distribution and homogeneity of variances (Kolmogorov–Smirnov and Levene tests). Where the data did not satisfy these criteria, nonparametric tests were performed (e.g., Spearman's rank correlations R_s, Mann–Whitney tests). Otherwise, t tests were used to compare parameter of parasitized and intact fruits. To examine whether globeflowers from the two sites differed in their chemical response to increasing larval numbers, the slopes of the correlations between the number of larvae and the flavonoid amounts were compared in Cherlieu and Galibier by performing an analysis of covariance (ANCOVA), testing for the effect of population, larvae number, and interaction on the amount of flavonoid. A significant interaction indicates that the slopes are different within each population.

Results

Phenolic Composition of Parasitized versus Unparasitized Fruits Concentrations of total phenolic compounds did not differ significantly in seeds from unparasitized vs parasitized fruits collected in Cherlieu (8.6±0.7 vs. 9.1±1.0 mg g^{-1} FW) (Mann–Whitney test, U=54,

$P>0.05$), or in the floral receptacle (7.3±1.0 vs. 8.5±0.6 mg g^{-1} FW) ($U=39$, $P>0.05$). In contrast, the carpel walls of fruits infested by one or several larvae exhibited higher concentrations of total phenolic compounds (7.8±0.4 mg g^{-1} FW) than intact, uninfested fruits (5.7±0.5 mg g^{-1} FW) ($U=28$, $P<0.01$). As a single larva contained only 0.024 mg of total phenolic compounds, this increase is not caused by larvae excretion (the larvae were discarded during dissection).

Further analyses were, therefore, carried out on the carpel walls only. Because the number of larvae developing in a globeflower increased with carpel number and with the mass of the carpels (Fig. 1) ($R_s=0.477$, $P<0.01$, $N=29$ at Cherlieu; $R_s=0.603$, $P<0.01$, $N=26$ at Galibier), we preferentially assessed phenolic synthesis by measuring the absolute amount per fruit rather than the concentration.

The two populations did not differ in terms of total amounts of phenolics in unparasitized carpels (Mann–Whitney test, $U=63$, $P>0.05$), but the concentration was higher in Galibier (7.8±0.6 mg g^{-1} FW) than in Cherlieu (5.4±0.3 mg g^{-1} FW) ($U=30$, $P<0.01$). This was because of the higher carpel mass in Cherlieu ($m=0.31\pm0.04$ g) compared to Galibier ($m=0.18\pm0.03$ g). The total amount of phenolics per fruit (Fig. 2) was correlated to larval number per fruit in each of the two study populations ($R_s=0.562$, $P<0.05$, $N=45$ at Cherlieu; $R_s=0.705$, $P<0.01$, $N=47$ at Galibier). The highest amount per fruit (6.0 mg) was recorded in Galibier in a fruit parasitized by six larvae and was four times higher than the mean amount observed in unparasitized fruits from the same site (1.3±0.2 mg). To determine which fraction was responsible for the increase of total phenolics with the increasing number of larvae, we carried out a chromatographic analysis adapted to detection of phenolic acids and flavonoids.

Seven compounds were recorded when using the chromatographic conditions for phenolic acid determination (Table 1). These compounds could not be identified as any of the most common phenolic aglycones used as standards, probably because they occur as glycosides. Because unparasitized and parasitized fruits did not differ in the relative area of each compound (Mann–Witney tests, $P>0.05$), no further attempt was made to identify them.

Five flavonoids had areas high enough to be recorded on the chromatogram (Table 2). Concentrations of the five compounds significantly increased with larval number per fruit in Galibier, whereas the concentration of only one flavonoid (F3) significantly increased in Cherlieu. This compound, characterized by a retention time of 5.4 min, also exhibited the

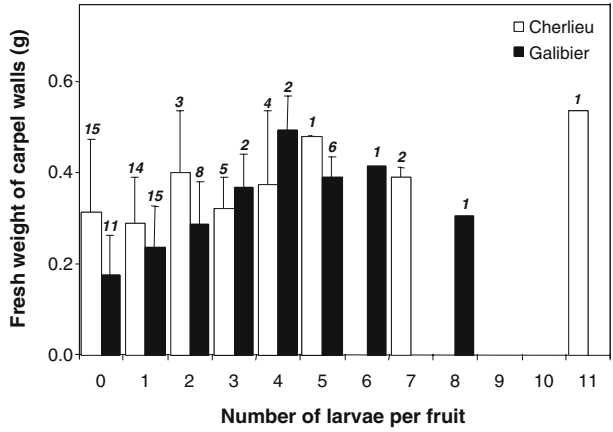

Fig. 1 Fresh weight (mean + SE) of carpel walls according to the number of larvae per fruit for the two populations: Cherlieu ($N=45$) and Galibier ($N=47$). Sample numbers (italics) are indicated above the bars

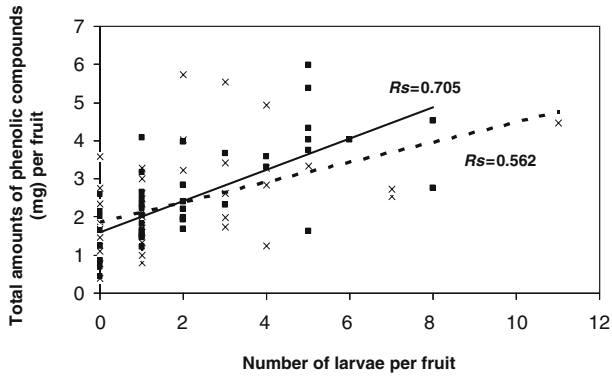

Fig. 2 Spearman correlation (*Rs*) between total amounts of phenolic compounds and the number of larvae in fruits of *T. europaeus* from low altitude (Cherlieu, x, dashed line) (N=45) and high altitude (Galibier, ■, solid line) (N=47) sites. Total amounts of phenolic compounds (Folin-Ciocalteu) were expressed in milligrams of gallic acid equivalent per fruit

highest area (68–79% of the total area). It was selected for structural identification and further identified as adonivernith (see below). Moreover, total amounts of phenolics and amounts of adonivernith were highly correlated (R_s=0.786 and 0.691, P<0.001, in Cherlieu and Galibier, respectively). F2 and F4 have the same UV spectra as F3, indicating the same flavone basis.

In unparasitized fruits, the amount of adonivernith per fruit was slightly higher in the population at low altitude (Mann–Whitney test, U=45, P=0.051) because of larger fruits, but no differences were detected when concentrations were compared between the two populations (U=68, P>0.05). In contrast, in parasitized fruits, the amounts of adonivernith (and the concentrations) were higher at the high altitude site: 117±13 μg in Galibier vs. 62± 6 μg in Cherlieu (U=245, P<0.005). The highest amount of adonivernith (331 μg) was observed in Galibier in a fruit parasitized by five larvae, and represented 17 times the mean amount (19 μg) in unparasitized fruits. In Cherlieu, the highest increase was only by a factor of 4 (from 38 μg for the mean amount in unparasitized fruits to 151 μg for the highest amount). The stronger chemical response to larval infection of plants at the high-altitude site was confirmed by significantly different slopes for the correlation between the number of larvae and the amounts of adonivernith induced in Galibier and in Cherlieu: R_s 0.851 vs. R_s

Table 1 Peak areas (mean ± SE) of phenolic acid like compounds obtained by chromatographic analysis (HPLC-DAD) of intact (N=5) and parasitized (N=5) carpel wall extracts

Phenolic Compounds	Peak Areas[a]	
	Without Larvae	With N>1 Larvae
P 1[b]	537±137[c]	1325±159
P 2	858±194	2021±177
P 3	574±87	825±102
P 4	782±260	1262±128
P 5	1071±289	1419±300
P 6	1867±403	914±273
P 7	1057±276	1241±244

[a] Peak areas were recorded at 280 nm.

[b] The order of the phenolic compounds in this table followed their order of elution.

[c] No significant differences were detected between the two groups.

Table 2 Retention time (rt), proportion of the peak areas of flavonoids (percent of total area), and spearman correlation coefficients (*Rs*) between number of larvae and peak areas for the five flavonoids obtained by chromatographic analysis (HPLC-DAD) of parasitized carpel wall extracts from cherlieu (*N*=45) and Galibier (*N*=47) populations

Flavonoids	R_t (min)	Percent Total Area [a]		R_s [b]	
		Cherlieu	Galibier	Cherlieu	Galibier
F1	3.5	8	4	0.010	0.491**
F2	4.9	6	5	0.346	0.694***
F3	5.4	68	79	0.479*	0.851***
F4	7.2	16	9	0.117	0.543***
F5	8.5	2	3	-0.156	0.532**

[a] Peak areas were recorded at 350 nm.

[b] Levels of significance: *P<0.01; **P<0.005; ***P<0.001

0.479, respectively (Fig. 3) (ANCOVA, larval number: $F_{1,\ 85}$=39.01 P<0.001; population $F_{1,\ 85}$=4.32, P=0.04; larval number by population interaction: $F_{1,\ 85}$=11.77, P<0.001).

Structural Elucidation of Adonivernith The UV spectra recorded from HPLC-DAD analysis gave λ_{max} 256(sh), 270, 350 nm, characteristic of the flavone luteolin. The positive ion mode MS spectra gave an $[M+H]^+$ ion at *m/z* 581, indicating a possible derivative of luteolin containing one hexose and one pentose substitute. ^{13}C and ^{1}H NMR were required to allow characterization of the linkage and configuration of the sugar moieties. The compound was identified as luteolin 8-β-D-glucopyranosyl-2″-*O*-D-xylopyranoside (or orientin 2″-*O*-D-xylopyranoside), and the spectral data are in good agreement with respective literature data (Wagner et al. 1975; Markham et al. 1987). This compound was previously named adonivernith from its first identification in *Adonis vernalis* (Hörhammer et al. 1960).

Mechanical Induction of Phenolic Metabolism To measure the specificity of the phenolic response as previously demonstrated, we carried out two experiments with mechanical damage of different intensities on flowering globeflowers and analyzed the production of adonivernith in the carpel walls (Fig. 4). Compared to the control plants, no differences were observed between the amount of adonivernith in the carpel walls pierced by a needle (*t* test, t=0.766, P>0.05), nor in those more seriously injured by using a scalpel (t=1.714, P>0.05), nor when the floral stem was pierced (t=0.563, P>0.05). None of these wounds influenced carpel mass.

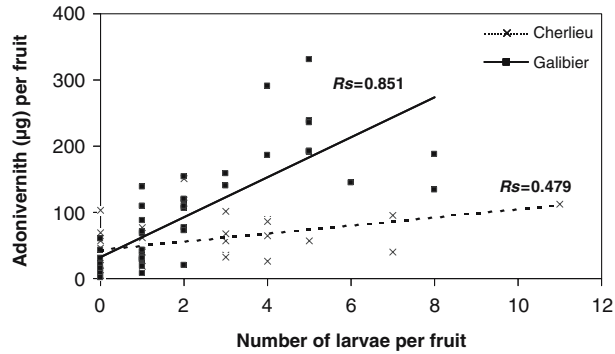

Fig. 3 Spearman correlation (*Rs*) between adonivernith amounts and the number of larvae in fruits of *T. europaeus* from low altitude (Cherlieu, x, dashed line) (*N*=44) and high altitude (Galibier, ■, solid line) (*N*=47) sites. Adonivernith amounts were expressed in micrograms of luteolin equivalent per fruit

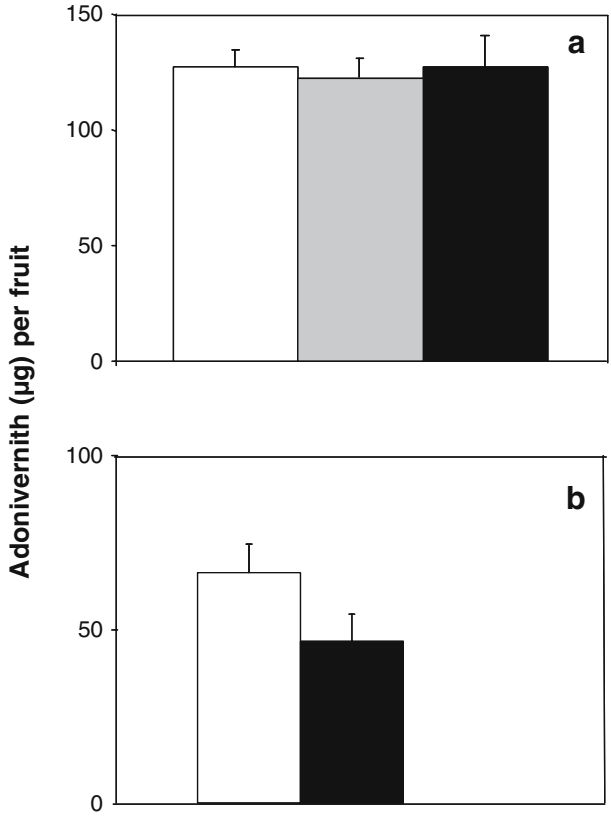

Fig. 4 Amounts (mean + SE) of adonivernith in carpel walls of *T. europaeus* mechanically damaged. (**a**) In Cherlieu, wounds (10 holes) were inflicted with a needle (= low wound) on the carpel (■ black, *N*=19) or on the stem (▒ gray, *N*=21) and compared to control plants (□ white, *N*=19). (**b**) In Galibier, wounds (five incisions) were inflicted with a scalpel (= severe wound) on the carpel (■ black, *N*=21) and compared to control plants (□ white, *N*=25). Adonivernith amounts were expressed in micrograms of luteolin equivalent. No statistically significant differences were observed

Adonivernith Occurrence in T. europaeus and Other Species The fruits of three other *Trollius* species (*T. chinensis*, *T. ledebouri*, and *T. pumilus*) did not contain adonivernith in detectable amounts. However, *T. chinensis* extracts contained two compounds characterized by close retention time (4.6 and 6.1 min, respectively) and similar UV spectra when compared to adonivernith, indicating the likely occurrence of the luteolin flavone.

In *T. europaeus*, adonivernith was not present in the seeds and the floral receptacle, but was found in all the other floral parts and in the leaves (Table 3). Its concentration in the yellow sepals (3.9 mg g^{-1}) was especially high. Except for staminodias, adonivernith was generally more concentrated in all the flowering parts than in unparasitized mature carpels previously analyzed (0.11 mg g^{-1}). The decrease in adonivernith concentrations during the maturation process (Table 4) was also observed when the complete flowers at different developmental stages (from buds to mature fruits) were analyzed.

Discussion

This study is the first to show that larvae of a specific mutualist can induce a chemical plant response, suggesting a means of partner control to prevent overexploitation. This is also one of a few studies on induced plant defenses that have been conducted in field conditions,

Table 3 Concentration of adonivernith in the different organs of *T. europaeus* plants (N=6, pooled) collected at 1-day flower stage

Plant Organs	Adonivernith (mg of luteolin eq./g)
Leaf	0.83
Floral receptacle	_[a]
Sepal	3.88
Staminodia	0.09
Carpel wall	0.24
Stamen	0.20
Seed	_[a]

[a] Below the detection limit

rather than in controlled environment. Therefore, it was not possible to set up a control for the abiotic environmental conditions (e.g., UV intensity) nor for the biotic stresses other than *Chiastocheta* parasitism (e.g., pathogens) that might influence plant phenolic metabolism (Koricheva et al. 1998; Reifenrath and Muller 2007). For instance, adonivernith concentrations in intact carpel walls ranged from 52 to 394 μg g^{-1} in Cherlieu, and from 7 to 355 μg g^{-1} in Galibier. Large variability in plant secondary metabolite production, presumably under both genetic and environmental determinism, is a well-documented phenomenon, and is consistent with previous findings that the chemical profiles of the roots of *Caltha leptosepala* and *Trollius laxus* are highly influenced by habitat characteristics (Kuhajek et al. 2004). Despite these field constraints, individual fruit analyses showed that *Chiastocheta* larval development enhances phenolic metabolism in the carpel walls of the globeflower, and that this increase in content of phenolics is mostly caused by a C-glycosyl flavone that we identified as the rare adonivernith.

The occurrence of luteolin and of C-glycosylflavones in *T. europaeus* leaves has previously been reported (Gonnet 1981; Lebreton 1986), but this is the first report of its presence in several organs of this plant. In the *Trollius* genus and in the closely related genus *Adonis* (Després et al. 2002), the classical flavonols and *O*-glycosyl flavonols are largely replaced by the less widespread flavones and C-glycosyl flavones (Jensen 1995). Adonivernith was first isolated from *Adonis vernalis* (Hörhammer et al. 1960) and more recently from *Trollius macropetalus* (Liu et al. 1992), but was also reported in the Poaceae *Setaria italica* (Harborne and Baxter 1999). *T. chinensis* and *T. ledebouri* have been more extensively studied than *T. europaeus*, and C-glycosyl flavones structurally related to adonivernith (like vitexin and orientin) have been found in their flowers (Liu et al. 2004;

Table 4 Adonivernith concentration (mean ± SE) (N=3) of *T. europaeus* flowers at different developmental stages

Developmental Stage of the Flower	Adonivernith (mg of luteolin eq./g)
Green floral bud	2.38 ± 0.76^{a}
1-day flower	1.09 ± 0.49^{ab}
7-day flower (nonpollinated)	0.71 ± 0.24^{b}
7-day flower	0.64 ± 0.11^{b}
14-day fruit	0.08 ± 0.02^{c}
21-day fruit	0.04 ± 0.04^{c}

[a] Different superscript letters indicate significant differences (Mann–Whiney test, P<0.05) between the developmental stages

Zou et al. 2005; Li et al. 2006). However, we found no adonivernith in these two species, nor in *T. pumilus*, indicating species specificity.

Adonivernith has been found in significant amounts in most organs of *T. europaeus* plants, especially in the sepals where it probably contributes to the bright yellow coloration. Phenolic compounds found in flowers play both attractive and defensive functions (Gronquist et al. 2001) leading to the question of the significance of the correlation between mutualism-linked traits (attractive colors) and antagonism-linked traits (defensive compounds) (Herrera et al. 2002). However, adonivernith induction by larval infestation seems restricted to the carpel walls. Other minor flavonoids also participate in the globeflower-induced response, but their contribution to the total flavonoid production is low. At least two of them are structurally close to adonivernith, i.e., luteolin-based C-glycosyl flavones, indicating possible metabolism intermediates.

Another argument to support the specificity of this chemical plant response to larval infestation is the lack of induced response to mechanical damage, whatever the intensity and the location of the wounding. *Chiastocheta* larval regurgitant might be required to trigger plant-induced response as found in other plants (Alborn et al. 1997). The increase in adonivernith production observed in parasitized fruits might be an efficient defense mechanism if it affects the survival or growth of the larvae. Another luteolin-based C-glycosyl flavone, maysin, induced after infestation of corn by caterpillars of corn earworms (*Helicoverpa zea*) reduces larval growth (Elliger et al. 1980; Wiseman et al. 1993). Such a growth inhibitor effect by adonivernith on *Chiastocheta* larvae has not been demonstrated. So far, experimental bioassays have been limited by the difficulty of breeding *Chiastocheta* larvae in the laboratory. Preliminary experiments have nevertheless been conducted during short time periods: *Chiastocheta* larvae exposed on paper imbibed with carpel extracts for 4 hr did not exhibit increased mortality, compared to larvae on watered paper. In the same manner, choice experiments showed no deterrence of those extracts. The observations in the field that larval survival is independent from the number of larvae developing in a fruit (Jaeger et al. 2001), and that individual larval fresh mass at the end of development decreases according to that number, although seeds are not limited (Després and Cherif 2004), supports the hypothesis that the induced compound inhibits larval growth rather than kills the larvae.

The present study also shows that the increase in adonivernith production is proportional to the number of larvae developing within the infrutescence. Recent empirical and theoretical studies suggest that density-dependent processes control mutualistic interactions (Holland and DeAngelis 2006). For example, yucca moth density was shown to be regulated by selective abortion of highly parasitized fruits (Pellmyr and Huth 1994). These results were later challenged by several studies that showed that fruit abortion was random in yucca (Addicott 1998; Shapiro and Addicott 2004); however, although random, fruit abortion might limit and regulate yucca moth population growth through density-dependent recruitment. Similar density-dependent population level processes are believed to occur in the senita cactus–senita moth mutualism (Holland and DeAngelis 2006). The density-dependent chemical response of globeflowers might limit fly population growth at densities where the interaction is still mutualistic, which could contribute to the interaction stability.

We also found that adonivernith overproduction is tissue specific: only the carpel walls of parasitized fruits show an increase in the chemical defense, whereas the seeds and the floral receptacle remain unchanged. Such carpel-specific accumulation of phenolic compounds has been observed in *Hypericum calycinum*, raising the question of the development of specific chemical defensive profiles by plant carpels (Gronquist et al. 2001). Although larvae of all *Chiastocheta* species feed on *T. europaeus* seeds, the larvae

from each species are spatially partitioned within a single infrutescence, either in the floral receptacle (the first ovipositing species), or mining their way through the inner or outer carpels (the five remaining species) (Després and Jaeger 1999; Pompanon et al. 2006). The first species infesting the fruit occupies the only undefended part of the fruit (adonivernith-free refuge), whereas all the other species appear to be more exposed to adonivernith. The tissue-specific differential induction of adonivernith after larval infestation creates a heterogeneous chemical environment within a single fruit ("chemical niches") that might have selected for different fly resistance strategies, such as escape to a phenolic-free refuge for the first visitor, and evolution of metabolic resistance for later visitors. This hypothesis remains to be tested by evaluating the differential resistance of early vs. late *Chiastocheta* species to adonivernith exposure. If verified, this mechanism might explain the unusually large number of congeneric flies coexisting on *T. europaeus* fruits.

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RESUME

Traits morphologiques et biochimiques impliqués dans la spécialisation de *Trollius europaeus* sur les pollinisateurs prédateurs de graines *Chiastocheta spp*.

Les interactions entre espèces sont un moteur d'évolution. Nous montrons ici quels sont les traits morphologiques et biochimiques du trolle d'Europe qui ont évolué au cours de sa spécialisation (*Trollius europaeus*) vis-à-vis des mouches pollinisatrices et prédatrices de graines (*Chiastocheta spp.*). La forme globulaire de la fleur est décisive dans l'attraction spécifique des chiastochètes. En comparaison avec une forme artificiellement ouverte, les fleurs globulaires, bien que souffrant plus de la prédation produisent plus de graines (4%), mais surtout elles exportent plus de pollen (85%). Un modèle de dynamique adaptative montre que l'évolution de la forme globulaire requiert non seulement une efficacité minimale de la pollinisation par les chiastochètes, par rapport à des pollinisateurs alternatifs qui ne consomment pas de graines, mais également une efficacité maximale : si les chiastochètes sont « trop » efficaces, en attirer beaucoup plutôt que quelques uns ne confère pas d'avantage. L'attraction des pollinisateurs se fait également par des signaux olfactifs. Plusieurs composés volatils émis par le trolle déclenchent une réponse électrophysiologique chez les chiastochètes (methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, linalool). Des observations de visites de chiastochètes en conditions naturelles ont montré que la variabilité des composés volatils présents dans les fleurs expliquait une part de la variabilité des visites reçues par ces fleurs, en comparaison avec des traits morphologiques et pigmentaires. Les interactions entre une plante et des prédateurs de graines sont conflictuelles : la plante à intérêt à soustraire les graines de l'appétit des larves. Un glycoside du flavonoïde lutéoline, l'adonivernith, s'accumule dans les parois des carpelles lorsque les dégâts causés par les larves augmentent, avec comme conséquence une baisse de l'intensité de prédation. Les six espèces du genre *Chiastocheta* étudiées induisent et réagissent différemment à l'adonivernith, cette molécule pourrait donc être impliquée dans la radiation sympatrique du genre. Les traits impliqués dans la spécialisation du trolle sur les chiastochètes sont donc à la fois mutualistes (morphologie globulaire et composés volatils de la fleur) et antagonistes (défense chimique contre les larves). Les contradictions de cette mosaïque de traits sont un moteur d'évolution.

Mots-clefs

mutualisme, coévolution, *Trollius europeaus*, *Chiastocheta*, dynamique adaptative, écologie chimique, pollinisation

ABSTRACT

Morphological and biochemical traits involved in the specialisation of *Trollius europaeus* on the seed-eating pollinators *Chiastocheta spp*.

Interactions between species are a major driving force in evolution. We show here which morphological and biochemical traits evolved during the specialisation of the European globeflower (*Trollius europaeus)* on seed-eating pollinator flies (*Chiastocheta spp*). The globular shape is a key factor in the specific attraction of chiastochetes. Globular flowers produce more seeds (4%, they suffer higher predation but are better pollinated) and moreover export more pollen (85%) than artificially open flowers. An adaptive dynamics model shows that the evolution of the globular shape requires a minimal pollination efficiency by chiastochetes relatively to alternative pollinators that do not eat seeds, but also a maximal efficiency: if the chiastochetes are "too" efficient, to attract a lot of them rather than a few confers no advantage. The attraction of pollinators is also mediated by olfactive signals. Several volatile compounds emitted by the globeflower trigger an electrophysiological response in chiastochetes (methyl salicylate, Z-jasmone, β-caryophyllene, germacrene D, E,E-α-farnesene, linalool). Field behavioural observations of chiastochetes visits have shown that the variability of the volatile compounds inside the flowers explains a part of the variability of the visits, together with morphological and pigmentation traits. Interactions between plants and seed predators are conflictual: the plants tend to reduce predation costs. A flavonoid close to luteolin, adonivernith, accumulates in the carpel walls when the damages caused by the larvae increase, leading to a reduction of predation intensity. The six *Chiastocheta* studied species have different exploitation patterns in the fruit, they induce and are affected by adonivernith in specific ways: this chemical defence could be involved in the sympatric speciation of the genus. The traits involved in the globeflower specialisation on chiastochetes are simultaneously mutualistic (globular floral morphology, floral colour and volatile compounds) and antagonistic (chemical defence against the larvae). The contradictions of this trait mosaic are a factor of evolution.

Key-words

mutualism, coevolution, *Trollius europeaus*, *Chiastocheta*, adaptive dynamics, chemical ecology, pollination