

Patterns of floral colour neighbourhood and their effects on female reproductive success in an *Antirrhinum* hybrid zone

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Abstract

The maintenance of genetic integrity of parental populations is often explained by selection against hybrids. However, the selection agents are usually unknown. The role of environmental biotic interactions is often suspected but has rarely been demonstrated. In plants for instance, mutualism with pollinators may be involved. After verification that pollen deposition is a limiting factor for fruit set, we used an individual-based study and a representation of pollinator colour perception to test the effects of local plant density and floral colour neighbourhood on female reproductive success in an *Antirrhinum* hybrid zone. In addition to flower colour and density effects, the composition of the floral neighbourhood was found to influence fruit set, suggesting that most plants were usually better fertilized when similar to their neighbours. However, the plants of one particular type were sometimes favoured when very different from their neighbours. The implications for hybrid zone dynamics are discussed.

Introduction

Secondary contact between partially reproductively isolated populations often leads to the formation of narrow hybrid zones (Arnold, 1997), where genetic differences between parental populations are maintained in spite of gene flow (Barton & Hewitt, 1985). The maintenance of genetic integrity of parental populations is often explained by selection against hybrids (but see Arnold & Hodges, 1995). One prominent theory (Key, 1968; Barton & Hewitt, 1985) assumes that hybrids are intrinsically unfit owing to genetic incompatibilities between the parental genomes. Many recent studies have shown that selection against hybrids may often be environment dependent (Hatfield & Schluter, 1999; Campbell & Waser, 2001; Johnston *et al.*, 2001; Fitzpatrick & Shaffer, 2004). The distinction between these two categories of models is useful, because they correspond to two very different selection mechanisms with distinct and testable predictions. A large number of studies have focused on how hybrid fitness relates to abiotic environmental

factors but few have examined in detail the influence of biotic factors (Rundle & Nosil, 2005). The role of the biotic environment in selection may arise from very different mechanisms operating at the intra- or interspecific levels such as mutualism, competition or predation, all of which having the potential, either singly or through interactions with other factors, to generate complex patterns of fitness variation within hybrid zones.

In animal-pollinated flowering plants, plant–pollinator interactions can greatly influence the dynamics of hybrid zones. Pollinators may behave differently in different parts of the hybrid zone, reacting to change in the mean and variance in floral phenotypes. Hybrid zones are structured, with maximum diversity of floral phenotypes at their centre and a gradual shift from one parental phenotype to the other as one moves across the zones (Barton & Hewitt, 1985; Arnold, 1997). Floral neighbourhoods vary greatly in terms of phenotypic diversity and frequency of particular types at a biologically meaningful spatial scale, which can be expected to affect pollinator behaviour. If pollinators prefer to visit common floral types (Levin, 1972), then plants with many morphologically similar neighbours could receive more visits than plants surrounded by dissimilar neighbours. Such frequency-dependent selection may strongly affect hybrid zone dynamics. If positive and symmetrical, i.e.

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favouring the most common morph at both ends of the hybrid zone, frequency-dependent selection may play a prominent role in maintaining distinctions between hybridizing populations (Mallet & Barton, 1989). For example, a lower reproductive success or a higher predation risk of rare hybrid phenotypes could contribute to the reproductive isolation of parental taxa (Turelli *et al.*, 2001). On the contrary, negative, frequency-dependent selection should favour introgression between lineages. As well as the floral diversity of the neighbourhood, local densities may also vary across hybrid zones (Barton & Hewitt, 1985) and pollinators may behave differently at different plant densities (Kunin, 1997; Mustajärvi *et al.*, 2001; Bernhardt *et al.*, 2008). Density-dependent selection has a less predictable effect but may complicate the selection pattern in hybrid zones.

Although frequency-dependent and density-dependent foraging behaviour seems usual in pollinators (Smithson & Macnair, 1997), few studies have examined their possible selective consequences in plant hybrid zones (but see Nagy, 1997; Hauser *et al.*, 2003). Here, we investigated the potential for biotic environment-dependent selection in an *Antirrhinum* hybrid zone between two subspecies displaying different flower colours and sharing pollinators. The magenta-flowered *A. majus pseudomajus* and the yellow-flowered *A. m. striatum* have parapatric distributions. In places where they meet, hybrid zones sometimes occur. In one of these contact zones, an exceptional variability in flower colour has been observed. On the basis of molecular evidence, Whibley *et al.* (2006) proposed that selection operates on flower colour combinations. However, the mechanisms of selection remain largely unknown. Several lines of evidence suggest that pollinators are likely candidates as agents of selection. First, *A. majus* is self-incompatible and pollen compatibility between subspecies is very high (Andalo *et al.*, 2010). The genetic composition of pollen loads arriving on stigma was not found to influence seed production and the female fitness component may thus be directly related to pollinator preference. Secondly, *A. majus* is exclusively pollinated by bees (Vargas *et al.*, 2010). Lastly, bees are able to perceive the expressed phenotypic diversity in flower colour within the centre of the hybrid zone (Tastard *et al.*, 2008). To test whether pollinators indeed act as selective agents within this hybrid zone, we verified that female reproductive success is pollen and pollinator limited by carrying out a field pollen supplementation experiment. Moreover, we investigated how individual fruit production varied with plant density and frequency of flowering types in the neighbourhood. We specifically addressed the following questions: (i) How do flower colour, flower colour neighbourhood, local conspecific plant density and their interactions influence fruit set? (ii) Are the selection processes homogeneous over the years?

Material and methods

Biological system

Antirrhinum majus is a hermaphroditic, self-incompatible, short-lived perennial (1–3 years) that produces annual inflorescences with zygomorphic, personate, flowers. The corolla is made of two lobes that close the flower, and only large bees (mostly *Bombus lucorum*, *B. hortorum*, *B. lapidarius* and *Xylocopa violacea*) are able to get access to the nectar. Here, we studied two Pyrenean subspecies of the *A. majus* complex (*Antirrhinum* subsection *Antirrhinum sensu* Rothmaler, 1956): *A. m. pseudomajus* with magenta flowers and *A. m. striatum* with yellow flowers. These subspecies are considered as sister taxa. They occur parapatrically in the eastern part of the Pyrenees (Fig. 1a) where they come into contact in a zone stretching 150 km along the Sierra del Cadi in Catalonia, Spain (Whibley *et al.*, 2006). A number of herbarium specimens show that this contact zone has existed for a large number of generations (C. Thébaud, pers. obs.), an observation consistent with the hypothesis that it originated through ancient secondary contact between populations that had diverged in allopatry (Rothmaler, 1956). In the Pyrenees, *A. m. pseudomajus* and *A. m. striatum* reach 1900 m altitude and form a sharp and clinal hybrid zone along two parallel roads wherever they meet closely along the contact zone, regardless of environmental features such as altitude, rainfall, soil type and human disturbance (C. Andalo and C. Thébaud, unpublished data).

Analysis of selection within the hybrid zone comes up against a few problems that need to be specifically addressed. First, when moving from one parental population to the other, flower colour varies continuously. Deciding where the hybrid zone starts and where it finishes is therefore a nontrivial task. Second, the diversity in flower colour in the hybrid zone is exceptional, with continuous variation among magenta, yellow, white, pink and orange phenotypes (Tastard *et al.*, 2008). Colour variation therefore needs to be quantified so that the natural history aspects of the system can be properly documented. For instance, account must be taken of pollen limitation of plant reproduction and analysis of fruit set variation with regard to local plant density, colour neighbourhood and floral colour phenotype. Each of these issues is addressed below.

Delineation of the centre of the hybrid zone

The hybrid zone located in the Toses valley was studied along a transect following a road stretching from yellow-flowered to magenta-flowered plants. In 2002, 2004, 2007 and 2010, plants ($N = 577, 598, 348$ and 2206, respectively) were mapped by Global Positioning System (GPS) across the hybrid zone and scored visually for the

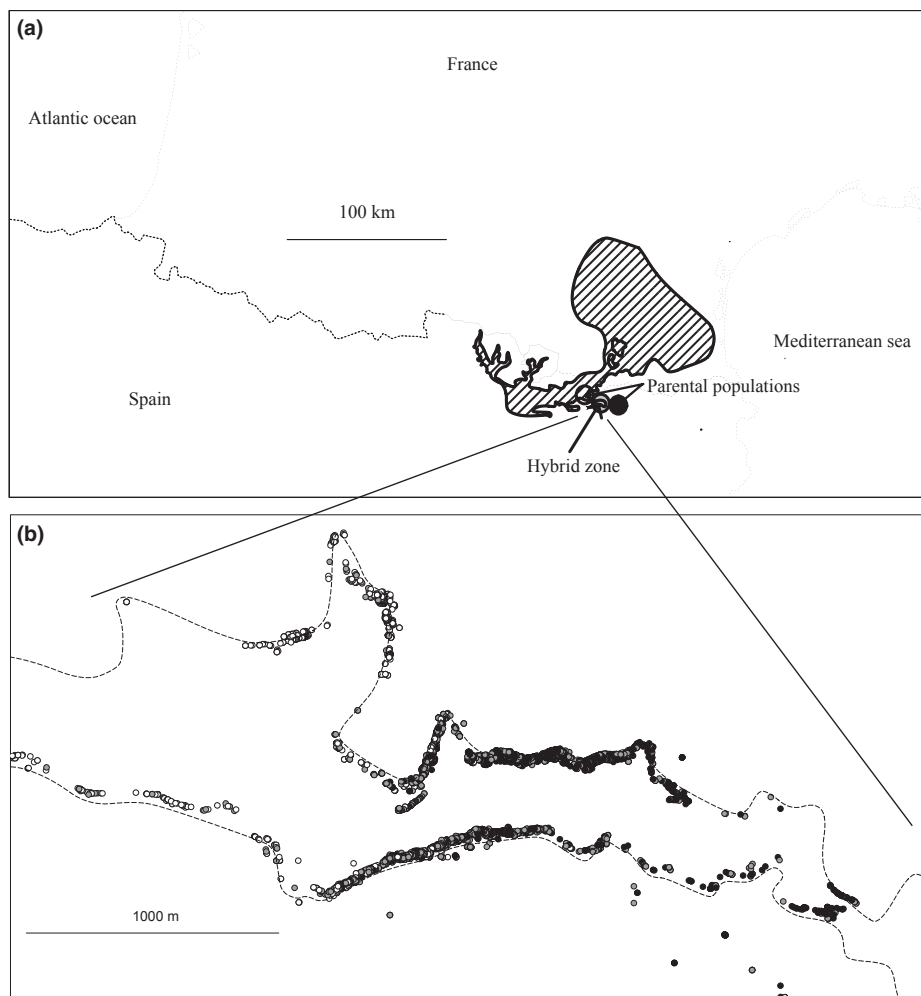


Fig. 1 Location of the hybrid zone in the Pyrenees (a). The hatched area represents the distribution of *Antirrhinum majus striatum*. Detailed map of the hybrid zone (b). *Antirrhinum. m. pseudomajus*, *A. m. striatum* and hybrid plants are symbolized by black, white and grey dots, respectively. The dotted lines represent the two roads along which the hybrid zone stretches along.

magenta and yellow components of their flower colour (range: 1–5 for the magenta score and 1–3 for the yellow score; Whibley *et al.*, 2006). Plant sampling was as exhaustive as possible in the centre of the hybrid zone every year. In 2010, more numerous plants were sampled because a new road was built and new open habitats favourable to *Antirrhinum* became available. Because in the hybrid zone most *Antirrhinum* individuals were observed near the disturbed roadsides (Fig. 1b), we used the linear distance of each plant location along one or the other road as an estimation of the position within the hybrid zone. To study comparable areas among years, we delineated the central transition zone between the two parental subspecies by fitting a sigmoidal cline to the colour scores and estimating its width and the position of its centre (Table 1, see Whibley *et al.*, 2006). These cline width and centre position estimations were made independently for each of the 4 years, for each colour

component and for each road. For each combination of year and road, the cline width and position estimations were then averaged over the two colour components (magenta and yellow) and the central transition zone delineated as an average cline width on each side of the average cline centre.

Assessing floral colour variation as perceived by pollinators

To analyse floral colours as perceived by bees, we used the ‘colour hexagon’, which offers a visual representation of bees’ colour perception (Chittka, 1992). Colour reflectance can be converted into colour space coordinates [Xcol and Ycol] by considering the opponent processes of colour perception in bees in order to assign to each plant a given locus in the hexagonal bee colour perception space. Colour loci that lie close to each other

Table 1 Summary of the analyses performed.

| Question/objective | Populations used | Years | Observational unit | Statistical method | Fitted models (main fixed effects) |
|---|-----------------------------|------------------------------|--------------------|---|--|
| Delineation of the centre of the hybrid zone | Parental Pop Hybrid zone | 2002 2004 2007 2010 | Plant | Nonlinear regression | Yellow or Magenta scores <i>Against</i> -Linear distance along road |
| Assessing floral colour variation as perceived by pollinators | Hybrid zone | 2004 2005 | Plant | Mean position within the bee colour perception space of each combination of the magenta and yellow scores | |
| Testing for pollen limitation in fruit set | Centre of the hybrid zone | 2002 2004 2007 2010 | Flower | Generalized linear mixed model | Fruit set <i>against</i> -Pollination treatment -Position within the bee colour perception space (Xcol, Ycol) -Year |
| Analysis of female fitness hybrid zone in the centre of the hybrid zone | Centre of the hybrid zone | 2002 2004 2007 2010 | Flower | Generalized linear mixed model | Fruit set <i>against</i> -Local plant density (D) -Mean colour difference in the neighbouring plants (M \bar{c} col) -Number of flowers per axis (Nbflo) -Colour position within the bee colour perception space (Xcol, Ycol) -Year |

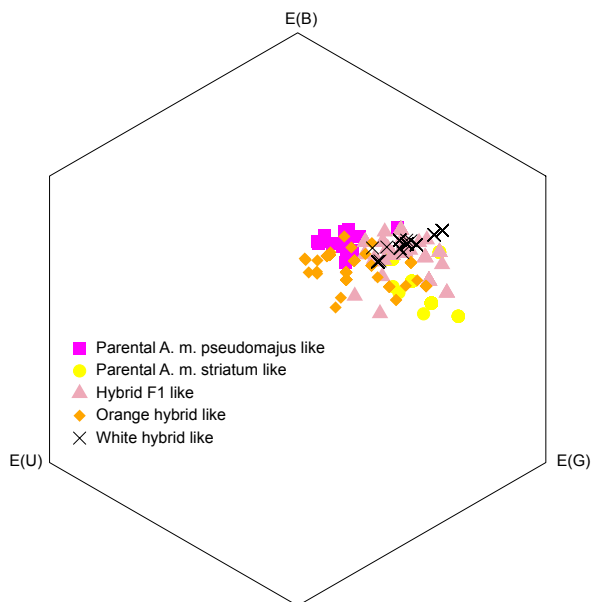


Fig. 2 Colour loci in the bee colour perception space for all plants from the central transition zone in 2002, 2004, 2007 and 2010. E(B), E(U), E(G) : percentage of excitation of the receptors sensible to wavelengths situated, respectively, in the blue, in the UV and in the green zone of the light spectrum.

within this space are perceptually similar, whereas distant colour loci are perceptually different to bees. We measured the colour reflectance of the lower lobe of the

flower's corolla from 52 individuals belonging to the sampling design of the central area of the hybrid zone in 2004. For these individuals, we calculated the position of their flower colour in the bee colour perception space. For all the other individuals within the central transition zone, we predicted an approximate location in the bee colour perception space on the basis of the colour visual scores. This was possible by using a predictive model that specifies the relation between human colour visual scores and position in the bee colour perception space (Table 1, see Tastard *et al.*, 2008). This model was built on a larger data set of 124 plants sampled within the hybrid zone in 2004 and 2005 so as to cover most of the colour range generated by hybridization (Tastard *et al.*, 2008). Interestingly, the vertical [Ycol] (vs. horizontal [Xcol]) position within the bee colour perception space seemed roughly related to the level of yellow (vs. magenta) pigmentation (Fig. 2).

Testing for pollen limitation in fruit set within the centre of the hybrid zone

In self-incompatible plant species, fruit production is usually strongly limited by reception of exogenous pollen (Knight *et al.*, 2005). However, under field conditions, fruit set may depend on both pollinator visitation rates and resource availability. To disentangle the influences of these two factors in the selection process, we performed pollen supplementation experiments within the central transition zone (Table 1). In 2002, 2004 and 2010, one or two flowers per plant (mean = 1, 1.80 and 1.46,

respectively) were hand-pollinated with pollen from five *Antirrhinum* neighbours on 12, 185 and 56 plants, respectively. In 2007, two to 13 flowers per plant (mean = 7) were hand-pollinated with pollen from five *Antirrhinum* neighbours on seven plants. For each hand-pollinated flower, an uncovered control flower at the same flowering stage was also marked in the same inflorescence. The fruit set of control flowers was therefore representative of natural pollination during the same short phenological window. To assess the effect of pollen deposition on female reproductive success, a generalized linear mixed model (GLMM) was fitted on fruit set using the `glmmPQL` function (Venables & Ripley, 2002) in R software. The error structure was assumed to be quasibinomial, and an individual plant random effect was included in the model. This model included a year fixed effect to take any time effect into account. In addition, the pollination treatment, the vertical and horizontal positions within the bee colour perception space and their interactions were tested as fixed factors. Furthermore, because our data might be spatially autocorrelated, we assumed that residuals were linked with correlation exponentially decaying with distance along the roads between observed points. A significant effect of pollen addition would indicate that pollen deposition is a limiting factor and that variation in fruit set may be explained, at least partially, by variation in pollinator visitation rate.

Analysis of female fitness in the hybrid zone

In July or August in 2002, 2004, 2007 and 2010, we counted the total number of fruits and flowers (which had not set any fruit) on one to three randomly selected dried axes (mean = 1.96) on as many plants as possible except in 2010 where fruit set was estimated on a random subsample of the plants ($N = 194, 198, 281$ and 208 in 2002, 2004, 2007 and 2010, respectively).

To assess the effects of local plant density and floral colour neighbourhood on female fitness, we estimated the final percentage of fruit set for each plant within the central transition area and, for each individual, we computed local plant density and the mean colour difference from the neighbouring plants. Local plant density [D] was estimated as the number of *Antirrhinum* plants within a fixed radius around each focal plant. We used the radius that maximized the density effect in the parental populations (7.6 m, see Supporting Information). The mean colour difference from the neighbouring plants [$M\delta col$] is the average colour difference between the location of the focal plant and the location of each of its neighbours within the bee colour perception space. A large colour difference from the neighbouring plants will be attributed to a focal plant with a rare flower colour. We then fitted a GLMM on the number of fruits over the total number of flowers per

axis [Nbflor] (Table 1). The existence of a relationship between this fitness component and certain characteristics of plants' neighbourhood provides an empirical test of our hypothesis that biotic selection processes operate within the *Antirrhinum* hybrid zone. Nevertheless, many other floral traits are suspected to modify this relationship in an unpredictable and time-dependent way. Two characteristics of plants' neighbourhood (local plant density and the mean colour difference from the neighbouring plants), two individual floral traits (the total number of flowers per axis and the colour position within the bee visual space), the year and all the interaction terms were thus tested simultaneously as fixed effects. The error structure was assumed to be quasibinomial, and an individual plant random effect was included in the model. Furthermore, residuals were considered as spatially autocorrelated with correlation exponentially decaying with distance along the road between observed points.

From this global analysis performed for all years, some interaction terms involving year were found to be statistically significant. Therefore, we analysed the data from each year separately. Because interactions between biotic and abiotic factors in shaping individual fitness are likely but unknown, we started with the full model that included all possible interactions. The minimal adequate GLMM was obtained by sequential removal of nonsignificant interactions of highest order. In order to remove any potential effect of a hidden environmental gradient along the hybrid zone transect, we also added plant position along roads and the corresponding quadratic term to the previous statistical models. Because the results were unchanged, these models with plant positions were not subsequently presented.

In 2004 and 2007, complex triple interactions involving local plant density, the mean colour difference from the neighbouring plants and the horizontal position within the bee colour perception space were significant. We thus delineated two categories of plants: those with low magenta pigmentation (horizontal position within the bee colour perception space below the median) and those with high magenta pigmentation (horizontal position within the bee colour perception space above the median). We did the same for local plant density. Partial GLMM were run for each combination of colour category (two levels: 'low magenta pigmentation' vs. 'high magenta pigmentation') and local plant density (two levels: 'low density' vs. 'high density'). The fixed part of this partial GLMM tested only the effect of the mean colour difference from the neighbouring plants. Furthermore, in order to assess whether the influence of the mean colour difference from neighbouring plants on fruit set may be explained by pollen deposition on stigmas within these four colour-density combinations, we again analysed the pollen supplementation experiment for each sub-data set.

Table 2 Generalized linear mixed model (GLMM) analysis of the effects of the number of flowers per axis (Nbflor), horizontal (Xcol) and vertical (Ycol) position within the bee colour perception space, local plant density (D), mean colour difference from the neighbouring plants (Mδcol) on fruit set in the central area of the hybrid zone in 2002 and 2010. Starting from the full model, the minimal adequate GLMM was obtained by sequential removal of nonsignificant interactions of highest order.

| Explanatory factors | 2002 | | | | 2010 | | | |
|---------------------|-----------|--------|------|---------------|-----------|--------|------|---------------|
| | Estimate* | SE | d.f. | P-value | Estimate* | SE | d.f. | P-value |
| Intercept | 0.6739 | 0.5848 | 190 | 0.2507 | -0.0829 | 0.5570 | 205 | 0.8818 |
| Nbflor | -0.0010 | 0.0067 | 82 | 0.8830 | 0.0012 | 0.0039 | 160 | 0.7532 |
| Xcol | 0.6763 | 0.8745 | 190 | 0.4403 | -0.5333 | 0.7640 | 205 | 0.4860 |
| Ycol | -5.6541 | 1.5229 | 190 | 0.0003 | -4.9882 | 1.4386 | 205 | 0.0006 |
| D | 0.0346 | 0.0113 | 190 | 0.0026 | -0.0000 | 0.0027 | 160 | 0.9743 |
| Mδcol | -5.0748 | 1.7172 | 82 | 0.0041 | -0.1913 | 1.7621 | 160 | 0.9137 |

*Values are on the logit scale. P-values of 0.05 or less are in bold type.

Table 3 Generalized linear mixed model (GLMM) analysis of the effects of the number of flowers per axis (Nbflor), horizontal (Xcol) and vertical (Ycol) position within the bee colour perception space, local plant density (D), mean colour difference from the neighbouring plants (Mδcol) on fruit set in the central area of the hybrid zone in 2004 and 2007. Starting from the full model, the minimal adequate GLMM was obtained by sequential removal of nonsignificant interactions of highest order.

| Explanatory factors | 2004 | | | | 2007 | | | |
|---------------------|-----------|---------|------|---------------|-----------|---------|------|---------------|
| | Estimate* | SE | d.f. | P-value | Estimate* | SE | d.f. | P-value |
| Intercept | -0.8895 | 1.3318 | 234 | 0.5049 | -1.8733 | 0.9864 | 343 | 0.0584 |
| Nbflor | 0.0106 | 0.0048 | 234 | 0.0300 | 0.0183 | 0.0043 | 343 | 0.0000 |
| Xcol | 0.0764 | 3.0417 | 194 | 0.9800 | 2.3740 | 2.1793 | 277 | 0.2770 |
| Ycol | -1.8920 | 4.3792 | 194 | 0.6662 | 2.9659 | 3.4488 | 277 | 0.3905 |
| D | -0.6530 | 0.2448 | 234 | 0.0082 | -0.3005 | 0.1405 | 343 | 0.0331 |
| Mδcol | -0.1775 | 7.0339 | 234 | 0.9799 | -5.2869 | 9.2091 | 343 | 0.5663 |
| Xcol × Ycol | -3.1680 | 10.7407 | 194 | 0.7683 | -22.0316 | 8.6897 | 277 | 0.0118 |
| Xcol × D | 1.7011 | 0.5695 | 234 | 0.0031 | 0.6043 | 0.2898 | 343 | 0.0378 |
| Xcol × Mδcol | -2.0274 | 15.7210 | 234 | 0.8975 | 1.2738 | 17.2832 | 343 | 0.9413 |
| Ycol × D | 1.5017 | 0.6307 | 234 | 0.0181 | 0.1798 | 0.3087 | 343 | 0.5608 |
| Ycol × Mδcol | -18.3025 | 18.9909 | 234 | 0.3362 | 11.5387 | 23.0355 | 343 | 0.6168 |
| D × Mδcol | 2.4241 | 0.9801 | 234 | 0.0141 | 2.9779 | 0.8506 | 343 | 0.0005 |
| Xcol × Ycol × D | -3.6818 | 1.6036 | 234 | 0.0226 | | | | |
| Xcol × D × Mδcol | -5.0526 | 2.4353 | 234 | 0.0391 | -5.7021 | 2.0491 | 343 | 0.0057 |

*Values are on the logit scale. P-values of 0.05 or less are in bold type.

Sensitivity of our results to the width of the central transition zone and to the neighbourhood size

To assess the robustness of our results, we generated 49 different data sets using several widths for the central transition zone and several neighbourhood sizes. The central transition zone was delineated as one half to two-fold (0.25 increments) the average cline width previously estimated on each side of the average cline centre. For each width of the central transition zone, we calculated the local plant density and the mean colour difference from the neighbouring plants using the following radii: 5.6, 6.6, 7.6, 8.6, 9.6, 14.6 and 19.6 m. For each data set, the same final GLMMs as described in Table 2 were run for 2002 and 2010. For 2004 and 2007, we found significant triple interaction involving the mean colour difference from neighbouring

plants, local plant density and the horizontal position within the bee colour perception space (Table 3). Again for each data set, we ran partial GLMM for each combination of colour category (two levels: 'low magenta pigmentation' vs. 'high magenta pigmentation') and local plant density (two levels: 'high density' vs. 'low density'). The fixed part of this partial GLMM tested only the effect of the mean colour difference from the neighbouring plants.

Results

Pollen supplementation experiments

In all years, pollen supplementation experiments conducted in the centre of the hybrid zone demonstrated that hand-pollinated flowers set fruits with much higher

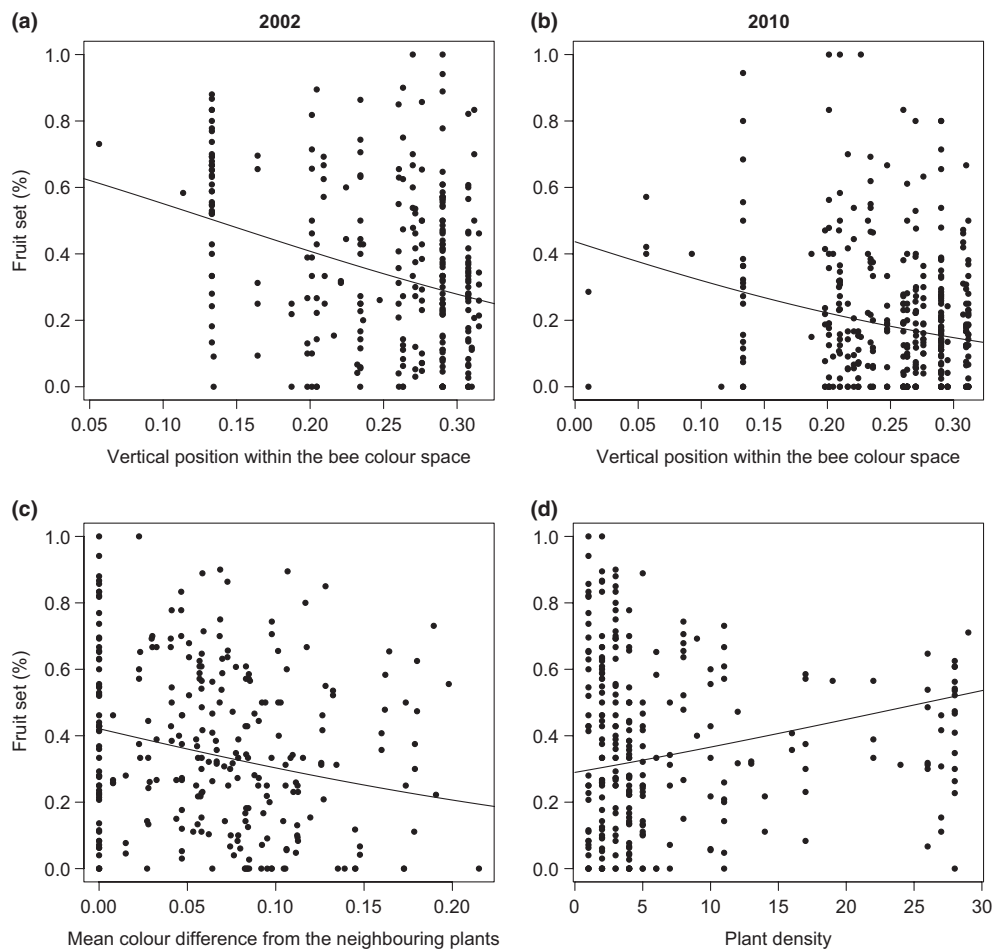


Fig. 3 Logistic regressions of fruit set over the vertical position within the bee colour perception space in 2002 (a) and 2010 (b), the mean colour difference from the neighbouring plants in 2002 (c) and local plant density in 2002 (d). Lines represent the expected fruit set probabilities.

probability than naturally pollinated flowers (fruit set being of 76.1% and 24.1%, respectively; $P < 0.0001$). The effect of pollination treatment on fruit set did not vary significantly with the position within the bee colour perception space. (The minimum P -value for the interactions between treatment and positions within the bee colour perception space was 0.1151).

Selection on flowers within the hybrid zone

In 2002 and 2010, we found that yellow-flowered individuals were better fertilized than the others (Table 2, Fig. 3a,b). Flower colour therefore influences fruit set, but its interactions with the characteristics of plants' neighbourhood vary from year to year. In 2002, fruit set decreased significantly with the mean colour difference from the neighbouring plants (Table 2, Fig. 3c) and increased significantly with local plant density (Table 2, Fig. 3d). In 2004 and 2007, we found a highly

significant triple interaction involving the mean colour difference from neighbouring plants, local plant density and the horizontal position within the bee colour perception space (Table 3). In low local plant density, fruit set tended to decrease as the mean colour difference from the neighbouring plants increased (Fig. 4). This confirms the results obtained in 2002, i.e. those plants experienced a better pollination success when locally frequent. In contrast, target plants in high-density patches tended to have a higher probability of producing a fruit when very different in colour from their neighbours (Fig. 4). For plants of the 'high magenta pigmentation' category, the trend was significant in 2004 and 2007. These results therefore indicate that the plants of the hybrid zone with 'high magenta pigmentation' located in dense patches reach higher pollination success when locally rare. Pollen supplementation had a significantly positive influence on fruit set (all $P < 0.001$) in high and low plant densities and for both magenta levels (Fig. 5).

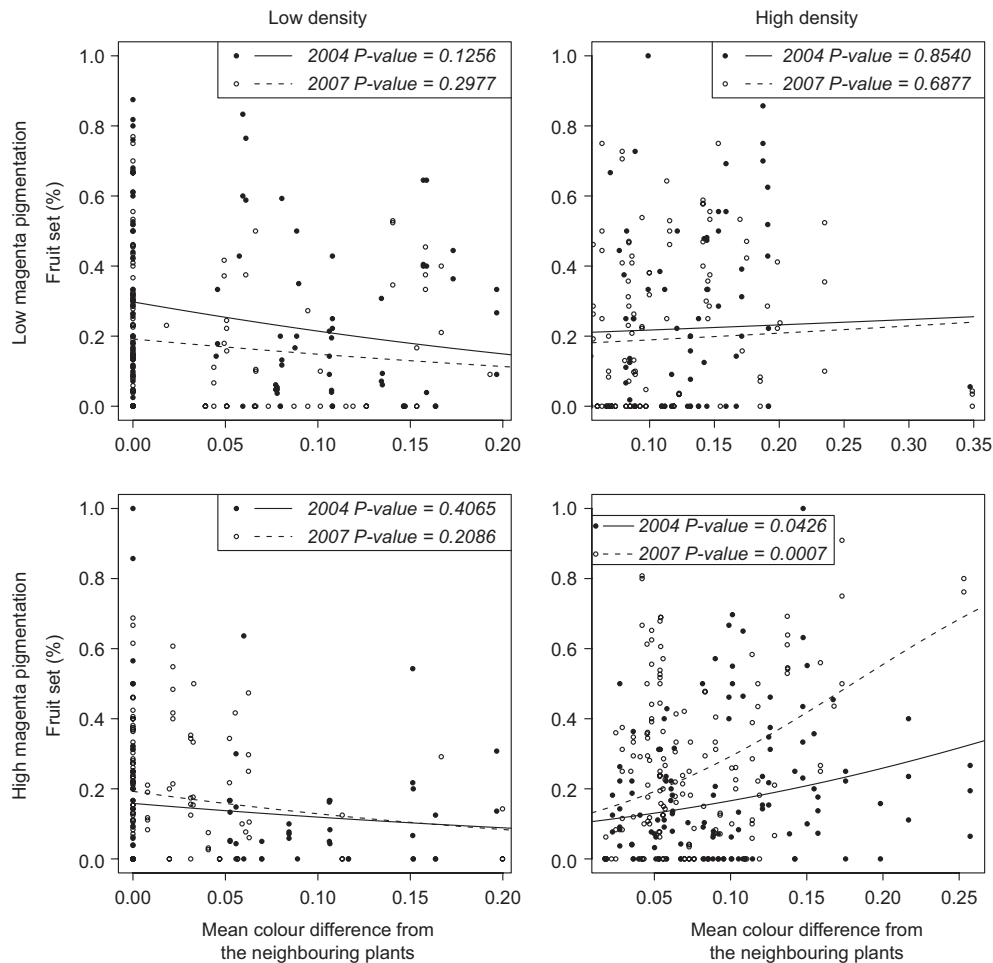


Fig. 4 Logistic regression of fruit set over the mean colour difference from the neighbouring plants for plants with high and low magenta pigmentation and in two plant density conditions, in 2004 and 2007. Lines represent the expected fruit set probabilities. The *P*-values were for the mean colour difference from the neighbouring plants effect tested in partial generalized linear mixed model.

The results were robust. Whatever the width of the central transition zone and the neighbourhood size considered, the signs of the effects described in Table 2 were unchanged and their *P*-value was below the significance threshold (0.05). The only exception was for an extremely narrow central transition zone (half the average cline width) where the local plant density effect in 2002 was not significant for some radii used to characterize the neighbourhood of each focal plant. In 2004 and 2007, using the partial GLMM run on each data set for each combination of year \times colour category \times local plant density, 81.4% of the analyses estimated the same sign of the mean colour difference from the neighbouring plant effect than those described in Fig. 4. For plants of the 'high magenta pigmentation' category located in high-density patches, this proportion of concordant analyses raised to 92.9%.

Discussion

In this study, we showed that female success of individual plants within an *Antirrhinum* hybrid zone may depend, at least in some years, not only on the individual's flower colour but also on the colour of the floral neighbourhood. Most of the time and for most individuals within the central transition zone, similarity with the floral neighbourhood increased the probability of producing fruits. As pollen deposition on the stigma appeared as a strong limiting factor for fruit set in the *Antirrhinum* hybrid zone, the selection patterns observed under open pollination may be generated by pollinator foraging behaviour. Given that most *Antirrhinum* plants from the hybrid zone produced nectar (C. Andalo and E. Tastard, unpublished data), the main negative effect of the mean colour difference from the neighbouring plants

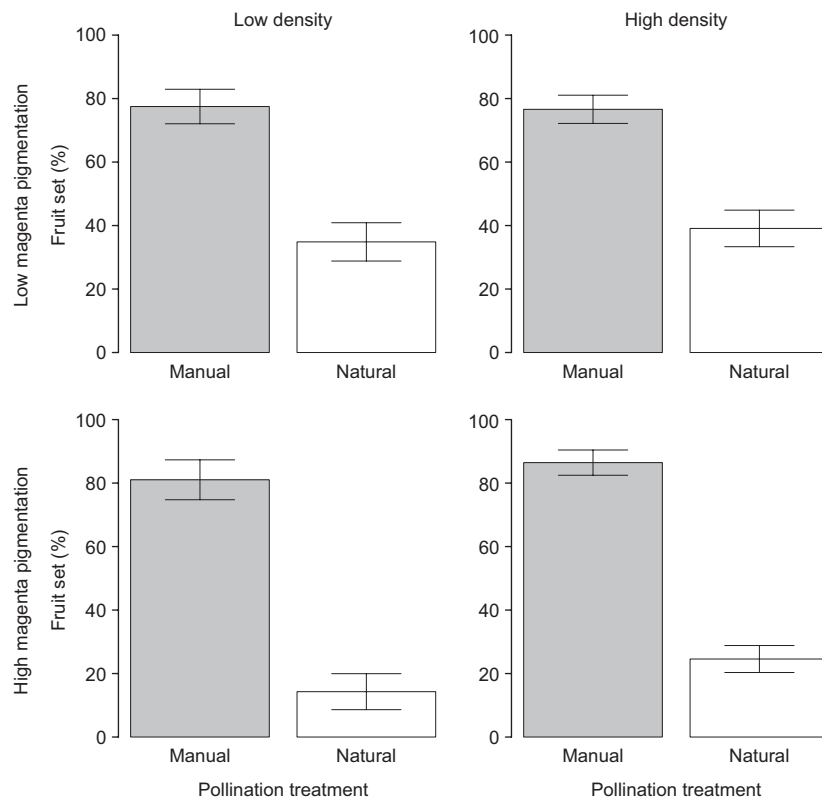


Fig. 5 Fruit set (\pm SE) for natural and manual pollination treatments in high and low plant density conditions and for plants with low and high magenta floral pigmentation in 2004 and 2007.

is in close agreement with the positive frequency-dependent selection detected in other studies using reared bumblebees foraging on artificial rewarding flowers (Smithson, 2001).

Interestingly, the floral colour neighbourhood effect was opposite on individuals in high-density patches and with highly magenta-pigmented flowers in 2004 and 2007. Briefly, magenta plants had greater pollination success within a mainly yellow patch than within a mainly magenta one. Furthermore, in 2002 and 2010, fruit set increased with the yellow pigmentation level of flowers. The positive influence of the mean colour difference from neighbours (expected under negative frequency-dependent selection) observed for plants with high magenta pigmentation is difficult to interpret. Negative frequency-dependent selection is expected for rewardless flowers (Gigord *et al.*, 2001) but no strong and consistent differences were found for nectar production between the two colour categories (C. Andalo and E. Tastard, unpublished data). Furthermore, when plant position within the hybrid zone was added as a dependent variable in the GLMM, the floral neighbourhood effect was still detected. A simple biotic or abiotic environmental gradient could therefore not explain the

fruit set variation pattern demonstrated here and pollinator-mediated selection is thus likely. Some floral feature differences between *A. m. pseudomajus* and *A. m. striatum* may produce a specific pollinator behavioural response and sign inversion in the floral colour neighbourhood effect. For instance, a repellent benzenoid molecule, acetophenone, is present in the magenta but not in the yellow subspecies floral scent (Suchet *et al.*, 2010). Furthermore, in close agreement with the advantage of yellow-pigmented flowers in 2002 and 2010, bees are known to have an innate preference for the yellow colour (Giurfa *et al.*, 1995; Spaethe *et al.*, 2001). Both observations are consistent with the hypothesis that yellow *Antirrhinum* flowers act as beacons (the magnet species effect; Thomson, 1978; Johnson *et al.*, 2003) that attract pollinators and thus facilitate the pollination of the rare magenta and intermediate hybrid morphs located in mostly yellow patches.

In the hybrid zone studied here, fruit set increased with high local plant density, whatever the colour phenotype, in 2002. This result supports the ideas that fruit set is, as already shown by the pollen supplementation experiment, mostly limited by pollen deposition instead of resource availability and that facilitation

among plants for pollinator attraction operates. Facilitation has been shown in other plant systems (Waser & Real, 1979; Thomson, 1981, 1982) and is not unexpected given the low number of pollinators usually observed within this particular hybrid zone (C. Andalo, C. Thébaud & C. Suchet, unpublished data) and in mountain habitats in general (Iserbyt *et al.*, 2008). Surprisingly, in 2004 and 2007 for the 'high magenta pigmentation' category, the influence of the mean colour difference from neighbours depended on local plant density. Using foraging bumblebees on artificial rewarding flowers, Smithson & Macnair (1997) found a preference for the commonest colour morph and this behaviour was not significantly affected by changing density. However, the flower densities observed in our natural system were far lower than those used in their experimental set-up.

From an evolutionary point of view, our results may have important consequences for the stability of the hybrid zone because the selection pattern may be related to positive frequency-dependent selection processes. Rare hybrid phenotypes close to the hybrid zone borders could have lower pollination success than the common parental phenotype. However, this floral neighbourhood influence on fruit set was in some years absent (e.g. 2010) or detected in complex interactions (2004 and 2007), and it is probable that other selection mechanisms operate concomitantly to maintain the abrupt change of colours observed. From the analysis of this single life-history trait, we therefore predict a pattern of asymmetric introgression between *A. m. pseudomajus* (the magenta subspecies) and *A. m. striatum* (the yellow subspecies) but with a direction that depends on the balance between the advantage of being yellow over that of being magenta in a mostly dissimilar colour neighbourhood. An advantage of yellow-flowered plants would lead to an introgression of *A. m. striatum* characters towards the former *A. m. pseudomajus* patches, whereas an advantage of magenta-flowered plants in mostly dissimilar colours would lead to an introgression of *A. m. pseudomajus* characters in the opposite direction.

Recent analysis of chloroplastic molecular variation (Khimoun *et al.*, 2011) shows that plants sampled from the hybrid zone and from the *A. m. pseudomajus* populations close to the hybrid zone were always found to have an *A. m. striatum* chloroplast haplotype. The most likely scenario to explain this asymmetric chloroplastic introgression is that the hybrid zone has moved towards the former *A. m. striatum* patches. Asymmetric introgression and species turnover between parapatric taxa occur because of demographic invasion of one species over another (Currat *et al.*, 2008) or because of differences in mating systems between hybridizing taxa (Sweigart & Willis, 2003). Alternatively, adaptive asymmetric introgression may also occur through directional selection (e.g. spread of herbivore resistance traits, Whitney *et al.*, 2006) or balancing selection (e.g. self-

incompatibility allele, Castric *et al.*, 2008) but examples in natural populations for diagnostic morphological traits are strikingly rare. The floral neighbourhood environment effect recorded here may provide a strong case for subspecies displacements by the means of selection.

The use of neighbourhood models and functional analysis of the phenotypic variation show how natural selection can operate in an *Antirrhinum* hybrid zone. Although similar methods have been employed to study the mating patterns in plant populations or spatial patterns of plant competition, to our knowledge, this is their first application to study the selection in a natural hybrid zone. The modelling framework used here can provide an alternative approach to classical transplant experiments with artificial populations (e.g. Campbell, 2003) that potentially disturb the natural dynamics of hybridization. In *Antirrhinum*, at least three main loci are involved in the natural flower colour variation (ROS EL, SULF, Whibley *et al.*, 2006). To further explain the movement of the magenta *A. m. pseudomajus* subspecies over the yellow *A. m. striatum* indicated by the chloroplast analysis (Khimoun *et al.*, 2011), we need to understand how the alleles that determine the magenta colour and those causing the absence of yellow spread within the yellow side of the hybrid zone. In other words, orange phenotypes should perform worse than the parental magenta phenotype within the hybrid zone to fully explain the subspecies movement. Unfortunately, the number of plants with orange flowers within the hybrid zone is too low to test this hypothesis properly. To overcome this sample size problem, the next step should be to directly estimate the mean female fitness of various allelic combinations in different biotic conditions. This remains a challenge because the SULF locus that mostly explains variation in yellow pigmentation within the hybrid zone has not yet been characterized molecularly and because the link between colour phenotype and genotype at this locus is confused by allelic dominance effects (E. Coen, pers. comm.). In this study, we also showed that natural selection in an *Antirrhinum* hybrid zone may be quite variable in time and that the local phenotypic composition of the neighbourhood influences individual female fitness. Over the 10 years of observation, the hybrid zone studied always appeared very sharp and one may wonder if the pollinator-mediated selection through female fitness demonstrated here is strong enough to account for this apparent stability of the cline. Other selective agents, such as several specialist insect herbivores that feed on *Antirrhinum* including one abundant seed predator (*Rhinusa hispida*, Coleoptera: Curculionidae), could also contribute to flower colour selection and to the entire hybrid zone dynamics. Seed predators are indeed known to use the same visual cues as pollinators, and floral pigments have been shown to have a pleiotropic defence function against herbivores (Rausher, 2008).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Analysis of fruit set in parental populations to choose the radius of the neighbourhood delimitation zone around each plant.

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