OIKOS

Are pollinators the agents of selection on flower colour and size in irises?

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Oikos
00: 1–13, 2017
doi: 10.1111/oik.04501

Subject Editor: Rein Brys.
Editor-in-Chief: Dries Bonte.
Accepted 8 November 2017

Plant–pollinator interactions are believed to play a major role in the evolution of floral traits. Flower colour and flower size are important for attracting pollinators, directly influencing reproduction, and thus expected to be under pollinator-mediated selection. Pollinator-mediated selection is also proposed to play a role in maintaining flower colour polymorphism within populations. However, pigment concentrations, and thus flower colour, are also under selective pressures independent of pollinators. We quantified phenotypic pollinator-mediated selection on flower colour and size in two colour polymorphic *Iris* species. Using female fitness, we estimated phenotypic selection on flower colour and size, and tested for pollinator-mediated selection by comparing selection gradients between flowers open to natural pollination and supplementary pollinated flowers. In both species, we found evidence for pollen limitation, which set the base for pollinator-mediated selection. In the colour dimorphic *Iris lutescens*, while pigment concentration and flower size were found to be under selection, this was independent of pollinators. For the polymorphic *Iris pumila*, pigment concentration is under selective pressure by pollinators, but only for one colour morph. Our results suggest that pollinators are not the main agents of selection on floral traits in these irises, as opposed to the accepted paradigm on floral evolution. This study provides an opposing example to the largely-accepted theory that pollinators are the major agent of selection on floral traits.

Introduction

Flower colour polymorphism within populations is quite uncommon (Kay 1978). Indeed, as flower colour is among the most important visual cues in pollinator attraction, variation in these traits may affect pollinator visitation rates, directly influencing reproductive success (Smithson and Macnair 1997, Fenster et al. 2004, Juillet and Scopece 2010). The evolutionary mechanisms underlying stable flower colour variation have been proposed to be the outcome of pollinator's preferences, such as assortative visits to a single colour morph in a bout (Jones and Reithel 2001), or innate

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differences in colour preferences by different pollinators (Lunau and Maier 1995, Dyer et al. 2016). Differential preferences of different pollinators to different colours can exert divergent pollinator-mediated selection on flower colour and cause pollinator-mediated reproductive isolation between species or ecotypes (Levin and Kerster 1967, Hoballah et al. 2007, Sobel and Streisfeld 2015). However, studies to explain within-population polymorphism are scarce.

Within-population flower colour polymorphism has been mainly described in orchid species where colour polymorphism is often associated with food deception (see Table 1 in Kagawa and Takimoto 2016 and Jersáková et al. 2006a). Food deceptive pollination has been described in more than 32 angiosperm families, including approximately one third of all orchid species (Renner 2006, Dormont et al. 2010a). Floral traits of deceptive plant species may be utilized by pollinators as a negative signal for deception (Ferdy et al. 1998). Therefore, variability in flower colour and morphological traits in deceptive plants prevents learning of visual cues that pollinators associate with non-rewarding flowers. Pollinators learn to avoid the deceptive flower morphs and visit the alternative ones, generating negative frequency-dependent selection (Gigord et al. 2001, Kagawa and Takimoto 2016). However, experiments in natural populations have failed to detect this mechanism in various species (see discussion in Imbert et al. 2014a).

In rewardless flowers, the most common form of pollination relies on exploiting the foraging behaviour of naïve pollinators (Jersáková et al. 2006a). Generally, food deceptive species are characterized by conspicuous early-spring flowering and are often pollinated by recently emerged insects, immigrants or vagrant pollinators searching for food and not familiarized with the available resources of the area (Jersáková et al. 2006a, Pellissier et al. 2010). In such a scenario, flower colour is expected not to be under pollinator-mediated selection.

However, various selective agents, other than pollinators, can be invoked to explain the maintenance of flower colour variation within populations. For instance, balancing selection due to both mutualists (pollinators) and antagonists (florivores, pathogens or seeds-predators) has been suggested to maintain colour polymorphism (Frey 2004, De Jager and Ellis 2014). Furthermore, since pigment synthesis is metabolically expensive, it is reasonable to expect that increased investment in pigment production is correlated with reduced investment in seed production (Chalker-Scott 1999, Campbell et al. 2012). In addition to their importance in determining flower colour, floral pigments such as flavonoids and anthocyanins also play an important role in plant defence against environmental stresses, such as UV radiation and drought (Chalker-Scott 1999, Winkel-Shirley 2002, Coberly and Rausher 2008, Tucić et al. 2009, Arista et al. 2013, Landi et al. 2015). Therefore, selective pressures, independent of pollinators, might also contribute to flower colour polymorphism maintenance (Narbona et al. 2017).

Irises, the flowers of the rainbow, are famous for the colour variation among and within species of this large Palearctic genus. Studies on two species of Louisiana irises, which differ in colour, identified segregation of pollinators as a reproductive barrier between these species (Martin et al. 2008). In contrast, continuous within-species colour gradient in two *Oncocyclus* irises was found not to be affected by pollinators' preferences (Lavi and Sapir 2015). In this study, we focused on two colour-polymorphic rewardless irises, *Iris lutescens* and *I. pumila*.

Iris lutescens and *I. pumila* are two early-spring flowering *Iris* species native to the western Mediterranean basin and to southeastern Europe. Both species are self-incompatible and exhibit two levels of flower colour polymorphism: two dominant phenotypes, purple and yellow, in the colour dimorphic *I. lutescens* (Fig. 1A), and three dominant phenotypes, yellow, blue and purple, in the colour polymorphic *I. pumila* (Fig. 1C; although six other rare colour phenotypes were identified). Floral morphology along with previous observations suggests that visiting insects are foraging for non-existent nectar rather than searching for a pollen reward, which is pressed against the upper surface of the gullet flower or tunnel (Imbert et al. 2014b). The most common insect visitors of both species are *Bombus* bees and other solitary bees (Fig. 1B–C). Considering that insect visitors are preferably foraging for non-existent nectar, it appears that these two *Iris* species exploit the sensory biases of newly emerged, naïve pollinators in order to set fruit. Therefore, consistent with previous experiments in *I. lutescens* (Imbert et al. 2014a, b), we hypothesized that flower colour is not under pollinator mediated selection,

Table 1. Frequency of flower colour morphs (FCM) and detailed sampling strategy for *I. lutescens* (Clape and Navas populations) and *I. pumila*. Open pollinated flowers are denoted as control, and hand-pollinated flowers are denoted as supplied.

Species	Coordinates WGS84	Morph	FCM	Sample size	
				Control	Supplied
I. lutescens					
Clape	43°13′45″N, 3°10′12″E	yellow	0.59	50	50
		purple	0.41	51	49
Navas	43°96′15″N, 3°54′39″E	yellow	0.30	47	47
		purple	0.70	61	60
I. pumila	44°81′74″N, 20°48′73″E	yellow	0.10	16	12
		blue	0.32	47	46
		purple	0.58	86	85

Figure 1. (a) The two colour morphs of *Iris lutescens* from a natural population. (b) Yellow morph variant being visited by an insect (*Vespoidea*) showing a typical nectar foraging behaviour (the insect goes into the flower tunnel searching for nectar) (c) The three most common flower colour morphs of *I. pumila* grown in experimental garden. Note the bumblebee visiting the purple morph. (pictures: A and B by D. Souto, C by A. Vuleta).

and instead expect the cost of pigment production to exert selection on flower colour.

To test for this hypothesis, it is necessary to consider floral pigment concentration as a quantitative trait in addition to the qualitative trait, flower colour. Therefore, pigment concentration was considered as a nested trait within flower colour. Such distinction enables to partition the selection on flower colour due to the effect of pollinators, which may select on colour morph (categorical) and the effect of environmental stresses, which may be the agents of selection on pigment concentration (continuous).

The aim of our study was to examine phenotypic selection on pigment concentration between different colour morphs, as well as on morphological traits. Experimental flower manipulations were carried out both in natural populations (*I. lutescens*) and in a common garden experiment (*I. pumila*). In *I. lutescens* we chose natural populations based on the dominance of either colour morph, while in the common garden experiment, colour morph frequencies mirrored those found in the plant's natural range (Tucić et al. 1989). Following standard protocols, we used supplementary hand pollination to experimentally remove pollinator induced pollen limitation. Differences in the relative fitness between treatments ($\Delta\beta_{poll}$ sensu Sletvold et al. 2016) allowed us to estimate the relative role of pollinators as selective agents, but permitting for other selective pressures to act on all floral traits (Lavi and Sapir 2015, Sletvold et al. 2016).

Material and methods

Study species

The experiment was conducted on two clonal rhizomatous perennial *Iris* species in parallel. *Iris lutescens* is a diploid (2n = 2x = 40) species, native to the western Mediterranean basin, extending from eastern Spain to northern Italy (Colasante 2015), while the tetraploid *Iris pumila* (2n = 4x = 32) is widely distributed in the lowlands of southeastern Europe, ranging from southern Moravia (Czech Republic) and western Italy to northern Anatolia (Turkey, Randolph 1955).

In both species, individuals produce a flowering stem approximately 10-cm tall with a single flower during the reproductive period (early March to May). Each flower has three upright petals (standards) and three pendant sepals (falls), with the style bending over the anther to form three pollination tunnels marked by colourful beards (Fig. 1). Although each stem produces a single flower, the rhizomatous nature of the species allows for each genet to produce several flowering stems in the same year. As genets ramify underground, telling apart flowers from the same genet is difficult; however, clone density for *I. pumila* has been reported to be approximately 0.85 per square meter in the most crowded areas (Tucić et al. 1998).

Pollination biology and its significance on the maintenance of flower colour polymorphism have been extensively

studied in *I. lutescens* (Imbert et al. 2014a, b). Flowers of different colour morphs do not differ in pollen and ovule production, vegetative characteristics, or floral morphology, except flower size (purple flowers are usually larger). While it has been demonstrated that bees are able to visually discriminate between the two colour morphs using Chittka's physiological models (Wang et al. 2013), observations in natural populations revealed no preference of *Apoid* bees or florivorous beetle *Cetonia hirsuta* for any particular colour morph (Imbert et al. 2014b).

Experimental design and phenotypic trait measurements

Experiment on I. lutescens

For *I. lutescens*, the experiment was carried out in two natural populations in southern France near Montpellier. One population is yellow dominant (Clape 43°13′45″N, 3°10′12″E; Table 1), while the other is purple dominant (Navas, 43°96′15″N, 3°54′39″E; Table 1). Sampling was carried out during the flowering peak of each population (mid-March 2014 for Clape, and mid-April 2014 for Navas). For each population, newly opened flowers were individually marked and morphological traits were measured. Although clones (genets) consist of multiple flowering stems, flowers selected for the experiment consisted of one flower per clone, and clones chosen for the experiment were at least two meters apart to avoid sampling flowers from similar genotypes. Measured traits included: 1) flower height to the nearest 0.5 cm from the ground to the top of the standard; 2) flower size to the nearest mm from the base of the fall to the top of the standard; 3) width of the fall (mm); and 4) width of the standard (mm). Plant height was measured using standard measuring tape while all other measurements were performed using digital callipers. To quantify pigment concentration, a fraction of petal was removed from each flower using a standard hole-puncher (diameter 6 mm) and was kept in an Eppendorf tube. Once in the lab (i.e. less than 10 h after manipulation in the field), tubes were stored at –80°C until further manipulation. Pigment extraction for each petal sample was carried out using 100 µl of 0.5% hydrochloric acid solvent in ethanol. Sample tubes were sonicated in a water bath for 10 min and stored overnight (maximum 17 h) at 4°C. Sample plates were prepared using 85 µl of supernatant and were quantified using a spectrophotometer using the UV-Vis function. Absorbance values were recorded at 350 nm and 540 nm to quantify total content offlavonoid and anthocyanin pigments, respectively (Wang et al. 2013).

In order to test for the net selection exerted by pollinators, relative to the general selection on a floral trait, we used the technique proposed previously by several authors (Sletvold et al. 2010, Bartkowska and Johnston 2012, Lavi and Sapir 2015, Thomsen and Sargent 2017, Trunschke et al. 2017). Briefly, this method utilizes the assumption that non-pollinator selection agents, such as nutrient availability and herbivores, are co-variate with plant's maternal fitness (that is, fruit or seeds). Supplementing excess pollen to the flower removes the potential effect of a floral trait on pollinator's choice and provides estimation of the 'background' selection. Using Lande and Arnold's phenotypic selection (1983), selection gradients are estimated for flowers supplied with excess pollen and for flowers left open to be selected by pollinators. Subtracting the latter from the former gives the net pollinator-mediated selection, $\Delta\beta_{poll}$.

In both populations, we randomly assigned the sampled individual flowers either to be supplied with excess pollen ('supplementary') or to be left open to pollinator's putative choice (Table 1). Pollen supplementation was done using a paintbrush with a pollen mix from many flowers in the same populations. Anthers were collected from flowers >10 m distance of each other, regardless of colour, because previous experiments did not identify any incompatibility between morphs (Imbert et al. 2014b). Morphological measurements for each sampled flower was done as detailed above. Approximately six weeks after manipulations, all marked flowers were surveyed for fruits. In the Clape population, 11 individuals (6 control and 5 supplied flowers) could not be found due to predation or tag destruction, while in the Navas population, only one supplied individual was lost. Fruit production was scored for each plant (1/0). Fruits were collected before maturation and seed dispersal to be able to count the number of seeds. At this stage, it is not possible to distinguish between fertilized and aborted ovules with confidence we can, however, determine the number of ovules per fruit.

Experiment on I. pumila

Iris pumila displays a variety of flower colour phenotypes ranging from white and yellow to dark purple and dark blue. Although nine colour phenotypes were identified, intense coloured flowers (yellow, purple and blue) are the most common ones (Tucić 1988, Tucić et al. 1989). Flower colour diversity and its causal relationship to abiotic factors has been monitored over a 24-year period at an open steppe, natural population, in the Delibato Sands (44°96′88″N, 21°02′38″E), in northern Serbia (Tucić et al. 1989). Frequencies of flower colour variants were relatively stable over years, reaching similar values for purple and blue flowers (0.49 and 0.40, respectively) and being smallest for yellow flowers (0.10, Tucić et al. 1989).

Due to access difficulty, we could not perform the *I. pumila* experiment in natural populations. Thus, the experiment was conducted on plants grown in an experimental garden, located in the backyard of the Inst. for Biological Research 'Siniša Stanković', Belgrade, Serbia. The plants originate from a natural population of *I. pumila* which occupies an open site in the Deliblato Sands approximately 45 km from the Institute. In 1997, seedlings obtained from crosses of selected genotypes growing in the wild were planted in the experimental garden, where they still grow as adult clones under relatively uniform environmental conditions. Preliminary observations confirmed the presence of natural pollinators, such as medium-size bees, in the common garden (Fig. 1C). The natural fruiting rate of the common garden experiment closely matches that of the wild population (48.4% in

2012 and 39% in 2013; n = 850 pots, several flowering stems per pot; Tarasjev 2005) which indicates presence of pollinators in the garden. In April 2015, the occurrence of the three main flower colour morphs in the experimental garden (Table 1) was similar to that found in the natural population they originated from (Tucić et al. 1989). Morphological traits measurements and pigment quantification were performed on newly opened flowers, as described for *I. lutescens* above (Table 1). The absorbance of pigment extracts was determined in microplates using UV/visible light spectrophotometer.

For the experiment, we used one flower per pot, each pot representing one genotype. As for the *I. lutescens* experiment, approximately half of the sampled flowers received supplementary excess pollen using a mix of pollen from a few randomly selected flowers from different pots, while the other half of the flowers were left for natural pollination (Table 1). In June 2015, fruit production was scored for each plant and unripe fruits were collected for determination of seeds and ovules number, as above.

To prevent any selection effects caused by pollinators learning to avoid these flowers, the sites were regularly monitored and all manipulations were performed during the flowering peak of each site.

Statistical analyses

Data were analysed for each species separately, and for *I. lutescens,* data were analysed for each population separately. First, we compared the probability of fruiting and seed set between treatments and colour morph (and their interaction) using a generalized linear model. Fruit production and seed number (number of trials being the total number of ovules per fruit) per fruit were considered as binomial variables. Pollen limitation has been proposed as conditional for pollinator-mediated selection to act on floral traits (Ashman et al. 2004). Thus, we calculated the extent of pollen and pollinator limitation as the relative fruit-set or seed-set in supplemented versus open flowers (Campbell and Husband 2007, Lavi and Sapir 2015), as follows:

$$\text{pollinator limitation} = [(\text{fruit-set}_{sup} - \text{fruit-set}_{open})/\text{fruit-set}_{sup}]$$
$$\text{pollen limitation} = [(\text{seed-set}_{sup} - \text{seed-set}_{open})/\text{seed-set}_{sup}]$$

In order to detect phenotypic selection on female fitness, we first calculated un-conditional maternal fitness for each plant using the *aster* model, as proposed by Geyer et al. (2007) and Shaw et al. (2008). *Aster* models evaluate the relationships between female fitness and explanatory variables (i.e. treatment and phenotypic traits) based on a graphical model that specifies dependencies between a fitness component and an earlier component. Thus, seed production depends on total number of ovules, which, in turn, depends on fruit production. Considering phenotypic selection analyses, *aster* models allow estimating female fitness for each individual over a few consecutive life-history stages. Fruit production and seed number were considered as Bernoulli variables, while total number of ovules per fruit fitted a normal distribution of unknown mean (variance being the sampled variance in each population).

In both species, the four morphological traits measured (flower height and size, sepal width, petal width) are correlated with each other (r-value ranges = 0.24–0.71), thus it is not relevant to consider that each trait as an independent variable. Furthermore, including each variable in *aster* models would artificially increase the likelihood to detect a significant p-value. Therefore, the four morphological traits were first analysed with principal component analyses using scaled data for each dataset separately. The first axis (PC1) explained 57%, 68% and 61% of the variance in *I. lutescens*, Clape and Navas populations, and *I. pumila*, respectively. In all three populations, variance of data in PC2 was < 1, thus, using Kaiser's criterion for variance > 1, we retained only PC1 as global morphological variable. Pigment concentration values were standardized to mean = 0 and $\sigma^2 = 1$, as were PC1 (mean being 0 before standardization, but σ^2 differed from 1). Because colour can act either as visual cue and environmental adaptation, we used pigment concentration as a trait nested within colour morph. Therefore, the full quadratic model used in the *aster* model was: treatment + colour + colour/flavonoid concentration + colour/anthocyanin concentration + PC1 + their interactions with treatment + (colour/flavonoid)^2 + (colour/anthocyanin)^2 + PC1^2 + their interactions with treatment. For the linear model, we removed all the quadratic terms. Model selection was based on conventional likelihood ratio tests with backwards elimination to retain the minimal adequate model. We focused in particular on interactions between treatment and these phenotypic variables. Once we determined the minimal model, predicted values for fruiting probability and seed-set were used to compute female fitness of each individual, as the product of probability of fruiting by seed-set. This estimation enables computing the relative fitness (individual fitness/mean fitness). Finally, we computed selection gradients by extracting partial regression coefficients (β and γ sensu Lande and Arnold 1983) from the linear models using relative fitness as the dependant variable and phenotypic traits significantly contributing to female fitness as explanatory variables. For γ-values, partial regression coefficients were doubled to obtain the extent of selection gradients (Stinchcombe et al. 2008).

All statistical analyses were performed using the R software ver. 3.2.1 (<www.r-project.org>). *Aster* models were implemented using the *aster* package of R (Geyer 2017).

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.44mt9> (Souto-Vilarósa et al. 2017).

Table 2. Mean values (SD) for morphological traits and pigment concentrations measured in both populations of *I. lutescens* and for *I. pumila*. Flower height is in cm; all other morphological traits are in mm. Pigment concentration values are presented in units of relative absorbance (see Methods for details). Different letters indicate significant differences between colour morphs within species and within populations ($p < 0.05$, one-way ANOVA).

						Pigment concentration	
Species	Morph	Flower height	Flower size	Fall width	Standard width	Flavonoids	Anthocyanins
I. lutescens							
Clape	yellow	11.6 (2.6) a	45.5 (8.2) a	18.4 (2.6) a	19.6 (2.7) a	1.74 (0.50) a	0.007 (0.01) a
	purple	11.3 (2.3) a	45.9 (7.2) a	18.4 (2.8) a	19.3 (2.7) a	1.09 (0.34) b	0.66 (0.28) b
Navas	yellow	15.6 (2.9) a	48.8 (8.3) a	18.6 (2.9) a	21.5 (3.3) a	1.99 (0.46) a	0.02 (0.02) a
	purple	16.8 (3.2) b	54.8 (9.4) b	20.9 (3.3) b	23.1 (3.7) b	1.29 (0.43) b	1.10 (0.33) b
I. pumila							
	yellow	11.0 (1.7) a	33.5 (7.5) a	13.9 (2.9) a	15.2 (3.6) a	1.93 (0.40) a	0.07 (0.01) a
	blue	10.8 (1.7) a	33.8 (7.5) a	13.7 (2.4) a	15.9 (3.3) a	1.25 (0.42) b	0.78 (0.28) b
	purple	10.9 (1.8) a	34.3 (7.0) a	13.5 (2.3) a	16.1 (3.0) a	0.52 (0.28) c	1.10 (0.35) c

Results

Phenotypic variation, fruit-set and seed-set in *Iris lutescens*

As expected, pigment concentrations greatly differed between flower morphs (Table 2). Purple flowers tended to be larger in the Navas population, while there was no difference in morphology between flower morphs in the Clape population (Table 2). Concerning principal components analyses, PC1 and PC2 explained 57% and 19%, respectively, of the total variance for the Clape population and 68% and 15%, respectively, for the Navas population. Following Kaiser's criterion (eigenvalue > 1), we only retained PC1 for the following analyses. For both populations, PC1 was positively correlated ($r > 0.60$) with all traits.

Fruiting was irrespective of flower colour ($\chi^2 = 2.4$, df = 1, $p > 0.12$, for Clape and $\chi^2 = 0.47$ df = 1 $p = 0.48$ for Navas, Fig. 2A), and only supplementary pollination had an effect of fruiting in both populations ($\chi^2 = 22.7$, df = 1, $p < 0.0001$, $p > 0.96$ for interaction for Clape, and $\chi^2 = 18.1$ df = 1 $p < 0.0001$, $p = 0.58$ for interaction for Navas, Fig. 2A). In Clape, 32 out of 95 flowers of the control group produced a fruit, while 65 out of 94 flowers produced a fruit following the supplementary treatment (33% and 69%, respectively). In Navas, approximately 50% of pollen supplied flowers fruited (54/106), while the natural fruiting rate was 23% (25/108).

In contrast, seed set depended on both treatment and flower colour (interaction $\chi^2 = 62.17$, df = 1, $p < 0.0001$ for Clape and $\chi^2 = 58.03$, df = 1 $p < 0.0001$ for Navas). In Clape, pollen supplementation did not affect seed set for the yellow morph, however it decreased seed set for the purple morph (Fig. 2B). In Navas, there was no difference between colour morphs for control flowers, but pollen supplements had a greater effect on purple flowers than on yellow flowers, leading to a difference between morphs (Fig. 2B).

Pollinator limitation estimates were quite similar in both populations (0.51 in Clape and 0.55 in Navas), while pollen limitation, based on seed-set, was only 0.04 in Clape and reached 0.22 in Navas.

Phenotypic variation, fruit-set and seed-set in *Iris pumila*

Pigment concentration differed significantly between all three flower colour morphs, while no difference was found for morphological traits (Table 2). Regarding morphological traits, PC1 and PC2 explained 61% and 16% of total variance, respectively. PC1 was positively correlated with all traits ($r > 0.7$). As for *I. lutescens*, only PC1 was retained in the following analyses.

Fruiting rate for control flowers was only 15.4% (23/149), while 44.8% of supplementary pollinated flowers produced fruit (64/143, Fig. 2A). Contrary to the highly significant treatment effect ($\chi^2 = 31.06$, df = 1, $p < 0.0001$), flower colour had no effect on probability of fruiting ($\chi^2 = 0.30$, df = 2, $p = 0.86$; interaction $\chi^2 = 1.01$, df = 2, $p = 0.60$, Fig. 2A).

As for *I. lutescens*, seed set of *I. pumila* depended on the treatment by colour morph interaction (interaction $\chi^2 = 19.84$, df = 2, $p < 0.0001$). Among control flowers, yellow morph showed greater seed set, compared to blue and purple. In contrast, supplied purple flowers had increased seed set compared to other colour morphs (Fig. 2B). Pollinator limitation, calculated based on fruit-set, was 0.66, while pollen limitation, calculated based on seed-set, was 0.04.

Phenotypic selection in *I. lutescens* in the Clape population

In the *aster* model for global fitness, the model including the quadratic terms was not significantly different from the one including only the linear terms ($p = 0.67$, Table 3). There was no significant interaction between treatment and phenotypic traits (Table 3). Morphological traits did not affect female fitness, and only anthocyanin pigment concentrations significantly explained female fitness variation (Table 3). Removing the nested effect of anthocyanin within colour did not significantly change the results (Table 3). Consistently with analyses on fruiting rate and seed-set, there was no difference between colour morphs (Table 3). Therefore, only

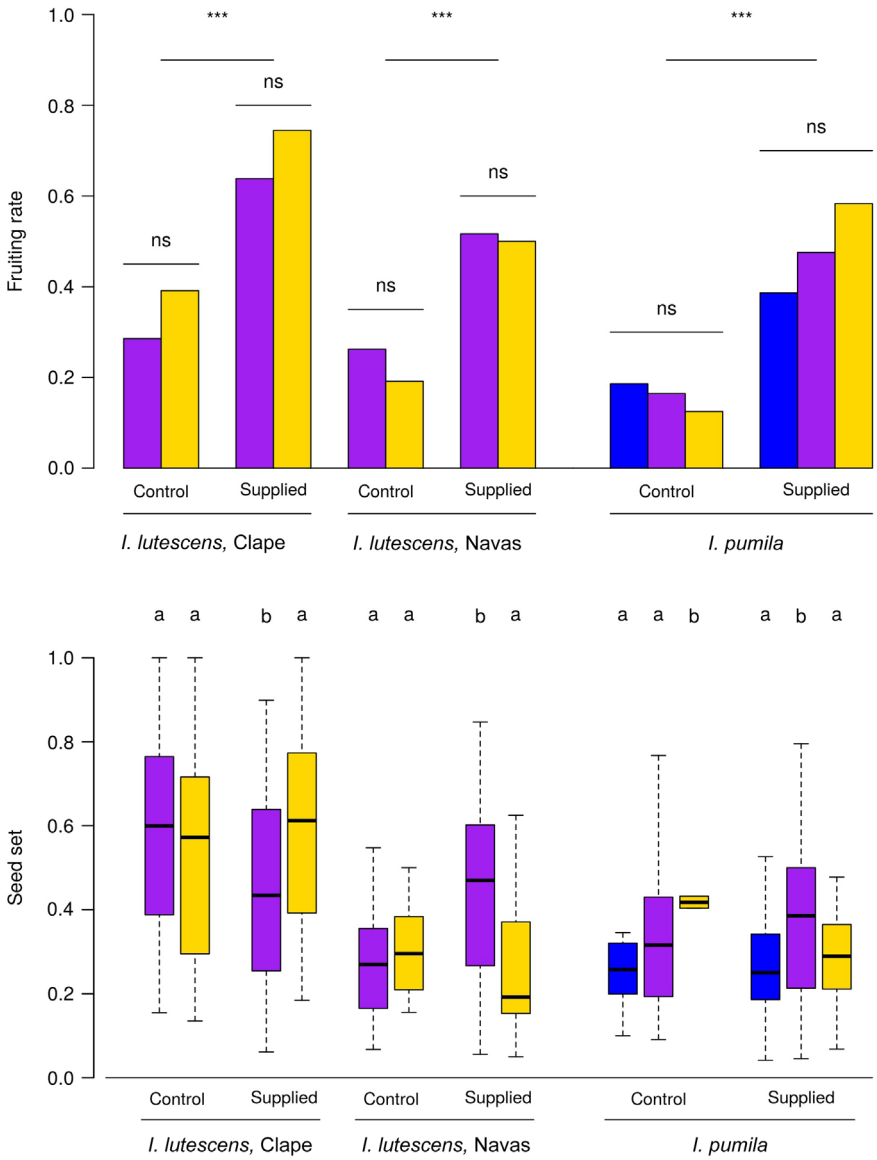

Figure 2. Fruiting rate (% of sampled flowers which produced fruit; upper panel) and seed-set (% of seeds per ovule found in all fruits; lower panel) for control and supplied flowers for each species and for each colour in each population (*I. lutescens*), asterisk denotes significant difference in fruiting rate between treatments; letters indicate significant differences between treatments for seed-set.

the treatment ($p < 0.0001$) and the anthocyanin concentration affected female fitness. Partial selection coefficient for anthocyanin concentration was negative ($\beta = -0.19 \pm 0.002$, Fig. 3).

Phenotypic selection in *I. lutescens* in Navas

Similar to Clape, the minimal retained model included only linear terms, and none of the interactions between phenotypic traits and treatment was significant (Table 3). For PC1, partial coefficient of regression was positive ($\beta = 0.23 \pm 0.007$, Fig. 4A), suggesting a positive effect on plant fitness, independent of pollinator choice, since the interaction PC by treatment was not significant. We also detected an effect of flavonoid concentration on female fitness, which was different between colour morphs (Table 3). Our data suggests disruptive selection, positive in yellow flowers and negative in purple flowers ($\beta = 0.35 \pm 0.01$ and $\beta = -0.20 \pm 0.01$, respectively; Fig. 4A), however, the effect was also independent of pollinators, indicating that there is differential, non-pollinator, selection on colour for this population.

Phenotypic selection in *I. pumila*

Contrary to *I. lutescens*, the retained model included quadratic terms (Table 3). Accordingly, and following stepwise model selection, all interactions between treatment and PC1 and the quadratic term for PC1 were not retained

Table 3. Summary of the *aster* models for each population of *Iris lutescens* and for *Iris pumila*. Minimal models were retained on a classical backward selection procedure. All phenotypic traits are scaled to mean = 0 and $\sigma^2 = 1$ before analyses. The full model was: treatment + colour + colour/flavonoid concentration + colour/anthocyanin concentration + PC1 + interactions with treatment + colour/(flavonoid)^2 + colour/(anthocyanin)^2 + PC1^2 + interactions with treatment. PC1 summarized flower size. For the linear model, all the quadratic terms were removed. Model selection performed by removing parameters step-wise and by testing for significance, using Δdeviance. Significant differences are in bold.

I. lutescens, Clape	Deviance	df	p
full model	–6589.8	26	
linear model	–6597.4	16	0.67
– interactions terms	–6601.6	10	0.65
– PC1	–6603.1	9	0.22
– colour/flavonoid	–6607.4	7	0.12
– colour/anthocyanin	**–6616.0**	**5**	**0.013**
– colour/anthocyanin + anthocyanin	–6607.4	6	0.09
– colour	–6610.2	5	0.21
Minimal model:	**treatment + anthocyanin**		
I. lutescens, Navas			
full model	–5668.2	26	
linear model	–5677.0	16	0.54
– interaction terms	–5681.0	10	0.68
– PC1	**–5687.0**	**9**	**0.014**
+ PC1 – colour/flavonoid	**–5690.7**	**8**	**0.008**
+ colour/flavonoid – colour/anthocyanin	–5685.7	8	0.09
Minimal model:	**treatment +PC1 + colour + colour/flavonoid**		
I. pumila			
full model	–6198.3	36	
linear model	**–6226.6**	**22**	**0.013**
– (PC1)^2 – treatment × PC1 – treatment × (PC1)^2	–6203.3	33	0.16
– PC1	**–6227.7**	**32**	**<0.0001**
+ PC1 – all terms concerning flavonoid	–6216.1	21	0.39
– treatment × colour/(anthocyanin)^2	**–6226.6**	**18**	**0.01**
+ treatment × colour/(anthocyanin)^2 – treatment × colour/anthocyanin	**–6229.0**	**22**	**0.006**
Minimal model:	**treatment + PC1 + colour + colour/anthocyanin + treatment × colour + treatment × colour/anthocyanin + colour/(anthocyanin)^2 + treatment × colour/(anthocyanin)^2**		

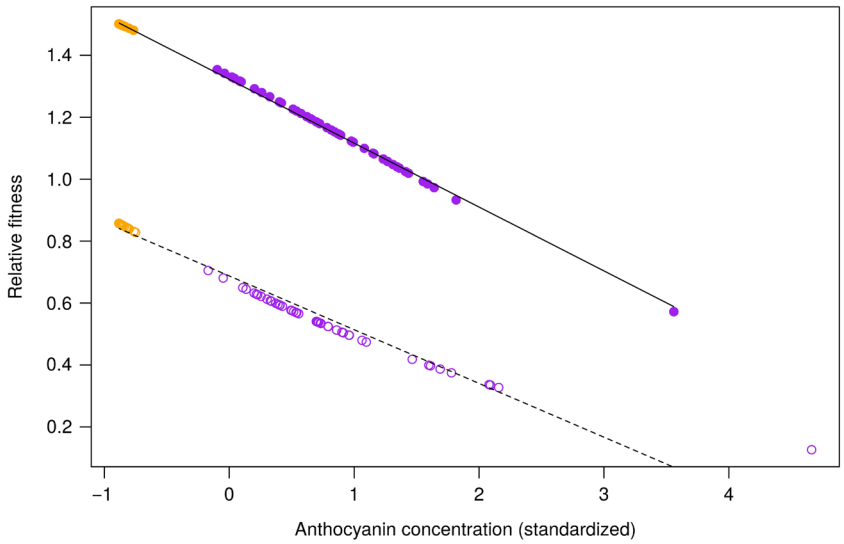

Figure 3. Predicted relative fitness (probability of fruiting×seed set) implemented from the *aster* model according to anthocyanin concentration for *I. lutescens* in the Clape population. Anthocyanin concentrations were standardized prior to analyses. Orange symbols are for yellow flowers and purple symbols are for purple flowers. Open symbols and dashed line are for control flowers (natural pollination) and closed symbols and solid line are for pollen supplied flowers.

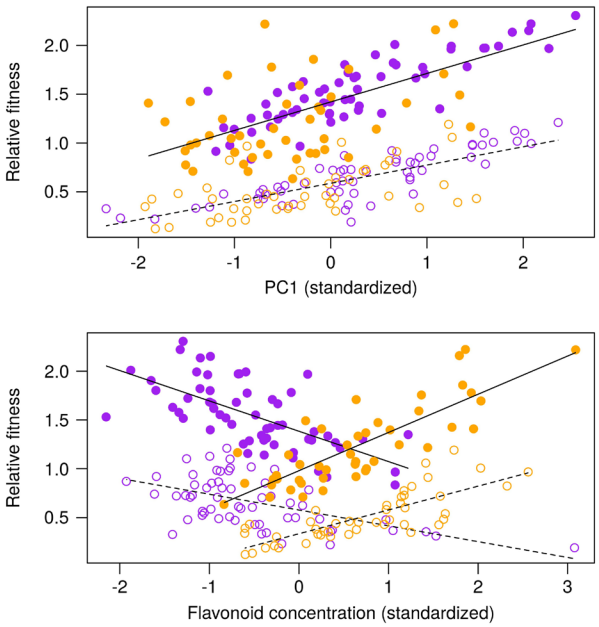

Figure 4. Predicted relative fitness (probability of fruiting×seed set) implemented from the *aster* model according to morphological traits (PC1, upper panel) and flavonoid concentration (lower panel) for *I. lutescens* in the Navas population. Phenotypic traits were standardized prior to analyses. Orange symbols are for yellow flowers and purple symbols are for purple flowers. Open symbols and dashed lines are for control flowers (natural pollination) and closed symbols and solid lines are for pollen supplied flowers.

(Table 3), as well as interactions between treatment and flavonoid concentration (Table 3). PC1 significantly affected female fitness ($p < 0.0001$, Table 3), with a positive partial coefficient of regression ($\beta = 0.40 \pm 0.02$). Furthermore, interactions between treatment and anthocyanins concentration (nested within colour) were significant (Table 3). Therefore, further analyses were performed for each morph separately using the same method, except for the yellow flowers because of the low sample size (Table 1).

For the purple morph, the minimal model retained from the previous analysis was not significantly different ($p = 0.09$) from the null model (the model including only the treatment factor and PC1). Therefore, for this morph, there was neither effect of treatment, nor effect of anthocyanin concentration on female fitness.

For the blue morph, the interaction between treatment and the linear term for anthocyanin concentration was significant ($p = 0.008$), as was the interaction with the quadratic term ($p = 0.006$). For the flowers supplied with pollen, only the partial coefficient of linear regression was significantly different from 0 ($\beta = 0.42 \pm 0.003$, quadratic term $= 0.003 \pm 0.03$), suggesting a positive and linear effect of pigment concentration on female fitness (Fig. 5). For the control flowers, the quadratic term was significantly different from zero, indicating a stabilizing selection ($\gamma = -0.90, \pm 0.12$, $\beta = -0.25 \pm 0.06$, Fig. 5).

Discussion

Flower colour is usually considered to be adaptive in pollination systems, and hence, under pollinator-mediated selection. Nevertheless, our results from two *Iris* species show that pollinators are not the main selection agents on flower colour in these species. In *I. lutescens*, similar to previous results from another *Iris* species (Lavi and Sapir 2015), phenotypic selection was found for pigment concentration, but explicit experimental test showed that this selection is not dependent on pollinators' choice. In the Clape population, we found a negative effect of anthocyanin concentration,

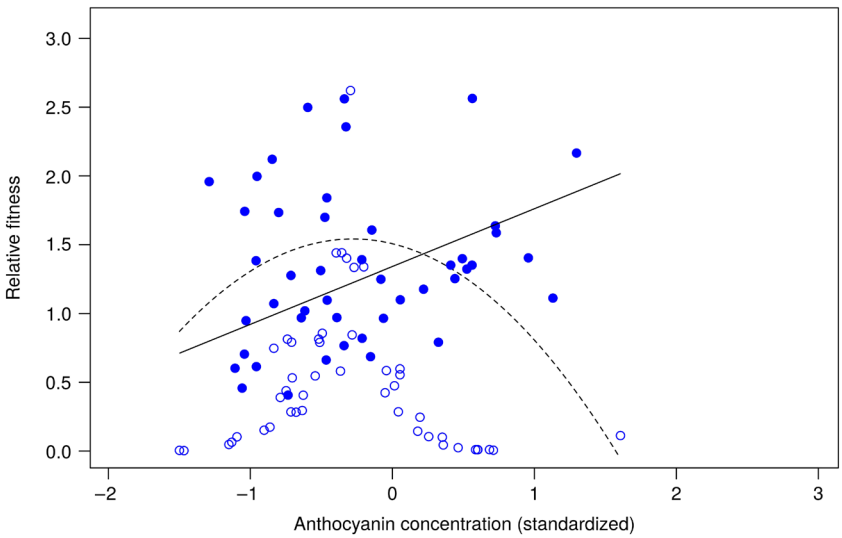

Figure 5. Predicted relative fitness (probability of fruiting×seed set) implemented from the *aster* model according to anthocyanin concentration for the blue morph of *I. pumila*. Anthocyanin concentrations were standardized prior to analyses. Open symbols and dashed line are for control flowers (natural pollination) and closed symbols and solid line are for pollen supplied flowers.

suggesting a cost for anthocyanin production. Consistently, purple flowers supplied with pollen had a lower seed-set than non-supplied flowers in this yellow-dominant population. In the Navas population, where purple flowers are the most abundant phenotype, our results showed a negative effect of flavonoid concentration for the purple flowers and a positive one for the yellow flowers, a pattern that can be interpreted as a disruptive selection. Because pigment synthesis is metabolically expensive, it is reasonable to expect that increased investment in pigment production might correlate with reduced investment in seed production (Chalker-Scott 1999, Campbell et al. 2012). Conversely, in *I. pumila*, we detected a positive correlation between anthocyanin concentration and female fitness, but only for the blue flowers. As flavonoids and anthocyanins play an important role in plant defense against various environmental stresses (e.g. UV radiation, herbivory, drought; Chalker-Scott 1999, Winkel-Shirley 2002, Coberly and Rausher 2008, Tucić et al. 2009, Arista et al. 2013, Landi et al. 2015), local variation in environmental pressures may account for the different effects of pigment concentration and female fitness in the three experiment conditions. These effects could also potentially vary within different years since environmental heterogeneity does not remain constant through time. Coupled with the long-lasting seed bank of the species, morph frequencies and reproductive success of individuals within different populations may depend more on environmental conditions than on pollinator attraction. As in Lavi and Sapir (2015), our experiment controlled only for the role of pollinators, hence, we do not have an explanation for the non-pollinator mediated selection for flower colour found here. Further studies are underway to test for the effect of abiotic environmental stresses, such as evapotranspiration and resource limitation

For *I. pumila*, the role of pollinators, particularly bumblebees, appeared to be more ambiguous since we detected a stabilizing selection mediated by pollinator's choice for the blue morph only. Some studies have reported that bumblebees have an innate colour preference for the blue range of wavelengths (Lunau and Maier 1995, Smithson and Macnair 1996), a pattern observed in other bees species (Dyer et al. 2016). Such innate colour bias is expected to guide them to explore blue flowers in preference to other natural objects within a landscape. Although learning process seems to be very important for bumblebees' flower colour choice, this innate preference towards blue wavelengths is maintained even after extensive training to other colors (Gumbert 2000). Similarly, as this experiment was performed in a common garden, pollinator composition might differ from those found in natural populations which may also influence these results. As for now, our results provide the basis for hypotheses regarding preferences of pollinators for intermediate colour values, result to be linked with the importance of the inaccuracy of colour discrimination by insects in the maintenance of flower colour polymorphism (Kagawa and Takimoto 2016).

Few studies have investigated putative phenotypic selection for flower colour in comparison to other flower traits (Parachnowitsch and Kessler 2010). Some studies have reported an effect of pollinator behaviour on flower colour (Jones and Reithel 2001, Irwin and Strauss 2005, Caruso et al. 2010, Hirota et al. 2013, Sletvold et al. 2016), supporting the general acceptance of pollinators as the selection agent on floral visual attraction traits. However, there are a several other studies that failed to document pollinator selection on flower colour (Parachnowitsch and Kessler 2010, Campbell et al. 2012, Parachnowitsch et al. 2012, Lavi and Sapir 2015). Together with our results reported here, we argue that pollinator-mediated selection on flower colour is, to the least, only part of the story, and pigment-derived colour in petals may be the outcome of other selection agents.

In food deceptive plant species, such as two *Iris* species analysed here, pollinator-mediated selection may maintain variation in floral traits by interfering with pollinator learning to avoid rewardless flowers (Smithson and Macnair 1997, Ferdy et al. 1998, Kagawa and Takimoto 2016). In natural populations, flower colour polymorphism is supposed to lead to negative frequency-dependent selection, thus an advantage to the rare morph. As pollinators learn to associate flower colour with reward quality, they avoid the most common rewardless morph, thus over-visiting the rarer morph (Smithson and Macnair 1997, Ferdy et al. 1998, Gigord et al. 2001, Kagawa and Takimoto 2016). Consistently with previous studies on *I. lutescens* (Imbert et al. 2014a, b), and other studies on rewardless species (Aragón and Ackerman 2004, Jersáková et al. 2006b), we did not detect any difference in fruit-set between colour morphs for both naturally and supplementary-pollinated plants, regardless of morph frequency at either site. Furthermore, as commonly observed in rewardless plants (Aragón and Ackerman 2004, Tremblay et al. 2005, Dormont et al. 2010a, Sletvold et al. 2016, Sonkoly et al. 2016), we documented a low natural fruiting rate and a high pollinator limitation level for both studied species. For comparison, in *I. tuberosa*, a Mediterranean species producing nectar, natural fruiting rate is greater than 60% (Pellegrino 2015). However, number of seeds showed differences between colour morphs in supplementary hand pollination treatments, suggesting intrinsic differences between colour morphs. While these differences could contribute to differential fitness, these results provide that these differences are not pollinator-related.

The strong pollinator limitation on flowers of both *I. lutescens* and *I. pumila*, and the absence of surrounding flower choice (Imbert et al. 2014a) are consistent with exploiting the naivety of recently emerged pollinators, as reported in some deceptive orchids (Jersáková et al. 2006b, Dormont et al. 2010b). Some examples of pollinator-mediated selection have been reported in plant species visited by a low diversity of pollinators (Sletvold et al. 2016). In the species studied here, pollinators are rather diverse and opportunistic (Imbert et al. 2014b), thus attraction depends mainly on innate preference for showy flowers. Nevertheless, we sampled during flowering peak period in order to have a robust sample size, but pollinator-mediated selection may be stronger during

different times. Pollinators which have learned to avoid rewardless flowers may further reduce reproductive success in late-flowering individuals. Similarly, early-flowering individuals may be subject to stronger selection by naïve pollinators, nevertheless, in a wide phenotypic study of *I. pumila*, Tarasjev (1997) reported that 40 to 90 percent of individuals in natural populations began flowering eight days after the onset of flowering (Tarasjev 1997). This rapid flowering could in fact dilute any effect early flowering may have on teaching insects to avoid these rewardless flowers.

Our results suggest lack of discrimination between different flower colour morphs by visiting insects. In the absence of other selective pressures on pigment concentration, such a situation should lead to monomorphic populations (Kagawa and Takimoto 2016). Most of the populations of *Iris lutescens* (Wang et al. 2016) and all populations of *I. pumila* are polymorphic, suggesting that monomorphic populations are the exception. In *I. lutescens,* a recent study showed that colour polymorphism is neutrally distributed in space (Wang et al. 2016), but association of yellow morph frequency with elevation in French populations suggests that environmental factors do affect flower colour variation in this species (Imbert unpubl.). Tucić et al. (1989) conducted extensive study to determine whether the clonal colour diversity existing in a natural population of *I. pumila* was causally related to variation in some climatic factors (temperature and rainfall). They identified temperature conditions as one of the factors which exerted influence on the extent of flower colour polymorphism. It was proposed that a combination of fluctuating temperatures contributed to environmental heterogeneity and thus, promoted stable coexistence of multiple colour variants in the natural population of *I. pumila* (Tucić et al. 1989).

Supplementary hand pollination experiment revealed strong pollinator limitation in both species, as hand-supplemented flowers produced significantly more fruits than those subject to natural pollination, regardless of colour and morphology. Pollen limitation has two components: lack of pollinators (i.e. reduced number of visits) and low efficiency of pollen vectors, requiring multiple visits to deposit enough pollen (Campbell and Husband 2007). In our study, pollen supply increased fruit production for both species. The significantly greater fruiting rate of hand-pollinated individuals demonstrate that these *Iris* species are pollen limited, while the lack of interaction between morphology and natural pollination indicates that pollinator attraction does not depend on floral traits. This is in line with a study on another Mediterranean *Iris* species*, I. tuberosa,* where pollinators did not discriminate between tall/short or large/small flowers and rather visited morphologically different flowers in equal proportion (Pellegrino 2015). Additionally, pollinator limitation appears to be common throughout the genus, as identified in *I. lacustris* (Planisek 1987), *I. versicolor* (Wheelwright et al. 2006), *I. bismarckiana* (Segal et al. 2006), *I. tuberosa* (Pellegrino 2015), *I. atropurpurea* and *I. haynei* (Lavi and Sapir 2015). Nevertheless, we only measured proximate pollen limitation due to pollinators. As both studied species are rhizomatous, fruit production could be costly and might appear to be resource limited over the plant's life (Ackerman and Montalvo 1990, Tarasjev 2005, Pellegrino 2015). Similarly, due to their self-incompatibility, geitonogamy and stigmate loading through pollinator visits to several flowers of the same genet could also account for low fruiting rates.

Finally, we found a positive relationship between floral morphology (as explained by PC1) and female fitness in both species. This result may not be surprising since generally, larger flowers produce bigger fruits, which in turn are able to produce more seeds (Primack 1987). However, similar to *I. tuberosa*, there was no pollinator-mediated selection for this trait (Pellegrino 2015). *Iris* flowers display some of the largest flowers in the Mediterranean basin, and our results indicate that although not pollinator-mediated, floral morphology seems to be under positive selection, favouring larger flowers. Similarly, the lack of clear pollinator choice for pigment concentration may possibly indicate that flower colour of the genus is rather labile, explaining the wide variety of flower colours different *Iris* species express.

Acknowledgements – We thank S. Rancus-Lazar for English editing.
Funding – This work was partly supported by Erasmus Mundus scholarship to DS, the Ministry for Education, Science and Technological Development of Serbia, grant no. 173007, a PhD grant of the China Scholarship Council to HW, and the Hubert Curien Program Pavle Savić to EI.
Author contributions – DSV and AV are first co-authors. DSV and AV wrote the manuscript with extensive comments and input by all authors during all stages. EI, HW and YS designed the study. DSV, HW, YS and EI collected and analysed the data for *I. lutescens* while AV, SMJ and SB collected and analysed data for *I. pumila*. All authors approved the final version of the manuscript.

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