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Research

Are pollinators the agents of selection for the extreme large size and dark color in *Oncocyclus* irises?

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Summary

- Pollinator-mediated selection is a major evolutionary driver of floral traits; yet, such selection has rarely been tested for floral extreme traits. The *Oncocyclus* irises have exceptionally large, dark-colored flowers, associated with night-sheltering pollination and heat reward by the dark flowers.
- We quantified phenotypic selection on stem length, floral size and color in two species of iris (*Iris atropurpurea* and *I. haynei*), using an experimental approach. We estimated selection gradients for both flowers open to natural pollination and for flowers receiving supplementary hand pollination, assuming that open-pollinated flowers are affected by all factors that could influence fitness, whereas supplementary pollination removes the possible influence of pollinators.
- We found evidence for pollinator-mediated selection to increase floral size and stem length in *I. atropurpurea*, but floral color in this species was not under pollinator-mediated selection. In *I. haynei*, no pollinator-mediated selection on any of the traits was detected.
- We conclude that the extreme floral size of *I. atropurpurea* has probably evolved as a result of pollinator behavior. Lack of such evidence for *I. haynei* and for the dark floral color in both species suggests that other non-pollinator agents are selecting for these prominent traits, or that phenotypic color variation in these irises is neutral.

Introduction

Plant–pollinator interactions are thought to have shaped patterns of floral diversity through the preferences of animal pollinators for a suite of floral traits (Darwin, 1862; van-der-Pijl, 1961; Grant, 1994; Fenster *et al.*, 2004; Aigner, 2006). Floral size attracts pollinators, which react to the association between advertisement size and the amount of reward (Cohen & Shmida, 1993; Kaczorowski *et al.*, 2005; Fenster *et al.*, 2006; Higginson *et al.*, 2006). Floral color is an advertisement trait also proposed to affect discrimination by different groups of pollinators (Stuurman *et al.*, 2004; Wilson *et al.*, 2004; Hegland & Totland, 2005; Wolfe & Sowell, 2006). In addition to selection for specific floral traits, pollinators can select for a genetically correlated suite of traits and create pollination syndromes (Armbruster *et al.*, 2004; Reynolds *et al.*, 2010; Murren, 2012). Overall, a suite of directional, stabilizing and correlational selection regimes on floral traits is expected to be mediated by pollinators.

Pollen limitation, which reduces a plant's female fitness, is proposed to facilitate pollinator-mediated selection (Ashman *et al.*, 2004; but see Parachnowitsch & Kessler, 2010). Experimental removal of pollen limitation by the hand pollination of flowers removes any selection imposed by pollinators, and enables an estimation to be made of the relative role of pollinators as agents of selection (Sandring & Ågren, 2009; Sletvold *et al.*, 2010). Although flowers open to pollination are exposed to all selection agents acting on floral traits (e.g. water loss; Galen, 2000; Strauss & Whittall, 2006), including pollinators, hand-pollinated flowers will be exposed to all selective pressures **except** those imposed by pollinators. Thus, differences in selection gradients between treatments allow the isolation of pollinator-mediated selection. In this study, we used this method to experimentally estimate pollinator-mediated selection on floral traits in the royal irises.

The species known as royal irises (*Iris* section *Oncocyclus*) are exceptional in their large flower size, which can reach 12 cm in diameter (Sapir *et al.*, 2002), rendering them among the largest flowers in the flora of the Middle East. Another extraordinary floral trait is their dark color: from dark pink and purple to dark brown and black (Fig. 1a,b). Their pollinators are night-sheltering male bees, mainly from the genus *Eucera* (Sapir *et al.*, 2005; Watts *et al.*, 2013; Fig. 1c). The reward to the night-sheltering pollinators is the high temperature absorbed by the dark-colored flowers in the hour after sunrise (Sapir *et al.*, 2006).

We tested hypotheses concerning the role of pollinators in shaping two floral traits in the royal irises, namely floral color and floral size. In a preliminary study, we found that male bees prefer dark-colored artificial night shelters over light-colored ones, with no preference for the size of the shelter (Y. Sapir, unpublished). Thus, we hypothesized strong pollinator-mediated selection on darker floral color. Floral size was hypothesized to be

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Fig. 1 (a) Flowers of *Iris atropurpurea* in the Netanya site; (b) flowers of *Iris haynei* in the Gilboa site; (c) male bee shelters within the pollination tunnel of *I. haynei* in Gilboa; (d) floral traits measured in this study, shown on *I. atropurpurea*.

under stabilizing selection, which results from positive pollinator-mediated selection for increasing advertisement and negative environmental-mediated selection for reducing water-evaporating petal area.

We tested these hypotheses in natural populations of two species: *Iris atropurpurea* and *Iris haynei*. For both species, we used an experimental approach to partition the selection mediated by pollinators from other selection agents, by removing pollen limitation using supplementary hand pollination (following Sandring & Agren, 2009). Statistically, analysis of covariance (ANCOVA) was employed to test the significance of the interaction between treatments (open vs supplementary pollination) and phenotypic trait in their effect on fitness. Significant interaction points to a significant difference between selection regimes of the phenotypic trait under the two treatments, which means that the removal of pollen limitation removes any selection imposed by pollinators, and implies that pollinators are the agents of selection.

Materials and Methods

Study system and sites

The large, dark-colored flowers of *Iris atropurpurea* Dinsmore and *I. haynei* Baker consist of three erect large inner tepals (standards) and three pollination tunnels, formed by the wide and colorful style bending above the outer tepals (falls; see Fig. 1a,b,d; Sapir *et al.*, 2002). These species are completely self-incompatible, and maternal fitness (fruit and seed production) depends entirely on the night-sheltering pollinators (Sapir *et al.*, 2005; Watts *et al.*, 2013). The plants also reproduce vegetatively by underground rhizomes, creating distinct patches (genets) of genetically identical ramets (*sensu* Harper, 1977).

Iris atropurpurea is endemic to the Israeli coast, and its distribution is limited to a narrow belt of *c.* 70 × 12 km along the coastal plain (Shmida & Pollak, 2008). This species grows only on sandy soils of the Pleistocene, a vulnerable habitat because of its location in Israel's most populated and urbanized region. The number of *I. atropurpurea* populations is declining, with about one-third of the populations already extinct (Sapir *et al.*, 2003). *Iris haynei* is endemic to northern Israel, distributed mostly in the Gilboa mountain ridge, where settlement development and forestry threaten the populations (Kintisch, 2005). In both *I. haynei* and its vicariant species, *I. atrofusca*, substantial phenotypic variation has been recorded within and among populations, especially with regard to floral traits (Arafeh *et al.*, 2002; Sapir *et al.*, 2002).

Iris atropurpurea was studied in the Netanya Iris Reserve (32°17′N, 34°50′E, altitude 37 m above sea level), located on a sandstone ridge covered by semi-stable sand dunes. The vegetation is dominated by the shrubs *Retama raetam* and *Lycium schweinfurthii*, with many annual species. The reserve aims to preserve the largest population of *I. atropurpurea* and is highly genetically and morphologically variable (see Supporting Information Fig. S1; Sapir & Mazzucco, 2012). *Iris haynei* was studied in Malkishua, on the Gilboa ridge (32°26′N, 35°24′E, altitude 440 m above sea level), on a southwest-facing slope. In this location, the irises grow on Eocene limestone, dominated by a pine forest planted *c.* 1960. Natural vegetation includes shrubs of *Pistacia lentiscus* and *Rhamnus lycioides*, and herbaceous plant species. To date, this is the largest known population of *I. haynei*, estimated to contain a few thousand plants. Relative to *I. atropurpurea*, this population is less variable (see Fig. S2).

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Experimental setup

Phenotypic selection on floral traits was quantified in the years 2010 and 2011, in February (*I. atropurpurea*) and March–April (*I. haynei*). A single patch (genet) contains between one and a few tens of flowers for *I. haynei*, or up to hundreds of flowers for *I. atropurpurea*. One flower was chosen randomly within each patch and was marked with a unique number. In a preliminary analysis, we measured two flowers in each of 94 patches across both sites (43 in *I. atropurpurea* and 51 in *I. haynei*). For all traits, we found that variation within patches was negligible (0.1–1.8% of total variation) relative to variation among patches (2–62% of total variation). Hence, we used a single flower to represent each patch.

Each day of field work, recently opened flowers (1 d after anthesis) were marked and measured. Of these flowers, half were randomly assigned as open pollinated and remained untreated, whereas the rest were designated for supplementary hand pollination. In the latter, stigmas were brushed with dehisced anthers from flowers > 10 m away. All three stigmas were brushed with the same anther, ensuring that > 800 grains were deposited on each stigma to guarantee sufficient pollen for maximal fertilization (Watts *et al.*, 2013). Flowers receiving supplementary pollination were not covered, allowing natural pollination as in the open-pollinated flowers. All the flowers of both treatments were measured for phenotypic traits.

Phenotypic traits

The traits measured were as follows: stem length, the height from the ground to the base of the outer tepal perpendicular to the ground; flower diameter, the distance across the widest part of the outer tepal; and floral length, the distance from the base of the outer tepal to the highest point of the inner tepal (standard; Fig. 1d). The product of the last two measures was used to approximate floral size, representing the two-dimensional projection of floral area.

To quantify flower color, one disk of 1 cm in diameter was cut from one of the outer tepals. The disk was placed in a tube containing 1 ml of methanol with 1% HCl (Weiss & Halevy, 1989) for pigment (anthocyanin) extraction. The tubes were kept at 4°C for at least 24 h before measuring the anthocyanin concentration by spectrophotometry. Previous studies on irises used a wavelength of 530 nm to measure anthocyanin concentration (Yabuya *et al.*, 1997). In a preliminary study, we found that, in both *I. atropurpurea* and *I. haynei*, the maximal absorbance is at 540 nm. Light absorbance was measured using an ULTROSPEC 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) in 2010 and a SpectraMax spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, USA) in 2011. These two devices gave different values of absorbance; thus, the values were scaled separately for each year (see Statistical analysis). Carotenoid pigments are probably also present in iris flowers (Tanaka *et al.*, 2008), and their traces were indeed found in the process of extracting anthocyanin pigments. However, these pigments were not measured in this study.

Fitness

Maternal fitness was estimated by seed-set (number of seeds per flower). About 6 wk after the middle of the flowering season, all marked flowers were surveyed for fruits. Fruits produced were collected and seeds were counted in the laboratory after complete ripening. Fruits that did not contain seeds, as well as flowers that did not set fruits, were recorded as 0 seeds. Relative fitness was calculated by dividing the number of seeds in each flower by the average number of seeds per flower.

Statistical analysis

To assess pollen limitation, we compared fruit-set (fraction of flowers that produced fruits) between treatments using a generalized linear model (GLM) with year as a fixed factor and with binomial distribution of errors. Seed-set was compared between treatments and years using GLM with a Poisson distribution of errors. These analyses were performed separately for each species. For each species and year, we calculated a pollen limitation index (PLI) following Campbell & Husband (2007):

$$\mathrm{PLI} = \frac{\mathrm{seed.set}_{\mathrm{Suppl.}} - \mathrm{seed.set}_{\mathrm{open}}}{\mathrm{seed.set}_{\mathrm{Suppl.}}} \qquad \text{Eqn 1}$$

This index compares the difference between seed-set following supplementary hand pollination and seed-set following natural pollination, divided by the former, and represents the potential for seed production when pollination is not limited. PLI values above zero indicate pollen limitation.

Selection was estimated following Lande & Arnold (1983), using multiple regression to estimate selection gradients (β). Traits were standardized to mean = 0 and variance = 1. We used standardized trait values as explanatory variables and relative female fitness (with mean fitness = 1) as the response variable, and analyzed the data using GLMs. Female fitness (as seed-set) was relativized separately for each treatment and year. The use of standardized trait values and relative fitness enabled the estimation of standardized partial linear regression coefficients that correspond to the strength and direction of the directional selection acting on each trait (Lande & Arnold, 1983). Non-linear selection gradients were estimated using GLM, which included quadratic (γ_{ii}) terms in addition to the linear term. Non-linear partial regression coefficients were doubled to obtain stabilizing or disruptive selection gradients (Stinchcombe *et al.*, 2008).

We used ANCOVA to determine whether selection gradients varied between pollination treatments (open-pollinated or supplementary pollination treatments). The interaction term obtained in such ANCOVA models is an estimation of the difference between the selection gradients exerted by pollinators (in the open pollination treatment) and by other selection agents (in the supplementary hand pollination treatment). Significant interaction suggests a change in the selection regime, providing evidence for pollinator-mediated selection.

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ANCOVA models were analyzed separately for each year and for each species. All statistical analyses were performed in the software R version 2.15 (R Development Core Team, 2012).

Results

Fitness and pollen limitation

In Netanya, 110 and 207 *I. atropurpurea* flowers were measured in 2010 and 2011, respectively. In Gilboa, 168 and 190 *I. haynei* flowers were measured in 2010 and 2011, respectively. Trait values are presented in Table 1.

In *I. atropurpurea*, fruit-set was significantly higher in 2011 than in 2010 ($P = 0.048$; Fig. 2a). No difference between years was found in *I. haynei* ($P = 0.124$; Fig. 2c). In both species, a significant increase in fruit-set was found in supplementary hand-pollinated flowers relative to open-pollinated flowers ($P < 0.05$; Fig. 2a,c). In both species and both years, supplementary hand pollination resulted in higher seed-set relative to open-pollinated flowers ($P < 0.001$; Fig. 2b,d). Pollen limitation was higher in *I. atropurpurea* (PLI = 0.57) and *I. haynei* (PLI = 0.45) in 2010 relative to 2011, with PLI = 0.38 and PLI = 0.33 for *I. atropurpurea* and *I. haynei*, respectively.

Pollinator-mediated selection in *I. atropurpurea*

Selection regimes were not equal between the 2 yr of the study. Significant interactions between treatments and phenotype, indicating different selection gradients, were found for

Table 1 Mean values (± SE) for morphological traits (stem length, floral diameter, floral length, outer tepal pigment concentration) measured in *Iris atropurpurea* and *I. haynei* in 2010 and 2011

Species	Year	Stem length (cm)	Floral diameter (mm)	Floral length (mm)	Outer tepal pigment (units of relative absorbance)
Iris atropurpurea	2010	31.5 ± 0.91	64.6 ± 0.78	78.8 ± 1.15	2.3 ± 0.05
	2011	31.3 ± 0.58	68.8 ± 0.58	80.9 ± 0.78	1.4 ± 0.03
Iris haynei	2010	34.9 ± 0.60	85.7 ± 0.73	106.2 ± 1.30	2.2 ± 0.03
	2011	39.7 ± 0.68	90.5 ± 0.67	114.8 ± 1.08	1.3 ± 0.03

Pigment concentrations are in relative absorbance measured by spectrophotometry with different devices in 2010 and 2011 (see text).

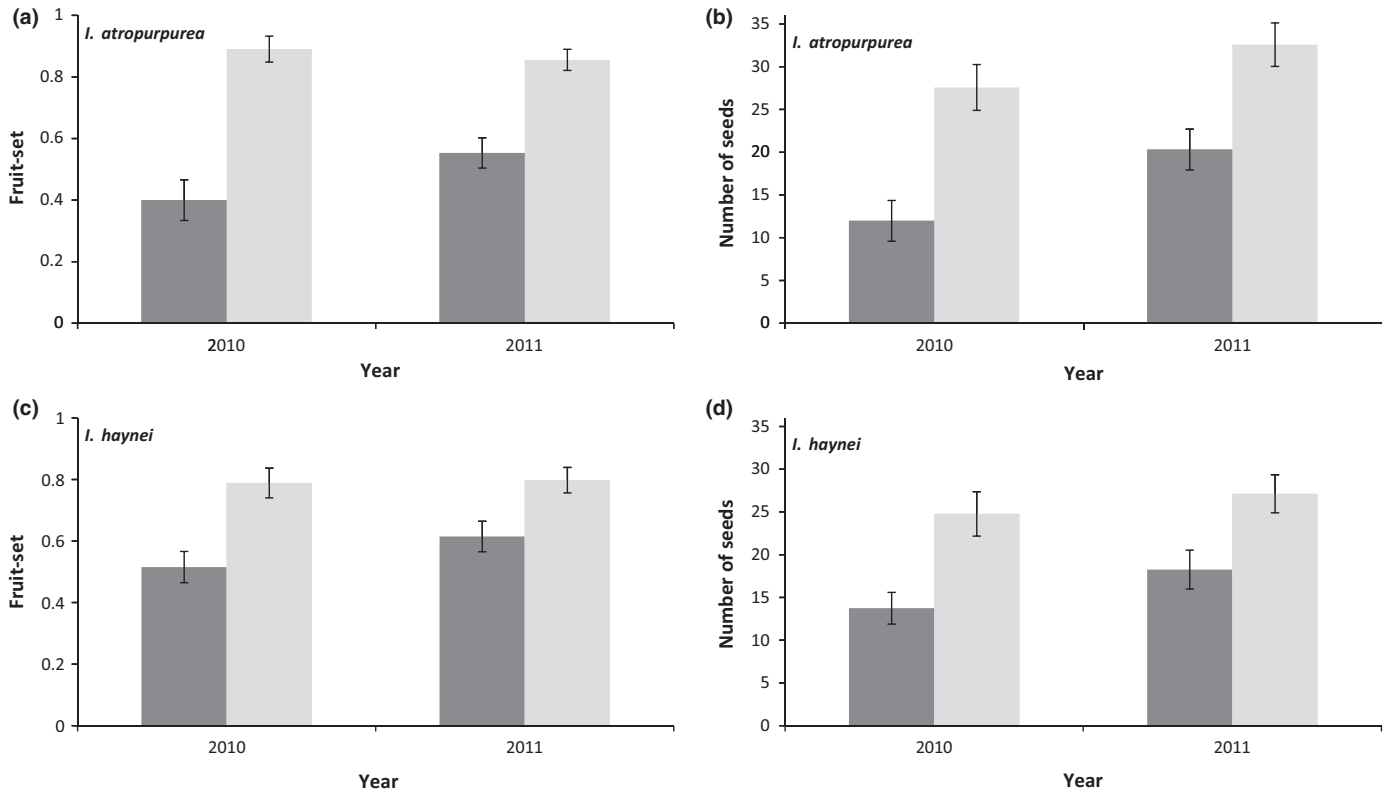

Fig. 2 Evidence for pollen limitation in two species of *Oncocyclus* irises in 2010 and 2011. Pollen limitation measured as fruit-set, the fraction of flowers producing fruits, and seed-set, the number of seeds in a pod. Dark gray bars, open pollination – flowers open to natural pollination; light gray bars, supplementary pollination – flowers receiving supplementary pollen by artificial hand pollination. (a) Fruit-set in *Iris atropurpurea*; (b) seed-set in *I. atropurpurea*; (c) fruit-set in *Iris haynei*; (d) seed-set in *I. haynei*. Bars are fraction of fruit-set (graphs a, c; ± standard errors) or mean of seed-set (graphs b, d; ± standard errors). All differences between the two treatments are significant ($P < 0.05$) in both years and both species.

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stem length in 2010 (Table 2), indicating pollinator-mediated selection. Flowers with longer stems had higher fitness in flowers open to pollination (Fig. 3a), whereas, in flowers receiving supplementary pollination, no effect of stem length was detected on fitness.

In 2011, stem length and flower size, but not color, were under positive pollinator-mediated selection, indicated by a significant interaction of the ANCOVA model, with positive selection gradient in flowers open to pollinators and non-significant selection gradients in flowers receiving supplementary pollination (Table 2; Fig. 3b,c). Both stem length and floral size were also under non-linear pollinator-mediated disruptive selection (Table 2; Fig. 3d,e).

Pollinator-mediated selection in *I. haynei*

No pollinator-mediated selection was detected in *I. haynei* in either year or on any of the three traits. Floral size was under positive directional selection, which, in 2010, was significant only for flowers receiving supplementary pollination, whereas, in 2011, it was significant only for flowers open to pollinators (Table 3; Fig. 4). Differences between selection gradients of the two treatments were not significant in either case (Table 3).

Discussion

Pollinators of the *Oncocyclus* irises are attracted to the heat absorbed in the dark-colored flowers in the morning (Sapir *et al.*, 2006). Hence, we expected that floral color would be the major target of pollinator-mediated selection, rather than floral size. Surprisingly, we found instances of pollinator-mediated selection only in *I. atropurpurea* and, even then, only on estimates of floral size (including stem length), with no evidence of such selection on floral color (Tables 1,2). This pollinator-mediated selection could be detected only through manipulative studies of supplementary pollination that enabled the identification of pollinators as the agents of selection.

The selection found on floral size suggests an advantage for larger flowers or longer stems in attracting pollinators. Numerous studies have shown directional selection on floral size (reviewed in Harder & Johnson, 2009), which was assumed to be mediated by pollinators. Here, we provide experimental evidence for pollinator-mediated selection on floral size in irises. However, this selection was not constant across both years and was found only in one species, suggesting that multiple factors act as selection agents on floral size in these two *Iris* species, and may obscure this selection, if it exists, in *I. haynei*. For example, such selection may act through reduced fitness of larger flowers that suffer from increased water loss (Galen, 2000; Strauss & Whittall, 2006). Such a hypothesis requires a field experiment to test for the effect of water availability on reproductive success in these irises, and the interaction of this effect with the effect of pollinators, by experimentally removing their effect, as performed here.

The extreme floral size of these irises is not sufficiently explained by the results of the current study. It is possible that selection on floral size acts through male fitness, which may form a different selection regime on floral traits (Horovitz, 1978). A few studies have used male fitness to estimate pollinator-mediated selection on floral traits (Young & Stanton, 1990; van Kleunen & Burczyk, 2008; Sahli & Conner, 2011), suggesting a significant role of male function in selection on floral size, which does not necessarily contradict selection via female fitness (Ashman & Morgan, 2004). Hence, it is plausible that floral size in irises is positively selected via male fitness, because of the advantage of large flowers having larger pollination tunnels enabling aggregations of night-sheltering pollinators, which facilitates pollen removal (Y. Sapir, M. Fishman & L. Hadany, unpublished).

The role of floral color in attracting pollinators has been studied extensively (e.g. Levin & Kerster, 1967; Waser & Price, 1983; Stanton *et al.*, 1986), and the evolutionary-genetic basis of this interaction has been studied in a few model systems (Bradshaw & Schemske, 2003; Hoballah *et al.*, 2007; Hopkins & Rausher, 2011). The dark color of the iris flowers studied here is rare among flowering plants. Dark-colored flowers are typically

Table 2 Selection gradients (± SE) on floral traits in *Iris atropurpurea* in Netanya

Trait	β open ± SE	β supp. ± SE	Interaction *P* value	γ open ± SE	γ supp. ± SE	Interaction *P* value
2010						
Stem length	**0.445** ± 0.177	−0.036 ± 0.170	**0.027**	1.800 ± 1.660	2.086 ± 1.888	0.547
Flower size	0.164 ± 0.177	0.182 ± 0.173	0.994	−0.340 ± 1.616	0.159 ± 2.301	0.884
Pigment concentration	0.203 ± 0.153	0.149 ± 0.166	0.814	−0.958 ± 1.994	0.018 ± 2.345	0.456
2011						
Stem length	**0.251** ± 0.092	0.098 ± 0.092	**0.023**	**3.469** ± 1.198	−0.423 ± 0.758	**0.005**
Flower size	**0.562** ± 0.083	0.146 ± 0.107	**0.003**	**3.334** ± 0.843	−2.847 ± 1.473	< **0.001**
Pigment concentration	0.116 ± 0.089	**0.173** ± 0.088	0.647	1.121 ± 0.590	1.329 ± 0.729	0.825

Linear (β) values are partial regression coefficients; positive or negative values imply positive or negative directional selection, respectively. Non-linear (quadratic; γ) positive or negative gradients imply disruptive or stabilizing selection, respectively. Quadratic partial regression coefficients were doubled to obtain selection gradients (Stinchcombe *et al.*, 2008). Selection gradients are presented for open pollination ('open') and supplementary pollination ('supp.') treatments. Significant ($P < 0.05$) gradients are in bold. Significance values are presented for the interaction between pollination treatment and trait in their effect on fitness. Significant interaction indicates a change in the selection gradient caused by the treatment, thus implying pollinator-mediated selection on this trait, given that open-pollinated flowers include all factors that could influence fitness, whereas supplementary pollination removes the possible influence of pollinators.

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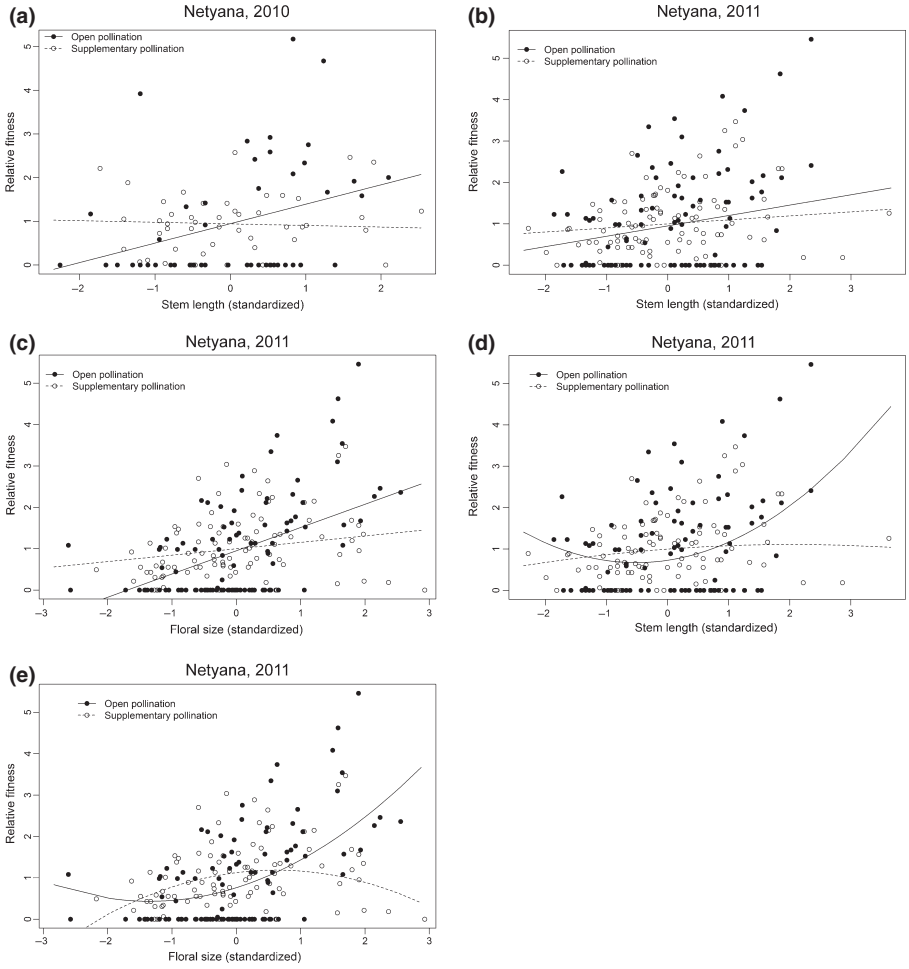

Fig. 3 Pollinator-mediated selection on floral traits in *Iris atropurpurea*. Lines represent the fitted function for each treatment (open/supplementary pollination) from the linear model, including three floral traits (stem length, floral size and floral color). Solid line and closed circles, values of flowers open to natural pollination; dashed lines and open circles, values of flowers receiving supplementary pollination. In all graphs, the differences between slopes of treatment effects were significant ($P < 0.05$). (a) Directional selection on stem length in 2010; (b) directional selection on stem length in 2011; (c) directional selection on floral size in 2011; (d) non-linear selection on stem length in 2011; (e) non-linear selection on floral size in 2011. See Table 2 for values and significance of selection gradients.

associated with deceptive pollination systems (Paulus & Gack, 1990; Albre *et al.*, 2003; Balkenius & Kelber, 2006), but the dark color of the *Oncocyclus* iris flowers is associated with heat reward to the night-sheltering pollinators (Sapir *et al.*, 2006). This predicts constant and strong pollinator-mediated selection for darker flowers in the two species studied.

Contrary to this expectation, we found no evidence for pollinator-mediated selection on floral color in these irises. Moreover, in 2011, we found positive directional selection on floral color in *I. atropurpurea* plants that were artificially hand pollinated. These results raise the question of the adaptive value of dark color in *Oncocyclus* irises. We propose two hypotheses to explain selection for darker flowers. First, the irises studied here may have reached their adaptive optimum for color trait, and the apparent variation among flowers (see Figs S1, S2) is neutral and does not affect pollinator choice. To test this hypothesis, one should expand the variation artificially, for instance by artificial selection (Conner, 2003; Delph *et al.*, 2004). Second, a high concentration of anthocyanin pigment could be beneficial to protect against herbivory (Coberly & Rausher, 2003; Frey, 2004; Strauss *et al.*, 2004). *Oncocyclus* irises are exposed to heavy herbivory pressure throughout their habitats in the semi-arid region of the Middle East, suggesting a hypothesis that herbivory stress acts as a selection agent on floral color. This can explain the positive directional selection found in *I. atropurpurea* when pollinator limitation was removed (in artificial hand pollination): without pollen limitation, darker flowers may have higher fitness because of less herbivory on the flowers. This hypothesis should be tested explicitly, with a controlled herbivory experiment (Cariveau *et al.*, 2004; Wang & Mopper, 2008).

Summary

Strong pollen limitation in the two *Iris* species studied here over 2 yr suggests that pollen limitation generates the basis for pollinator-mediated selection on floral traits. We found pollinator-mediated selection on floral size and stem length only in *I. atropurpurea*, whereas pollinator-mediated selection on floral color was not detected in either species. Our results suggest that pollinators play an important role as selection agents on size-related advertisement traits in these irises. The lack of pollinator-mediated selection on floral color contradicts our hypothesis and is in contrast with the interpretation from previous studies. It suggests that selection acts through male-mediated fitness, or that other non-pollinator agents are selecting for this prominent trait, or that the phenotypic color variation in these irises is at its adaptive peak, and hence neutral. We hypothesize that additional selection agents are acting on floral traits in these irises and further experiments are planned, to test it.

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Table 3 Selection gradients (± SE) on floral traits in *Iris haynei* in Gilboa

Trait	β open ± SE	β supp. ± SE	Interaction *P* value	γ open ± SE	γ supp. ± SE	Interaction *P* value
2010						
Stem length	0.093 ± 0.123	−0.050 ± 0.136	0.479	−1.781 ± 1.271	−3.071 ± 1.686	0.678
Flower size	0.264 ± 0.118	**0.352** ± 0.147	0.639	−2.270 ± 1.838	1.581 ± 1.891	0.115
Pigment concentration	0.104 ± 0.134	0.081 ± 0.124	0.899	1.946 ± 2.425	2.266 ± 1.707	0.914
2011						
Stem length	0.131 ± 0.112	0.187 ± 0.119	0.876	−1.455 ± 1.226	−1.233 ± 1.718	0.842
Flower size	**0.257** ± 0.123	0.138 ± 0.109	0.470	−3.085 ± 1.969	1.452 ± 1.885	0.095
Pigment concentration	0.087 ± 0.108	0.102 ± 0.106	0.924	−0.060 ± 1.037	−0.554 ± 0.988	0.731

Linear (β) values are partial regression coefficients; positive or negative values imply positive or negative directional selection, respectively. Non-linear (quadratic; γ) positive or negative gradients imply disruptive or stabilizing selection, respectively. Quadratic partial regression coefficients were doubled to obtain selection gradients (Stinchcombe *et al.*, 2008). Selection gradients are presented for open pollination ('open') and supplementary pollination ('supp.') treatments. Significant ($P < 0.05$) gradients are in bold. Significance values are presented for the interaction between pollination treatment and trait in their effect on fitness. Significant interaction indicates a change in the selection gradient caused by the treatment, thus implying pollinator-mediated selection on this trait, given that open-pollinated flowers include all factors that could influence fitness, whereas supplementary pollination removes the possible influence of pollinators.

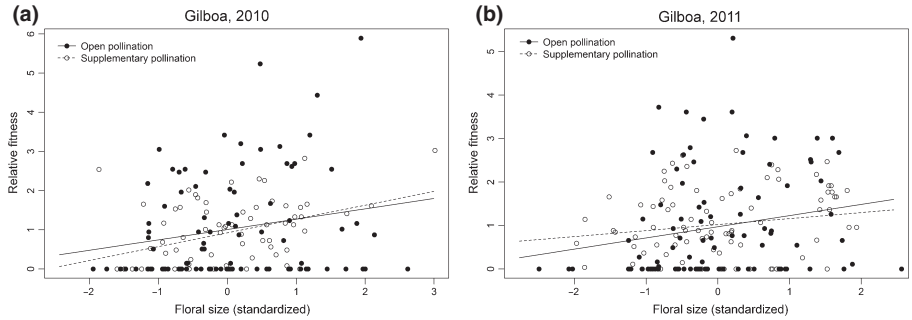

Fig. 4 Pollinator-mediated selection on floral traits in *Iris haynei*. Lines represent the fitted function for each treatment (open/supplementary pollination) from the linear model, including three floral traits (stem length, floral size and floral color). Solid line and closed circles, values of flowers open to natural pollination; dashed lines and open circles, values of flowers receiving supplementary pollination. In both graphs, the differences between slopes of treatment effects were not significant ($P > 0.05$). (a) Linear selection on floral size in 2010, significant only for supplementary pollination treatment ($\beta = 0.352$, $P = 0.018$); (b) linear selection on floral size in 2011, significant only for open pollination treatment ($\beta = 0.257$, $P = 0.039$). See Table 3 for values and significance of selection gradients.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Color and size variation in flowers of *Iris atropurpurea*.

Fig. S2 Color and size variation in flowers of *Iris haynei*.

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