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Floral pigmentation patterns provide an example of Gloger's rule in plants

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Ecogeographic rules explain spatial trends in biodiversity, species interactions and phenotypes[1]. Gloger's rule and its corollaries state that pigmentation of endothermic animals will increase from more polar to equatorial regions due to changing selective pressures including heat, humidity, predation and UV irradiance[2–4]. In plants, floral pigmentation varies within and among taxa, yet causes of wide-scale geographic variation are lacking. We show that Gloger's rule explains patterns of variation in UV-absorbing floral pigmentation in a widespread plant, *Argentina anserina* (Rosaceae). Specifically, the floral pigmentation pattern unique to the UV spectrum (UV 'bullseye') increases with proximity to the Equator in both hemispheres, and larger bullseyes are associated with higher UVB incidence. Experiments confirm UV as an agent of selection and bullseye size as a target. Results extend the generality of an ecogeographic rule—formulated for animals—to plants, implicating UV as a selective agent on a floral trait generally assumed to enhance plant–pollinator interactions. Global change is expected to alter UV irradiance in terrestrial systems[5], potentially intensifying the importance of UV-mediated selection to floral evolution. Because floral UV reflectance and pattern enhance pollinator attraction[6,7], altered selective regimes could disrupt coevolved plant–pollinator interactions, weakening an important ecosystem service[8].

A major goal of ecology is to identify principles that unify our understanding of patterns of biodiversity. A list of ecogeographic rules has been formulated to explain positional or environmental variation in morphology or life history in terrestrial and aquatic systems across the globe[1]. Yet, their applicability to both animal and plant kingdoms, and their mechanistic drivers, often remain open questions[1]. Gloger's rule and its corollaries state that endothermic animals in more equatorial regions will have darker pigmentation than those in more polar regions[3,4]. Increased pigmentation towards equatorial latitudes in animals including humans[9], neotropical and Old World primates[10,11] and house mouse[12] can be seen as a manifestation of this rule. This pattern is predicted to derive from protective functions afforded by greater pigmentation against abiotic or biotic stressors that increase with decreasing latitude, such as heat, humidity, predation and UV irradiance[3,4,13]. We extend tests of Gloger's rule and the underlying protective hypothesis to flowering plants. We focus on a common floral pattern—an ultraviolet (UV) 'bullseye' where petal bases are UV absorbing whereas petal tips reflect UV[14,15] (Fig. 1a). This floral pattern exists in at least 36 families of angiosperms and is known to result from variation in UV absorbing pigments[15] or in some cases, variation in cell shape[16].

Although diversity in flower colour and pattern has been traditionally ascribed to divergent pollinator-mediated selection[17], more recent work has implicated abiotic factors (for example heat, drought and UV irradiance) as selective agents as well[18]. Abiotic selection is often thought to act indirectly on flower colour via pleiotropic effects of genes that mediate stress tolerance[18]. However, the micro-environment of the flower can vary with petal pigmentation, so abiotic factors such as ambient UV irradiance could instead impose selection directly. Specifically, flowers with more UV reflectance, either due to larger reflective petal areas or due to higher intensity of UV reflection, will have floral environments with higher UV irradiance that can adversely affect the viability of gametes produced within them (Fig. 2). So in contrast to conventional wisdom that the UV bullseye floral pattern functions to enhance a pollinator's distance perception of flowers[7] and/or orientation to floral rewards, we propose that flowers with larger bullseyes (larger areas that absorb UV; Fig. 2b) may also protect pollen from UV damage after anthesis[19,20] and thus bullseye size is under selection mediated by UV irradiance. As UV irradiance is higher at lower latitudes[21], we predict that UV-mediated selection will contribute to latitudinal clines in the bullseye size, such that bullseyes will increase with increasing proximity to the Equator, supporting Gloger's rule.

We combined field-collected phenotypic data from 34 populations of silverweed cinquefoil, *A. anserina* (Rosaceae), a native plant distributed in temperate zones of both hemispheres. Sampling represented latitudinal transects in four regions, three in the northern hemisphere (Pacific Coast, USA; Rocky Mountains, USA; Great Lakes, USA) and one in the southern hemisphere (New Zealand). Silverweed cinquefoil has bowl-shaped flowers that appear uniformly yellow to the unaided human eye, but have a UV-absorbing bullseye (Figs 1a and 2a) that is due to the presence of flavonol glycosides at the petal bases[15]. We measured floral bullseye size by scoring the relative area of petals that absorb UV[22,23] (UV proportion) on 456 flowers by digital analyses of UV photographs. We determined whether mean population bullseye size is larger closer to the Equator after accounting for variation explained by regional transect and altitude. We then addressed whether UV irradiance predicts variation in bullseye size after accounting for other potential climatic agents of selection (temperature, precipitation) and region. Latitude explained 39% of the variation in bullseye size after accounting for region and altitude ($F_{1,22} = 28.4$, $P < 0.0001$, Table 1a). Within each of the four geographic regions, bullseye size increased with increasing vicinity to the Equator (Fig. 1b), that is a larger proportion of the petal area was pigmented with UV-absorbing compounds at lower latitudes, which is in line with Gloger's rule. The relationship between bullseye size and latitude did not differ among regions (region × latitude $F_{3,22} = 1.87$, $P = 0.16$; Table 1a; Fig. 1b), whereas the relationship between bullseye size and altitude did (region × altitude, $F_{3,22} = 5.21$, $P < 0.01$; Table 1a), suggesting that latitude was a more consistent predictor of bullseye size variation than was altitude. Three bioclimatic variables that can covary with latitude (UVB irradiance, temperature, precipitation) together explained 33% of the variation in bullseye size after accounting for region (Table 1b), but UV irradiance was the only significant bioclimatic predictor ($F_{1,19} = 11.82$, $P < 0.01$,

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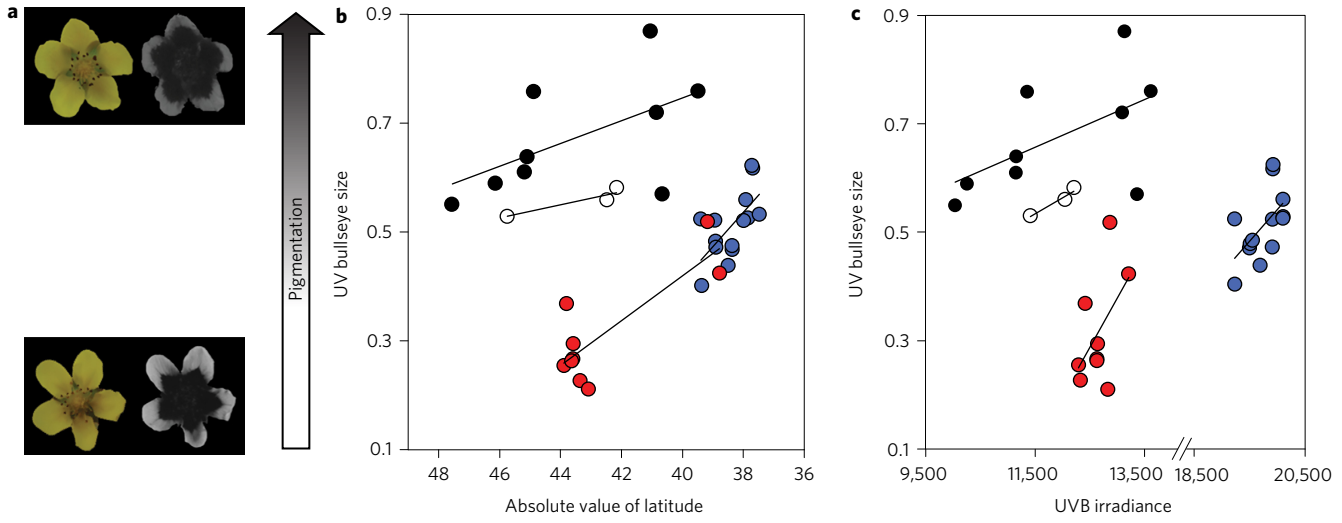

Figure 1 | Size of the floral UV bullseye increases with proximity to the Equator in silverweed cinquefoil (*A. anserina*). a, Representative images of *A. anserina* flowers in visible (colour) and UV (greyscale) spectra displaying a range of UV bullseyes sizes in New Zealand. In UV images, darker areas of flowers absorb UV, whereas lighter areas reflect UV. **b**, UV bullseye size (*y*-axis) was measured as the UV-absorbing proportion of petal area, and proximity to the Equator is the absolute value of latitude (*x*-axis). The *y*-axis is reversed such that populations on the left are at high latitudes and those on the right are at lower latitudes. **c**, UVB irradiance experienced during the flowering season (J m^{-2} per day) predicts variation in bullseye size (see Table 1b), and the relationship between bullseye size and UVB irradiance is positive in all regions. In **b** and **c** colours represent region (black, Pacific Coast; white, Great Lakes; red, New Zealand; blue, Rocky Mountains).

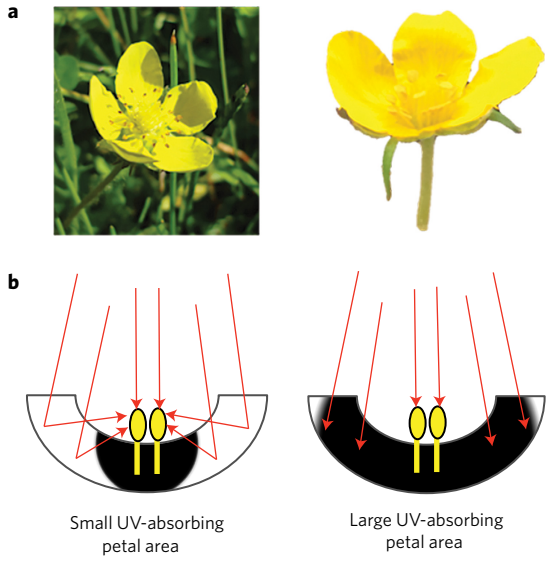

Figure 2 | Hypothesis for how variation in the UV bullseye influences floral microenvironment. a, *In situ* (left) and cross-sectional view (right) of the bowl-shaped architecture of an *A. anserina* flower. **b**, Schematic of the hypothesized effect of UV-absorbing bullseye size on the reflectance of UV light within a flower. A flower with a smaller UV-absorbing petal area (left) reflects UV light from petal tips onto pollen-bearing anthers (yellow structures), whereas a flower with a larger area of UV absorption (right) absorbs UV light across a larger area, reducing diffuse reflection and thus, UV exposure of pollen.

Table 1 | The effects of latitude, altitude and bioclimatic variables on mean population bullseye size.

(a)

Effect	df	Type I SS	MS	*F*	*P*
Region	3,22	0.59	0.20	63.30	**<0.0001**
Altitude	1,22	0.00	0.00	1.38	0.25
Latitude	1,22	0.09	0.09	28.35	**<0.0001**
Region × Altitude	3,22	0.05	0.02	5.21	**<0.01**
Region × Latitude	3,22	0.02	0.01	1.87	0.16

(b)

Effect	df	Type I SS	MS	*F*	*P*
Region	3,19	0.59	0.20	48.89	**<0.0001**
Temperature	1,19	0.02	0.02	3.81	0.07
Precipitation	1,19	0.01	0.01	1.38	0.25
UVB irradiance	1,19	0.05	0.05	13.45	**<0.01**
Region × Temp.	3,19	0.05	0.02	4.27	**0.02**
Region × Precip.	3,19	0.01	0.00	0.79	0.51
Region × UVB irrad.	3,19	0.01	0.01	1.76	0.2

Both general linear ANOVA models first accounted for regional variation.

Table 1b), explaining 24% of the variation. The slope of the relationship between UVB irradiance and bullseye size was similarly positive in each region (region × UVB Irrad., $F_{2,19} = 1.76$, $P = 0.20$; Table 1b; Fig. 1c). Together, these results demonstrate that geographic variation in a UV flower pigmentation pattern follows Gloger's rule, and that UV irradiance is the most important climatic factor underlying the global pattern.

We tested the prediction that UV irradiance favours individuals with larger bullseyes by measuring phenotypic selection on bullseye size using pollen viability as a fitness parameter under experimentally modified levels of ambient UV (absent, present, Fig. S1). Four to six flowers per plant were harvested from 71 plants with varying bullseye size that had been grown in a glasshouse for several years. From each plant, half of the flowers were exposed to UV irradiance and half were protected from UV irradiance. After exposure, we scored *in vitro* pollen germination as a component of male fitness in selection analyses. In the absence of UV irradiance, directional selection favoured the smallest floral bullseyes (Fig. 3a). Conversely, exposure to UV favoured individuals with intermediate sized bullseyes (Fig. 3b). Analysis of covariance showed that although the directional component of selection did not differ between UV treatments (treatment × bullseye size, $F_{1,126} = 2.40$, $P = 0.12$; Table 2), the non-linear

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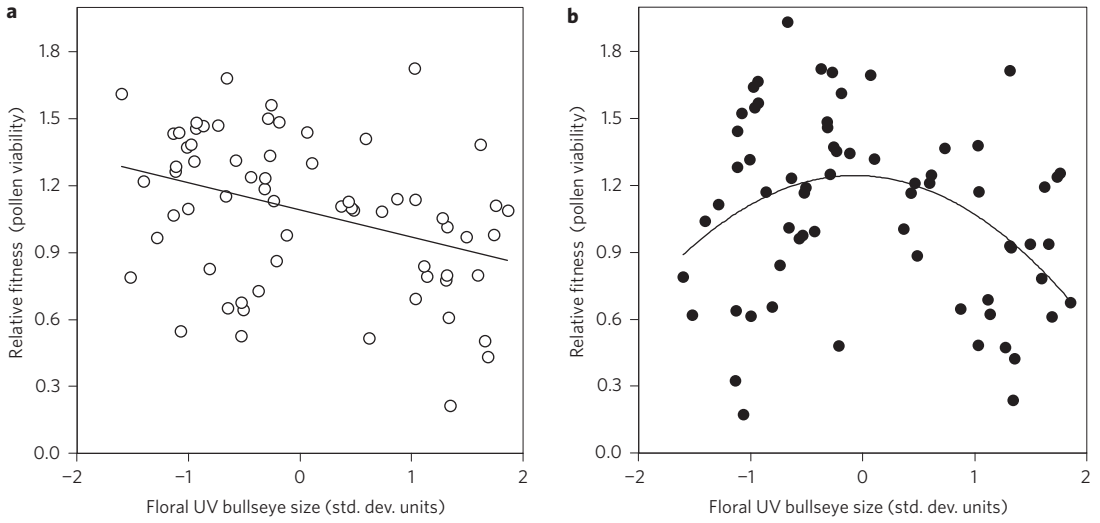

Figure 3 | Optimal UV bullseye size increases in the presence of ambient UV. a,**b**, Pollen viability (relative) as a function of UV bullseye size (standard deviation units) in the absence. (**a**) and presence (**b**) of experimental UV exposure. In the absence of UV, directional selection was observed (pollen viability = −0.12 × bullseye + 1.1; $R^2 = 0.13$; $P = 0.002$). In the presence of UV, stabilizing selection was observed (pollen viability= −0.15 × bullseye^2 − 0.02 × bullseye + 1.2; $R^2 = 0.14$; $P = 0.009$).

component did (treatment × bullseye size^2, $F_{1,126} = 3.80$, $P = 0.05$; Table 2). An explicit test of the location of the trait optimum, Mitchell-Olds–Shaw (MOS) test[24], confirmed that the fitness function was unimodal in the presence of UV ($P = 0.04$, optimum at −0.12 SD units; Fig. 3b), but not in the absence of UV ($P = 0.98$; optimum at −1.55 SD units, near the minimum of −1.62; Fig. 3a). Both parametric and non-parametric[25] analyses show that UV exposure favoured flowers with greater pigmentation (optimal bullseye size increased by 1.43 SD units; Fig. 3 and Supplementary Fig. 2). Given that the experimental levels of UVB were only 43% of what flowers typically experience at noon in July under natural conditions, even larger bullseyes might be favoured in nature, if all else is equal.

To verify that the bullseye is a target of selection[26] by UV irradiance, we recorded the effect of varying bullseye size on pollen viability using artificial flowers. We created flowers that had one of three discrete bullseye sizes (small, medium, large; Fig. 4) but were otherwise phenotypically identical in overall size and in human-visible colour. Anthers from glasshouse-grown plants were placed in each type of artificial flower and we exposed the flowers to UV-present and UV-absent environments as above. Viability of pollen was scored *in vitro* after exposure. Overall, the presence of UV reduced pollen viability by ∼12% (treatment, $F_{1,21} = 8.74$, $P < 0.01$), but the effect of UV treatment depended on bullseye size (treatment × bullseye size, $F_{2,41} = 3.23$, $P = 0.05$; Fig. 4). In the absence of UV irradiance, pollen viability was unaffected by flower type (all pairwise comparisons, $P = 1.0$; Fig. 4). However, in the presence of UV irradiance, pollen from the small bullseye flower had the lowest germination rate (28% lower than large bullseye with UV present, $P = 0.02$, Fig. 4), whereas pollen from the largest bullseye had a germination rate equivalent to that seen in flowers in the absence of UV ($P > 0.99$; Fig. 4). From this we conclude that bullseye size modifies the floral UV environment, with larger bullseyes providing greater protection of pollen from UV exposure than small ones (as in Fig. 2). The combination of the repeated geographic pattern and the results from experimental manipulations of both the UV environment and the bullseye trait strongly support the prediction that UV irradiance imposes selection on the size of the UV bullseye in a manner consistent with Gloger's rule.

All tests of Gloger's rule until now have been with animals, and the majority of studies have not fully explored the mechanistic underpinnings of the rule. This study extends Gloger's rule by showing that it applies to a floral phenotype in plants, and identifies UV irradiance as an agent of natural selection that drives latitudinal variation. Interestingly, UV irradiance is likely to be the main driver of darker human skin pigmentation towards equatorial regions[9,27], may drive latitudinal trends of darker eye masks towards the Equator in Neotropical primates[10] and may account for overall pigmentation differences in Old World Macaques[11]. Our result that latitude explains 39% of bullseye variation after the effects of region and altitude are accounted for, is not as strong as the latitudinal association found for human skin reflectance values ($r = 0.83$–0.97 depending on wavelength[27]), but is more on par with the association in Thai macaques ($R^2 = 0.32$–0.74 depending on body location[11]), and is much stronger than that seen in the house mouse (3.3% of variation in coat colour[12]), although we should note that these comparisons combine traits measured in different manners. Latitudinal clines in colour may be common in flowers. For instance, a recent study found that the proportional representation of dark blue to lighter red flowers in scarlet pimpernel increased at lower latitudes[28], a pattern of increased darkness also consistent with Gloger's rule, although not noted in the original paper. Moreover, flowers with exposed anthers may have evolved greater resistance to UVB pollen damage than those with structures that protect anthers[29], highlighting the role of UV as a driver of floral evolution. For flowers with exposed pollen, our data indicate that the evolution of increased UV-absorbing pigmentation in petals is another avenue for protecting pollen from UVB. We have shown that UV irradiance can exert selection on optimal bullseye size, but we caution that tests should additionally determine whether changes in pollinator assemblage with latitude[30] could also play a role in floral pigmentation variation. The shape of selection via other fitness parameters (for example seed production) and the potential cost of producing highly pigmented flowers (as suggested in Fig. 2a) may also factor into the optimal bullseye size at a given location.

These results add a novel dimension to the accumulating evidence for the action of non-pollinator forces in the diversification of floral traits by underscoring UV irradiance as an important climatic agent. Past ozone depletion has led to increased UV irradiance, especially at mid-latitudes and polar regions[21], and future changes in climate and ozone levels will continue to alter UV exposure in terrestrial systems[5]. The current study provides insight into how floral colour phenotypes

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Table 2 | The effects of UV exposure (treatment) on directional (bullseye size) and quadratic (bullseye size squared) selection on bullseye size.

Effect	$F_{1,126}$	P
Treatment	2.71	0.10
UV bullseye size	1.11	0.30
UV bullseye size^2	5.91	**0.02**
Teatment × bullseye size	2.4	0.12
Treatment × bullseye size^2	3.8	**0.05**

Random effects of population, date of exposure and their interactions with treatment included in the ANCOVA model were non-significant ($0.36 < P < 0.94$) and are not shown. Bold values are significant at the $P < 0.05$ level.

may respond adaptively to global change. These changes, however, may counter preferences of pollinators. For instance, whereas an increase in UV irradiance favours greater floral UV absorption, potentially leading to loss of UV pattern, the opposite—greater UV reflection and/or the presence of UV pattern—increases pollinator attraction to flowers of silverweed cinquefoil and other species[6,7]. Thus, global pigmentation responses may generate a mismatch between pollinator preferences and floral phenotypes, further complicating the health of an important ecosystem service[8]. Tests of Gloger's rule for floral colour variation among taxa in a phylogenetically controlled manner, as well as colour variation among flowering plant communities or among plant organs, will be important for testing the extent to which this animal rule applies to plants.

Methods

Floral traits and bioclimatic variables. To quantify floral UV bullseye size, we scored UV proportion (the relative area of petal UV absorption) from UV photographs of field-collected flowers (see ref. 23 for details). Collections were made by walking linear transects through the entire extent of populations and collecting a flower after every 2 metres. We scored UV proportion for 456 plants from 34 pristine natural populations from the Pacific Coast[23] ($n = 9$), the Rocky Mountains ($n = 13$), the Great Lakes[23] ($n = 3$) and New Zealand ($n = 9$) between June 2011 and January 2013 (Supplementary Table 1). We scored 2–27 flowers per population (mean population size = 13.4) depending on population size and proportion flowering. On all, or a subset of flowers in 28 populations (Supplementary Table 1), we recorded spectral reflectance at the petal apex and base using a UV-VIS spectrophotometer (Ocean Optics USB4000 or Jaz spectrophotometer, Dunedin, FL, USA), and calculated UV chroma ($R_{300–400}/R_{300–700}$) using CLR software (version 1.05, R. Montgomerie). Only UV chroma at the petal tip was correlated with UV proportion, but neither tip nor base chroma were associated with latitude (Supplementary Section 3). For this reason we only pursued subsequent laboratory experiments to examine the effects of UV proportion on pollen viability.

Using WorldClim[31] (2.5 × 2.5 min resolution) and DIVA-GIS (v. 7.5), we obtained mean annual temperature (BIO 1) and precipitation (BIO 12) for each population. From gIUV[32] we obtained UVB exposure from the UVB5 layer (sum of monthly mean UVB during highest quarter). This GIS layer maps UVB measurements in 15-arc-minute steps globally, taken from the Ozone Monitoring Instrument aboard the NASA EOS Aura Spacecraft from 2004–2013[32].

Test of Gloger's rule and abiotic associations with UV bullseye size. We used the absolute value of latitude to represent a population's distance from the Equator. We used analysis of covariance (ANCOVA) to model average population UV bullseye size as a function of the categorical variable of region, the continuous terms of altitude and latitude, and the interactions between region and the continuous terms.

To determine which bioclimatic variable(s) predicted bullseye size, we modelled mean population bullseye size as a function of region, temperature, precipitation, UVB and the interaction between region and bioclimatic variables using ANCOVA. Both ANCOVAs were performed with PROC GLM in SAS (SAS 9.3; SAS Institute Inc., 2011).

UV-mediated phenotypic selection on bullseye size

Plant material. To assess selection across a range of bullseye sizes we sampled individuals from a stock population of silverweed cinquefoil grown in the glasshouse for several years composed of plants originally collected from 14 sites across the three North American transects. Three weeks before flowering, plants received ~122 mg of slow-release fertilizer (Nutricote). During experiments, plants grew in a growth chamber with 11-h days at 15.5 °C/10 °C (day/night). We measured bullseye size on three or four clones per individual in a previous study[23], or took measurements directly on the flowers during the experiment.

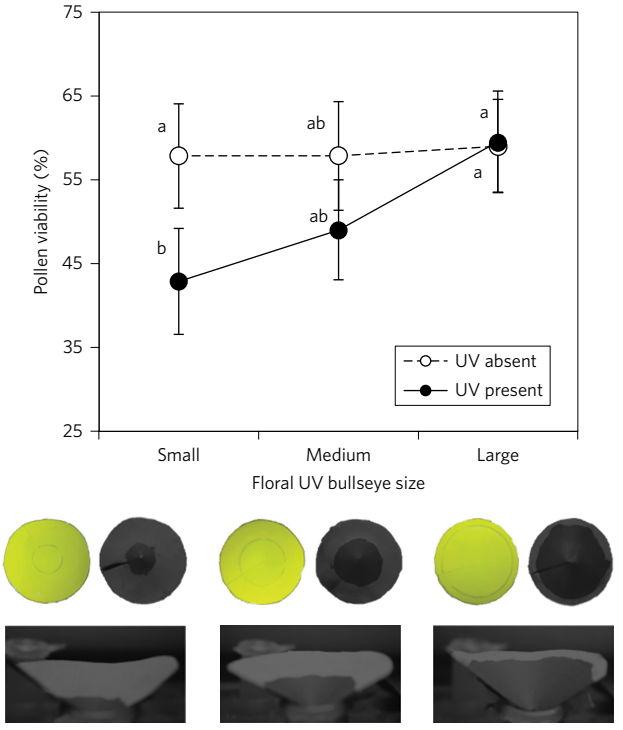

Figure 4 | UV bullseye size is a target of selection via UV irradiance. Mean percentage viability of pollen (±1 s.e.m.) placed in artificial flowers with small, medium or large UV bullseyes in the presence (filled circle, solid line) or absence of UV (open circle, dashed line). Means that do not share a common letter are significantly different at $P < 0.05$ as determined by Tukey-Kramer *post hoc* tests. Top-down human-visible and UV images (top), and cross-sectional UV images (bottom) of artificial flowers are shown on the *x*-axis (darker areas are UV absorbing and lighter areas are UV reflecting).

UV exposure and pollen germination. From 71 plants we removed flowers and placed them in water-filled microcentrifuge tubes in a 'UV chamber' illuminated with full-spectrum lighting. The first flower from each plant was randomly assigned to a UV absent or present treatment (Supplementary Fig. 1). Flowers were haphazardly arranged and exposed to 6-h light/12-h dark/6-h light to mimic diurnal rhythms. After exposure, we left pollen to germinate *in vitro* and scored germination blinded. See Supplementary Section 2 for lighting, UV treatment and pollen germination details.

The second flower from each plant received the opposite treatment of the first flower. We scored germination on four to six flowers per plant and calculated mean pollen viability for each treatment by individual. We removed five pollen-sterile individuals (<0.05% germination regardless of treatment) from the dataset prior to analysis.

Phenotypic selection analyses. Pollen viability was used as a component of male fitness. We calculated relative pollen viability (viability$_x$ / mean viability) in each treatment and standardized bullseye size (*Z*-score). We regressed fitness on bullseye size and then on bullseye size and its squared term to test for directional and stabilizing or disruptive selection, respectively[33]. To assess whether selection differed between treatments we used ANCOVA with fixed effects of treatment, UV bullseye size, UV bullseye size^2 and treatment × trait interaction terms. A significant interaction between treatment and bullseye size or its squared term indicates significant differences in directional or stabilizing selection between the treatments, respectively. We used population of origin, the average date of flowering for each individual within each treatment (*Z*-score), and their interactions with treatment as random effects to account for any population-level variation in pollen viability and temporal variation in UV exposure as bulbs aged. Date is analogous to a random blocking factor that can be used in selection analyses (for example ref. 34). The between–within option (DDFM = BETWITHIN) was used to estimate the denominator degrees of freedom as this is recommended for designs with many random effects and unbalanced data (UCLA: Statistical Consulting Group; http://www.ats.ucla.edu/stat/sas/notes2/). Next, we examined non-parametric relationships between relative fitness and bullseye size using cubic splines with λ-values that minimized GCV scores[25], ($\lambda_{UV\ absent} = 10$, $\lambda_{UV\ present} = -2$; Supplementary Fig. 2). Finally, we tested whether fitness optima in each treatment was at an intermediate value of bullseye size using the MOS test in R's vegan

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package[24]. This tests whether the relationship between a dependent and independent variable is unimodal, or simply increasing/decreasing quadratic (null expectation), by testing whether the peak is at the minimum or maximum of the independent variable (trait value).

UV bullseye as a target of selection. To determine if bullseye size is a target of UV-mediated selection, we tested the effect of UV light on pollen placed in artificial flowers of varying bullseye size. We constructed conical paper flowers (Rite in the Rain, Tacoma, WA) painted with yellow UV-reflective[7] and UV-absorbing paints ('UV Yellow', Reel Wings Decoy Company Inc. and, '509 Sunny Yellow', Plaid Enterprises, Inc., Norcross, GA). Three bullseye sizes spanning natural variation were created: small (~20% UV proportion), medium (~50% UV proportion) and large (~80% UV proportion). Artificial flowers were otherwise identical (Fig. 4).

Twenty-two individuals from the stock population were pollen donors for the experiment. From each, we removed six dehisced anthers from a single flower, and placed each anther onto a microscope cover slip placed inside one of six artificial flowers (two small, two medium, two large). One set of three flowers (small, medium, large) received a UV-present treatment and the other a UV-absent treatment. Pollen was germinated *in vitro* and germination scored.

To test the effects of bullseye size, UV treatment and their interaction on pollen viability, we used a mixed general linear model (SAS PROC MIXED) with treatment, bullseye size and their interaction as fixed effects. Pollen donor identity and its interactions with fixed effects were random effects. We assessed pairwise differences between bullseye size and UV treatment combinations using Tukey–Kramer *post hoc* adjusted *p*-values.

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Author contributions

M.H.K. and T-L.A. designed the research; M.H.K. performed experiments and analyses; M.H.K. and T-L.A. wrote the paper.

Additional information

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Competing interests

The authors declare no competing financial interests.

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