DOES SELECTION OR PLASTICITY BY UV-B RADIATION DRIVE VARIATION IN UV-ABSORBING AREA OF FLOWERS?

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Thesis outline

This thesis will be set out in five chapters. The first outlines the introduction and the rationale of the research. Chapters 2-4 describe and discuss the methods and results of the three component studies in detail. Chapter 2 is written in the style of a manuscript for New Phytologist, and Chapters 3 and 4 are written in the style of the Journal of Experimental Botany. The intent is for these chapters to be edited and submitted for publication at a later date. The final chapter summarizes and discusses the findings of the three studies as a whole, before concluding the thesis.

Contributions

I confirm that all stages of this Masters by Research degree are my own work with contributions from Carl Soulsbury (CDS) and Sandra Varga (SV).

All chapters:

Data collection: 2017-2018: **LG**, CDS, SV

Project supervision: CDS, SV

Chapter 1: Introduction

Literature search: **LG**

Text: **LG**

Editing: CDS, SV, **LG**

Chapter 2: UV-B as a driver of spatial and temporal patterns of UV bullseye phenotype

Study design: CDS, SV, **LG**

Data analysis: **LG**

Data interpretation: CDS, SV, **LG**

Manuscript: **LG**

Manuscript editing: CDS, SV, **LG**

Chapter 3: UV bullseyes respond plastically to elevated UV-B in *Brassica rapa*

Study design: CDS, SV, **LG**

Data analysis: L**G**

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Chapter 4: The Impact of ultraviolet radiation on flowering phenology and pollen viability in *Brassica rapa*

Study design: CDS, SV, **LG**

Data analysis: **LG**

Data interpretation: CDS, SV, **LG**

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Chapter 5: Conclusion

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Chapter 1: Introduction

Selective and plastic responses of floral phenotypes to ultraviolet radiation

Ultraviolet radiation

Since the initial discovery of the Antarctic 'ozone hole', a zone in the Southern Hemisphere that has depleted as result of anthropogenic release of chlorofluorocarbons (CFCs), the effect of stratospheric ultraviolet radiation (UV) on plants has received increasing research attention (Thompson *et al.*, 2011). This is because ozone absorbs ultraviolet light (composed of both short wavelength (UV-B 280-315 nm) and long wavelength (UV-A 315–400 nm), and decreased ozone leads to increased UV-B transmission reaching the Earth's surface. The effect of UV-B on plants is of particular concern therefore, because it is the most energetic part of the daylight spectrum, and high exposure makes plants vulnerable to both DNA and protein damage (Sharma *et al.*, 2012; Taylor *et al*., 1997).

Global distributions of UV-B radiation depend on several factors such as cloud albedo, pollutants, atmospheric CFCs (and other ozone-depleting substances), aerosols, and most importantly, ozone (Pyle, 1997; Herman, 2010). Total ozone varies strongly with latitude over the globe, with the largest values occurring at middle and high latitudes during all seasons; Fig. 1). Large-scale air circulation in the stratosphere however slowly transports tropical ozone toward the poles, with ozone accumulating at middle and high latitudes (i.e. the ozone layer becomes the 'thickest' in this regions). In contrast, the values of total ozone are low in the tropics in all seasons (with the exception of the ozone hole) because the thickness of the ozone layer is smallest along the equator (WMO, 2010). Seasonal variations in ozone also occur; at high latitudes and during the spring, total ozone is at its maximum as a consequence of the transported ozone from the tropics during the autumn and winter (Herman, 2010a). Over the summer and early autumn months however, this transport of ozone is weaker, and is also overall much weaker in the Southern Hemisphere (WMO, 2010; Fig. 1). Finally, because seasonal changes in sunlight and ozone transport are smaller in the Tropics than in Polar Regions, total ozone changes through the seasons are overall smaller in these regions. Overall, natural ozone levels vary strongly with latitude and longitude within a season and are due to geographical variations in mixes of air between regions of the stratosphere that have high ozone values and those that have low ozone values (WMO, 2014).

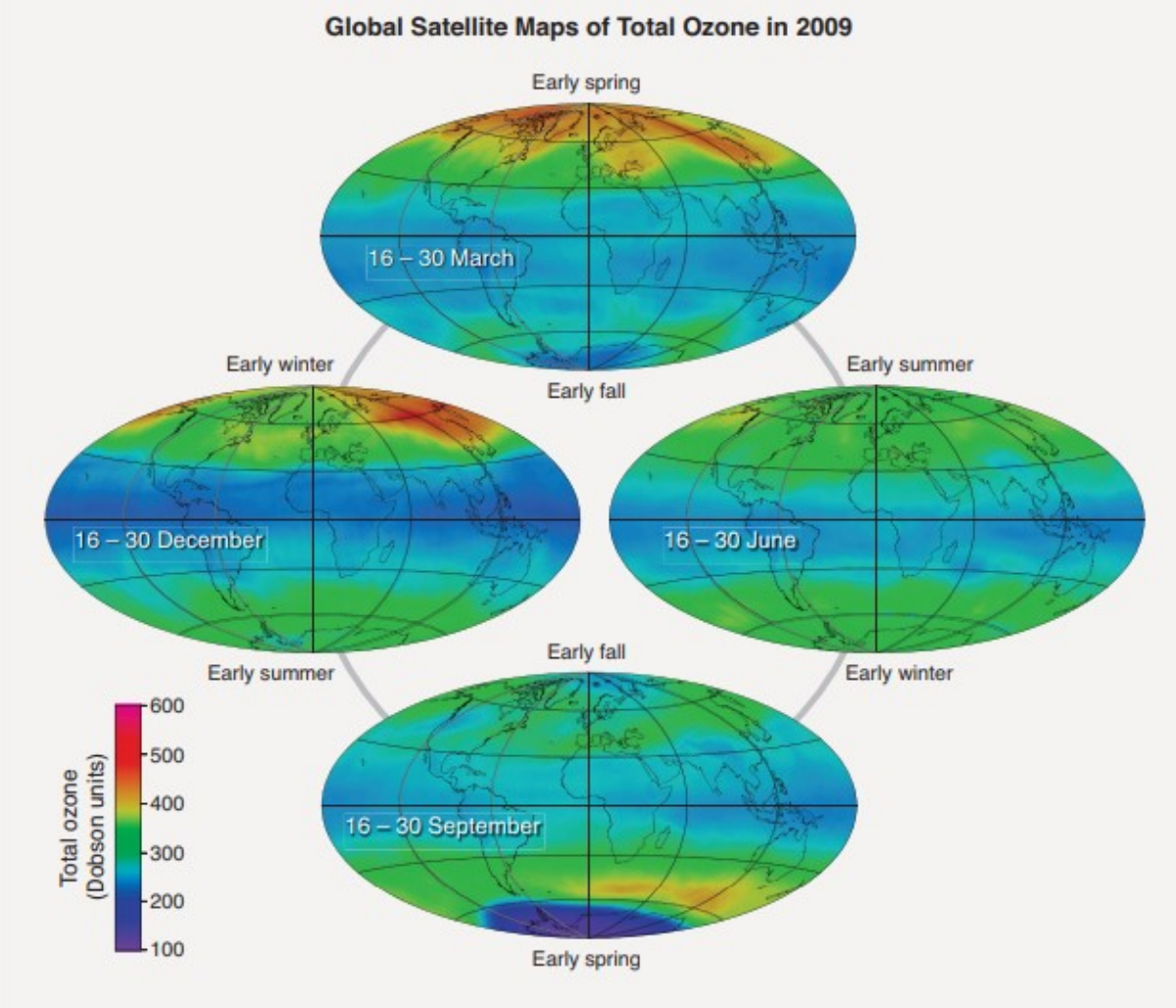

Figure 1. Total ozone varies with latitude and season, with largest values at high latitudes and the lowest values in tropical regions. Total ozone shows little variation in the tropics (20°N–20°S latitudes) over all seasons, whereas total ozone outside equatorial regions varies more strongly with time on a daily to seasonal basis. Such variation in ozone is the result of ozone-rich air moving from the tropics and accumulating at higher latitudes. Figure taken from WMO (2010).

In order to estimate global patterns and fluctuations of atmospheric UV-B radiation, contemporary studies have utilised multisatellite ozone datasets (Watanabe *et al.*, 2011, 2012; Beckmann *et al.*, 2014). In general, there is an inverse relationship between ozone changes and UV-B irradiance changes, and overall, UV-B has substantially increased over the past 30 years, with particular effects in high and low latitudes (Herman, 2010b; Fig. 2). As ozone varies with latitude, so too does UV-B. It decreases with increasing latitude (outside of the equatorial zone) as result of decreases in maximum daily noon solar elevation angles and

increasing ozone (Herman, 2010b). Significantly high clear-sky UV irradiances have been occurring in the tropic latitudes and at high mountain altitudes when the sun is directly overhead (Herman, 2010b), and mid-latitude UV-B in the Southern Hemisphere is greater than the UV-B levels in the corresponding latitudes of the Northern Hemisphere, due to overall lower ozone in the Southern Hemisphere (Herman, 2010a; Fig. 2). The Southern Hemisphere also has fewer atmospheric aerosols, pollutants which can scatter and absorb UV-B, increasing the amount of radiation reflected back to space and decreasing the amount reaching the ground. Cloud albedo can similarly reduce latitudinal levels of UV-B radiation, and broad seasonally repeating cloud patterns also cause changes on daily and monthly time scales as the weather changes (Pyle, 1997).

Overall, surface-level UV-B is variable on both long-term (annual, decadal) and short-term (i.e. daily, monthly, seasonal) scales, and these fluctuations are underpinned by both broad (global) and very localised geographic trends in ozone-level. Global climate change in general represents a challenge for evolutionary ecologists in estimating how organisms will respond to sometimes quite rapid increases in UV-B, as well as other abiotic factors such as temperature, CO_2, and precipitation, on a global to locale-scale (Parmesan, 2006). For plants in particular, global increases in UV-B, temperature, precipitation, and CO_2 have received much scientific attention due to their multiple effects on physiology (photosynthesis, respiration, growth and tissue composition in plants), species distributions (shifting climate zones are expected to induce range-shifts towards higher latitudes/elevations), phenology (life cycle events can cause desynchronisations between plants and pollinators), and finally adaptation (species with shorter generation times have the potential to undergo microevolutionary change) (Hughes, 2000; Walther *et al.*, 2002). In this study, we focus on evolved and plastic responses to UV-B, and consider how the changes in UV-B outlined above potentially challenge plants: do plants respond plastically to contemporary (and sometimes rapid) global climatic change, and do they respond adaptively over time to prevailing UV-B conditions (Walther *et al.*, 2002; Parmesan, 2006; Franks *et al.*, 2014)?

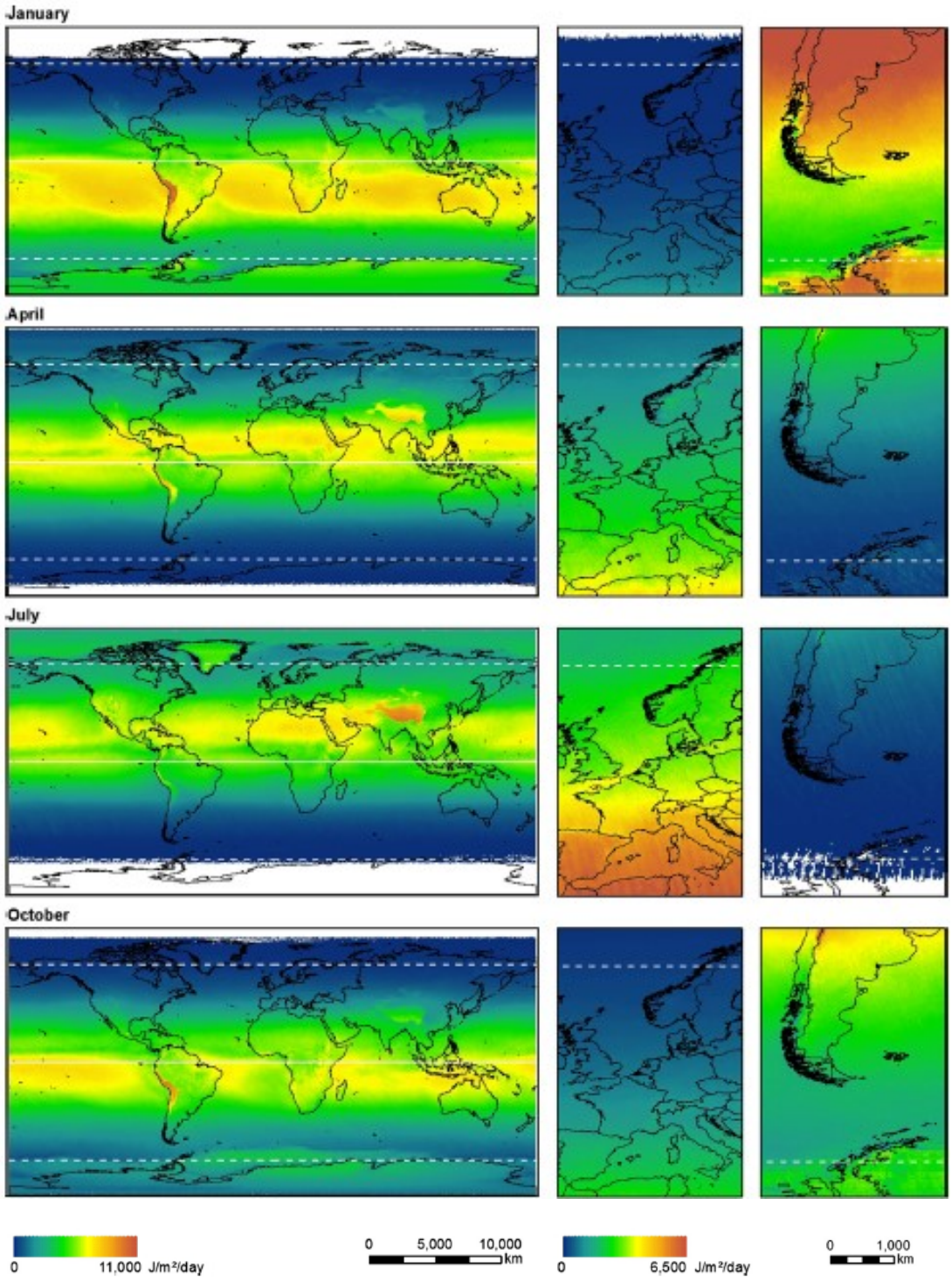

Figure 2. Examples of four monthly mean UV-B values (January, April, July, and October) taken from Beckmann *et al*. (2014). High intra-annual seasonality can be seen in both hemispheres. Smaller insets to the right of the global maps indicate finer-scale variation in the data for the same latitudinal ranges on the Northern and Southern hemispheres (Beckmann *et al.*, 2014).

Plants and UV-B

Because plants need to capture sunlight for photosynthesis, they are unavoidably exposed to UV-B. However the fact that naturally occurring populations of plants rarely show signs of severe UV-damage (Paul & Gwynn-Jones, 2003) indicates that they have evolved effective mechanisms for protection and repair (Sharma *et al.*, 2012). Early studies quantifying the effect of UV-B on plants were predominantly growth chamber, greenhouse, and laboratory experiments, and evidence of UV-B damage in the vegetative parts of plants has been abundant over the past three decades since. The major findings of these studies seemed to place UV-B damage into two main areas: reduction in vegetative plant morphology (or more broadly, growth), and changes in plant phenology (timing of anthesis, flowering duration, flower production). In several growth chamber experiments, higher simulated levels of UV-B have subsequently corresponded with overall reduced plant growth such as smaller leaf area and reduced biomass (as compared with plants from lower UV-B, or ambient UV-B treatments) (Terramura & Saile, 1990; Mepsted *et al*., 1996; Rozema *et al*., 1997). In addition, these reductions in vegetative growth, overall biomass accumulation, photosynthetic ability, and evidence of oxidative stress in vegetative tissues (Rozema *et al.*, 1997; Jansen *et al.*, 1998; Brosché & Strid, 2003; Frohnmeyer, 2003) were interpreted as 'harmful' stress (Hideg *et al.*, 2013). Harmful stress entails substantial cellular damage, impeded growth, and overall has a significantly negative effect on plants and their development. Although it has been argued that chamber and laboratory experiments only offer crude and inflated evidence of detrimental UV-B-induced responses, due mainly to unrealistically elevated levels of radiation (Hideg *et al.*, 2013), they are arguably valuable given that they indicate the pervasive influence of UV-B on plant growth. For example, Kakani *et al*. (2003) showed that in cotton plant (*Gossypium hirsutum*) seedlings when irradiated with UV-B, there was a reduction in plant height, leaf area, and branch length, and later in the plant life cycle, floral morphology for UV-B irradiated plants also differed from control plants. Specifically, petal area, petal length, and number of anthers per flower were significantly reduced in plants that had been exposed to high intensity UV-B as seedlings (Kakani *et al.*, 2003). Observations such as these were important since they highlighted that reproductive success could become compromised either via the reduced production and availability of pollen for pollinators (Sampson & Cane, 1999), or via reduced saliency of smaller flowers to pollinators (Llorens *et al.*, 2015). In Ziska *et al*. (1992), phenological responses to enhanced UV-B in *Oenothera* (Onagraceae)*, Plantago* (Plantaginaceae) and *Hypocheris* (Asteraceae) were also suggestive

of a strategic stress response, and again this accompanied overall vegetative tissue reduction (root and shoot biomass). The authors hypothesised that significantly earlier anthesis (and increased number of flowers produced) of plants exposed to elevated levels of UV-B (compared to control plants) was suggestive of efforts to increase reproductive output before yielding to the stressor (Wada & Takeno, 2010). Finally, the effects of detrimental UV-B on a number of vegetative parameters in early chamber-simulated levels of UV-B have also been replicated in a number of field studies. For example in cultivars of soybean (*Glycine max*), the only way in which the magnitude of response differed between chamber-radiated and wild-radiated plants was in relation to seed production (seed yield was reduced for chamber plants, Teramura & Murali, 1986). Similar results have also been found in natural populations of Antarctic pearlwort (*Colobanthus quitensis*) and Antarctic hair grass (*Deschampsia antarctica*), two species native to Antarctica, whereby shorter epidermal cells and leaves were observed in plants grown under framed filters (*in situ*) permitting the transmission of ambient UV-B; again these reductions in growth are consistent with laboratory and chamber-simulated findings (Ruhland & Day, 2000). These studies highlight that importantly, simulated UV-B conditions can be used to make estimates of plant responses *in situ* to naturally occurring UV-B, particularly when the biologically effective UV-B levels are used.

More contemporary studies have now begun to i) consider to extent to which UV-B serves as a harmful stressor on plant morphology, physiology, and development, and ii) address whether UV-B impacts upon on floral tissues. UV-B radiation can in some cases be characterised as regulatory or 'eustress', functioning to regulate or maintain plant metabolism and other important processes (Hideg *et al.*, 2013). When a plant experiences mild to moderate stress for example (i.e. the environmental conditions do not become too unfavourable), metabolism can adjust, and enables acclimation to the new environment (Hideg *et al.*, 2013). A good example of these types of responses includes increased accumulation of UV-absorbing compounds (such as flavonoids) in the pollen walls of plants grown in UV-B treatments that are significantly higher than typical ambient levels (Demchik & Day, 1996). Similarly, beneficial levels of UV-B can also be a significant regulating factor of petal colour. For example, enhanced UV-B intensity increased the accumulation of UV-B protecting pigments (anthocyanins and flavonoids) in both *Rosa hybrida* (Hennayake *et al.*, 2006) and *Anigozanthos* flowers (Ben-Tal & King, 1997), stimulating the production of petals with more intense colour than petals of flowers grown in UV-absent conditions. Whether these UV-B induced plastic changes in petal pigmentation influence overall plant

reproductive fitness has not been experimentally tested; however it has been suggested that aside from the mediating properties of increased UV-absorbing pigments, colour change in petals may function to modify floral temperature or increase the saliency of inflorescences to pollinators (Llorens *et al.*, 2015), both of which may improve reproductive fitness.

UV-B and floral traits

An increasing number of studies have now started to consider the effect of UV-B on floral characteristics, and as previously mentioned, a great many quantify either advances or delays in flowering time (Caldwell, 1968; Ziska *et al*., 1992; Mark *et al*., 1996; Sampson & Cane, 1999) or the number of flowers a plant produces when exposed to increased UV-B (Musil, 1995; Klaper *et al*., 1996; Saile-Mark *et al*., 1997). Shifts in plant phenology, and in particular flowering phenology, may therefore have important consequences for pollinators tracking the availability of floral rewards, and by extension, overall plant fitness (Sampson & Cane, 1999). The impact of UV-B on reproductive plant parts, and in particular pollen viability, has also been studied for a long time after realising that pollen walls may transmit up to 20% of UV-B (Sadler & Uber, 1942). The vegetative tissues and female reproductive parts of plants by contrast, are significantly better protected than pollen via the accumulation of UV-absorbing compounds in cell vacuoles and/or cell walls of the epidermis i.e. these compounds act to screen UV-B radiation (Caldwell *et al.*, 1983; Flint & Caldwell, 1984). UV-B has been frequently shown to significantly reduce both *in vitro* and *in vivo* pollen germination/viability (Chang & Campbell, 1976; Pfahler, 1981; Flint & Caldwell, 1984). Whilst the female reproductive systems of plants protect pollen from UV-B once it has penetrated the stigmatal surface (Caldwell *et al.*, 1983; Flint & Caldwell, 1984), upon anthesis, pollen is significantly less protected. The extent to which pollen grains are vulnerable to UV-B damage post-anthesis can be determined by pollen type (binucleate or trinucleate; Flint & Caldwell, 1986; Torabinejad *et al.*, 1998) and species, as well as the time taken for the pollen tube to penetrate and elongate into the stigmatal wall (Feng *et al.*, 2000). In maize (*Zea mays*) for example, pollen germination rate decreases over time, however when exposed to UV-B radiation, this decline significantly increases with each successive time interval since initial pollen exposure (Wang *et al*., 2010).

In a very recent study, Zhang *et al*. (2014) examined whether species containing UV-B vulnerable pollen were associated with protective floral architecture. Alpine plants

naturally inhabit environments with increased UV-B since altitude and UV-B positively correlate (Herman, 2010b; Koski & Ashman, 2015a), therefore the authors hypothesised that species with unprotected pollen grains on UV-B exposed anthers would be less sensitive to UV-B radiation than species with pollen grains protected by flower structures such as bracts or petals (Zhang *et al.*, 2014). Via *in vitro* pollen experiments, they demonstrated that pollen more sensitive to UV-B was indeed more likely to belong to species that featured protective floral structures. Very few studies have been able to demonstrate that floral phenotypes can be directly selected upon by abiotic factors, and Zhang *et al*. (2014) suggested that UV-B may play an instrumental role in the evolution of protective floral forms in alpine plants.

Adaptable morphology in response to UV-B radiation may become increasingly relevant for species occupying global regions associated with high UV-B as ozone levels decrease and more UV-B reaches the earth's surface (Zhang *et al.*, 2014). Recent research into the taxonomically widespread UV 'bullseye' pattern has indicated that the bullseye phenotype may also be an evolved response to UV-B (Koski & Ashman, 2015b). UV bullseyes are characterised by the presence of flavonoid glycosides at the bases of petals that act as UV-absorptive pigments, and petal apices reflect UV-B radiation (Harborne & Nash, 1984). From an evolutionary perspective, the floral bullseye variation we see (via UV photography) and that visible to UV perceptive insects and pollinators, has been traditionally well-explained by pollinator-mediated selection (Koski & Ashman, 2016). The bullseye has been shown to aid pollinators by: enhancing pollinator perception of flowers (Chittka *et al.*, 1994; Briscoe & Chittka, 2001; Sheehan *et al.*, 2016) orientation (Dinkel & Lunau, 2001), foraging efficiency (Lunau, 1992), visitation (Rae & Vamosi, 2013), and ultimately plant fitness (Morgan, 1992). Nevertheless, flowers producing UV bullseyes with greater ultraviolet proportion (the area of UV-absorptive pigment relative to the petal area; 'UVP') are now thought to also be the object of UV-B mediated selection because they have the potential to significantly improve male fitness in elevated UV-B environments. Flowers with more UV-B reflectance are thought to experience decreased pollen viability via diffuse reflection of UV-B onto pollen-bearing anthers; conversely, flowers with larger bullseyes protect pollen by absorbing UV-B radiation (Fig. 3). The extent of protection is such that the germination rate of flowers in UV-B conditions, but with large bullseyes, is comparable with that of flowers grown in UV-B absent conditions (Koski & Ashman, 2015b). When in UV-present conditions, the larger the bullseye, the greater the pollen viability. Whilst only quantified in artificial flowers, the findings of this study were important for highlighting: (i)

UV bullseyes (and variation of) can have a protective function for gametes, (ii) that the unique floral bullseye pattern *can* respond to an abiotic factor, and (iii) that UV bullseyes are good candidates to explore the extent to which its phenotypic variation reflects long-term UV-B mediated selection, or a more dynamic (plastic) response to UV-B conditions.

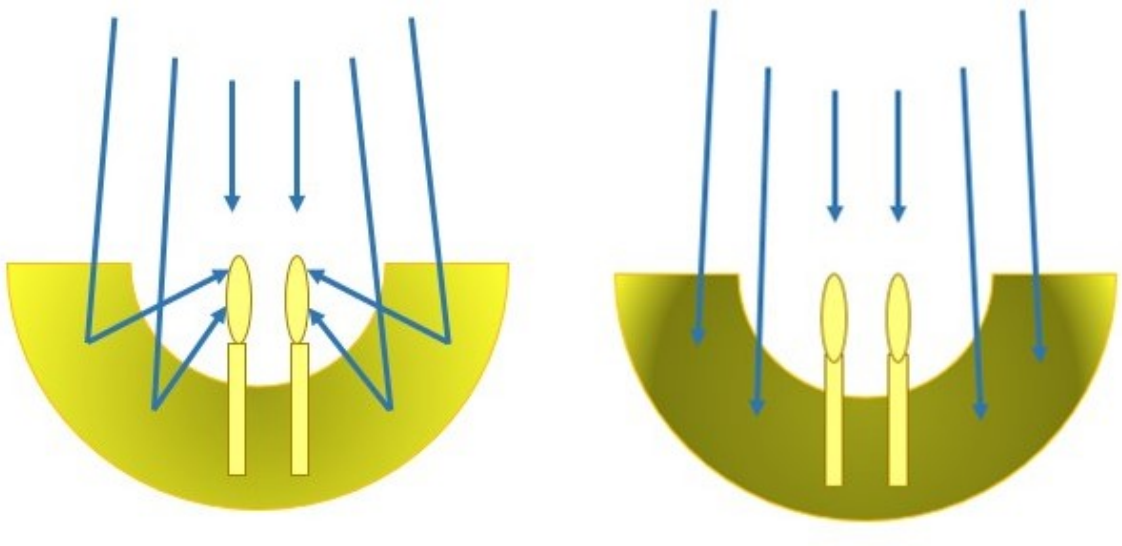

Figure 3. Koski and Ashman (2015b) hypothesis for the effect of UV-absorbing bullseye variation on floral micro-environment. Left: flowers with smaller UV-absorbing bullseyes are hypothesised to reflect UV light from the petal tips to the pollen bearing anthers. Right: flowers with larger UV-absorbing bullseyes are thought to absorb UV light over larger areas, decreasing the effect of diffuse reflection onto the pollen-bearing anthers.

UV bullseyes: the product of selection or plasticity?

Whilst several species of plants are known to produce bullseyes, an understanding of UV bullseye variation on a global scale is lacking. Evidence for Gloger's rule – that is the increase of dark pigmentation with decreasing latitude, affording greater protection against biotic and abiotic stressors such as temperature, humidity, predation and UV irradiance (Burt, 1981; Caro, 2005; Millien *et al*., 2006) – was recently found in *Argentina* (*Potentilla*) *anserina*, whose bullseyes increased in area in latitudinal clines towards the equator (Koski &

Ashman, 2015b). Although latitude explained variation in bullseye size in four geographic regions, UV irradiance also emerged as a significant bioclimatic predictor of UV pattern across the sampled regions. This potential for UV-B irradiance as a direct selective force was further explored in 177 species of *Potentilla*; on a macroevolutionary scale, UV-B irradiation predicted the degree of UV pigmentation (Koski & Ashman, 2016). These findings, although progressive in the field of floral trait evolution, are limited in their approach: they represent a small number of species encompassing only four global regions.

Overall, floral phenotypes can be under selection from a variety of biotic and abiotic factors (Rausher, 2008), however it has become evident that UV-B radiation is an important selective force. At present we know that both geographical location and contemporary UV-B conditions explain patterns of bullseye size, however we do not know whether these bullseyes have increased significantly over time, therefore indicating an evolved response. Since global UV-B radiation has changed dramatically over the last 50 years, we might expect this change to have a concomitant effect on floral phenotypes. The degree to which phenotypic changes materialise due to selection or plasticity (or both) in many species is an active research approach within the field of evolutionary ecology (Franks *et al.*, 2014). Currently however, there are few studies that have quantified whether plant reproductive/floral parts can respond plastically to UV-B radiation, and none (to my knowledge) have assessed whether floral UV phenotypes are plastic. This therefore represents a large paucity in our knowledge regarding UV-B patterns and floral trait evolution. In this thesis I therefore aim to assess the extent to which changes in UV bullseye size reflects phenotypic plasticity *vs* long-term selection using experimental approaches. Determining whether UV-B is a selective driver of bullseye variation represents an opportunity to better understand and predict i) how plant populations will respond to ongoing changes of UV-B radiation and ii) how this will affect plant-pollinator interactions.

Research aims

In this thesis, I investigate if UV bullseye variation is evidence of both plastic and selective responses to changes in UV-B intensity. In Chapter 2, I begin by focussing on the effect of long term changes in UV-B on the size of bullseye in several globally distributed angiosperm species. With the use of pressed specimens, contemporary field-collected specimens and published data, I create a dataset of UV bullseye areas over the last 250 years. I then model

the variation in bullseye size, identifying bioclimatic variables (UV-B, precipitation, temperature, altitude, latitude) that best predict global changes in bullseye size.

In Chapter 3, I then address whether UV bullseyes respond plastically to simulated levels of UV-B in plant chamber experiments. I grow *Brassica rapa* plants – species known to produce bullseyes- in three different UV-B intensity treatments, and under short and long UV-B exposure periods. Measuring the UV-proportion of petals pressed from flowers following anthesis, I quantify whether plants are capable of dynamic change when exposed to UV-B, and furthermore if duration of exposure significantly affects this.

In chapter 4, I measure pollen viability as a means of assessing whether UV-B influences plant male fitness. I use pollen harvested from the *B. rapa* plants grown in the chamber experiments, and furthermore determine whether UV-B intensity and exposure period prior to flowering impact upon pollen viability.

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II

A MANUSCRIPT WRITTEN IN ACCORDANCE WITH THE GUIDELINES OF NEW PHYTOLOGIST

UV-B as a driver of spatial and temporal patterns of UV bullseye phenotype

Summary

It has become increasingly apparent in recent years that abiotic factors can determine floral colour and pattern diversity. Broad-scale geographic patterns of ultraviolet (UV) bullseyes have been found for species of *Potentilla*, indicating that UV-B selection has to potential to act on a macroevolutionary scale. Whether UV-B radiation accounts for the extent of UV-absorbing pigmentation in species of other families is currently unknown. In addition, if UV-B emerges as an important selective agent upon UV bullseye size in flowers, we expect that the ultraviolet-absorbing proportion (UVP) of petals will increase over time as a result of increasing surface-level UV-B. Measuring the UVP of both historical and contemporary pressed specimens within Brassicaceae, Geraniaceae, Ranunculaceae, and Rosaceae, we found that increasing UV-B significantly increased bullseye size. We also establish that post-1950, UV bullseye size increases over time as a result of latitudinal and temporal interactions. We suggest that increased UV-pigmentation over time reflects anthropogenic-induced reduction in the ozone layer that subsequently resulted in a dramatic increase in UV-B transmittance. These results represent a critical starting point upon which our knowledge about spatio-temporal variations in UV bullseyes could be refined using hind-casted UV-B levels that account for both locational aerosol and clear-sky fluctuations; the use of such data would indicate more refined spatio-temporal changes in floral UV pigmentation.

Key words: abiotic selection, UV bullseye, floral evolution, temporal change, UV pattern, phenotypic variation.

Introduction

Floral colour diversity typically arises from pollinator-driven selection, and this is strongly supported by several lines of evidence. These include temporal matches (in geological time) between the radiation of angiosperms and major groups of pollinators (Grimaldi, 1999; Rosas-Guerrero *et al.*, 2011; Van der Niet & Johnson, 2012), strong associations between suites of floral traits and specific pollinator groups (Schemske & Bradshaw, 1999; Fenster *et al.*, 2004), and rapid events of diversification in lineages of animal-pollinated plants (Ricklefs & Renner, 1994) and are just a few examples of how pollinators are hypothesised to be the main architects of floral form (Herrera *et al.*, 2006). In combination with pollinators as agents

of selection, floral phenotypic variation can also be accounted for by indirect selection upon floral colour via pleiotropic effects (Rausher & Fry, 1993; Armbruster, 2002). In many cases, floral traits may be selected upon by non-pollinating agents (Ellis & Johnson, 2009). For example, heat stress and drought typically favour anthocyanin-producing petal morphs over white-flowered morphs (Warren & McKenzie, 2001; Coberly & Rausher 2003), and when moisture availability is a limiting factor, red morphs have greater fitness than white morphs in *Phlox drummondii* (Polemoniaceae) (Levin & Brack 1995).

Ultraviolet (UV) bullseyes are common floral patterns visible in UV light (Koski & Ashman, 2015a). The bullseye pattern is characterised by UV-absorbing petal bases and UV reflective petal apices (Guldberg & Atsatt, 1975; Harborne & Nash, 1984), resulting from UV-absorbing pigment variation (i.e. flavonoid pigments, Harborne & Nash, 1984) or variation in epidermal cell shape (Gorton & Vogelmann, 1996). From an evolutionary mechanistic perspective, the floral bullseye variation we see (via UV photography) and that visible to UV perceptive pollinators, has been well explained by pollinator-mediated selection (Koski & Ashman, 2016). UV bullseyes appear to function in several ways: to aid pollinator perception (Chittka *et al.*, 1994; Briscoe & Chittka, 2001; Sheehan *et al.*, 2016), orientation (Dinkel & Lunau, 2001), foraging efficiency (Dinkel & Lunau, 2001), visitation (Rae & Vamosi, 2013), and ultimately plant fitness (Morgan, 1992). Our understanding of the selective pressures and mechanisms responsible for UV patterns in flowering plants has markedly shifted with contemporary research however. This is because in recent studies, it has become evident that abiotic factors can determine floral colour and pattern diversity. For example, in one desert annual species (*Linanthus parryae,* Polemoniaceae)*,* the maintenance of blue and white colour morphs was better explained by temporal fluctuations in rainfall than pollinator preferences (Schemske & Bierzychudek, 2001). Blue-flowered plants typically had a fitness advantage in years of low spring precipitation, for white flowers the inverse was observed (Schemske & Bierzychudek, 2001). In *Ipomoea purpurea* (Convolvulaceae), flavonoids present in pigmented plant morphs ameliorated the effects of high temperature stress on fertilisation success, whilst white (non-pigmented) morphs experienced significantly lower fertilisation (Coberly & Rausher, 2003). Although only evidence of abiotic factors impacting upon floral colour diversity on microevolutionary scales (i.e. the maintenance of colour morphs in a limited number of populations, for one species), studies such as these have been important for indicating that selection may be acting indirectly on flower colour, and pattern, via the pleiotropic action of genes that determine

vegetative pigmentation (Rausher, 2008; Koski & Ashman, 2016). It is by this mechanism for example, that UV patterning or more specifically UV bullseyes, may have evolved (Ellis & Johnson, 2009). This is because the products of the flavonoid biosynthetic pathway that protect plant tissues from abiotic stressors also form the basis of UV-absorbing pigments in floral tissues (Harborne & Nash, 1984; Rausher, 2008).

Abiotic factors may also be acting on UV bullseyes directly. The hypothesized mechanism of direct, UV-B-mediated selection upon floral pigmentation was developed by Koski and Ashman (2015a) and in *Argentina* (*Potentilla*) *anserina* (Rosaceae) flowers, larger UV-absorbing bullseyes protected pollen from UV-B damage. The authors sought to identify whether geography and abiotic factors could explain UV floral phenotypic distribution on a global scale, sampling specimens from 34 populations across 4 regions that represented latitudinal transects. They also aimed to identify the mechanism responsible for global patterns of UV pigmentation by testing whether UV-B could be mediate by larger areas of UV-absorbing pigment. The microenvironment of *A. anserina* flowers was shown to vary with petal pigmentation, such that flowers with increased UV reflecting areas (as opposed to UV-absorbing pigmented areas) experienced greater detrimental diffuse reflection of UV radiation onto pollen-bearing anthers. In contrast, flowers with larger bullseyes produce pollen with greater viability. This is because UV is thought to select for larger absorbing areas of petals that protect pollen from damage after anthesis (Koski & Ashman, 2015a).

The fact that broad-scale geographic patterns of UV bullseyes were also found for populations of *A. anserina*, and a further 177 species of *Potentilla* in Koski and Ashman (2016), indicates that abiotic selection has to potential to act on a macroevolutionary scale. Whilst geography (altitude, region, latitude), and particularly latitude accounted for significant variation in bullseye size, a more accurate understanding of abiotic-factors-as-selective-drivers was derived from direct associations between bioclimatic variables and UV floral phenotypes. This is because bioclimatic variables correlate or co-vary with altitude and latitude. Temperature, precipitation, and UV radiation for example increase with decreasing latitude, whilst with increasing altitude, temperature decreases and UV-B exposure increases (Körner, 2007). Across several species of *Potentilla* UV radiation emerged as a significant and consistent bioclimatic predictor of bullseye size variation (Koski & Ashman, 2015a, 2016). This finding in particular is important for highlighting UV-B (amongst other abiotic factors) as an underlying driver of geographic variation of floral phenotypes (specifically UV pigmentation), and few other studies have been able to demonstrate this.

In this study we aim to build upon previous findings by quantifying the extent to which UV bullseye variation reflects temporal changes in UV radiation on a global scale. The UV-absorbing area of flowers containing bullseyes has already been shown to vary between populations with differing UV exposure (Koski & Ashman, 2015a,b; 2016), and genetic studies have identified that the ultraviolet proportion of flowers is both heritable (Yoshioka *et al.*, 2005; Koski & Ashman, 2013) and controlled by specific regulatory quantitative trait loci (Brock *et al.*, 2016). At present, we have evidence of UV-B mediated selection within and among species of one genus (Koski & Ashman, 2016), and UV bullseye variation is predicted by contemporary levels of UV radiation only. Recent findings have indicated that global UV has changed dramatically over the last 50 years in relation to changes in atmospheric aerosol and loss of the ozone layer (Herman, 2010). Although UV irradiance can vary on a daily basis due to the combined effects of geographical patterns of cloud coverage (Herman *et al.*, 2009), atmospheric pollutants, and daily ozone UV-B absorbance, short-term UV fluctuations only have a small effect on zonal or regional average irradiances. Long term ozone depletion however has been significant for overall global and zonal average changes in surface-level UV-B (Herman, 2010), with the largest zonal average increase in UV irradiance occurring in the southern hemisphere. Such a change may be predicted to have a concomitant effect on floral phenotype.

In this study, we measure interspecific variation in UV bullseyes of pressed herbarium and field-collected specimens, across four angiosperm families: Rosaceae, Ranunculaceae, Geraniaceae, and Brassicaceae. Using both geographical measures and bioclimatic data, we identify abiotic factors that predict UV bullseye variation. Finally, we investigate whether UV bullseyes have changed over time, and consider whether global increases of UV-B radiation over the last century account for these changes. Our understanding of the ecological correlates with UV floral patterns stands to be significantly advanced by expanding the number of species, genera, and families included in the present study. Furthermore, using specimens collected as early as 1840, and from globally distributed locations, we substantially enhance the degree of spatial and temporal coverage achieved by previous studies.

Methods

Plant family inclusion

To achieve broad spatial and temporal coverage of UV pattern variation, four focal angiosperm families were selected: Rosaceae, Brassicaceae, Geraniaceae, and Ranunculaceae. These families were chosen because their taxa are broadly distributed geographically, they contain species with appropriately sized flowers (and with dissected petals - further details below), and UV patterns within these families have been documented. Within Rosaceae, an extensive number of species have been documented with UV patterning and uniformly UV-absorbing flowers from at least nine genera (Eisner *et al.*, 1973; Utech & Kawano, 1975; Harborne & Nash, 1984; Burr & Barthlott, 1993; Naruhashi & Sugimoto, 1996; Narushashi & Ikeda, 1999; Koski & Ashman, 2013; 2014; 2015a,b; 2016). Additionally, for species of the *Ranunculus* genus within Ranunculaceae, UV patterning is also well documented (Eisner *et al.*, 1973; Utech & Kawano, 1975; Zhang *et al.*, 2017). UV patterning is scarcely reported in species of Geraniaceae, but is nevertheless present in three genera (*Erodium*, *Geranium*, *Pelargonium,* Burr & Barthlott, 1993). Finally within Brassicaceae, species from 15 genera photographed in UV light have shown UV patterning (Utech & Kawano, 1975; Horovitz & Cohen, 1976; Yoshioka *et al.*, 2005), and within *Brassica rapa* (and cultivars of *B. rapa*) variation in the ultraviolet proportion of petals (the area of UV-absorbing pigment relative to total petal area) is accounted for by underlying genetic architecture (Yoshioka *et al.*, 2005; Brock *et al.*, 2016).

Phenotypic, geographic, and bioclimatic data collection

Herbarium specimen selection

Several studies have used pressed herbarium specimens to either categorise or quantify UV floral patterns across several plant families (Horowitz & Cohen, 1972; Eisner *et al*., 1973; Parker & Bohm 1975; Koski & Ashman, 2013; 2016; Scogin *et al.*, 1977). We utilised the online herbarium catalogues of Kew Botanical Gardens, The Natural History Museum, and The Linnean Society to search for all UK archived pressed specimens. All available specimens were compiled into a large dataset for preliminary screening including information on geographical location and collection year. To obtain perceivable and accurate measures of petal ultraviolet absorbing area (UV_{area}) and UVP (the relative area of petals that absorb UV), only species with average petal size >3 mm total length were included, and similarly flowers of species with petals that are not fused were also chosen. Preliminary UV photography trials with specimens of varying sizes indicated that often clarity became compromised due to the

inherent nature of short UV wave lengths (Primack, 1980; Arribas, 2012); the inclusion of species with separate petals therefore ensured clearly defined petal boundaries.

In the statistical package R (R Development Core Team & R Core Team, 2017), specimens were screened such that only those with the following recorded data were suitable for UV photography: i) geographical location, ii) date of collection, and iii) accepted taxonomical name. All collection locations recorded as either a named place or as absolute values of latitude and longitude were transformed into digital degrees using Google Maps® (2018). Using digital images published in the herbarium catalogues, the specimens were further screened: only specimens containing flowers, and of those, containing adaxially facing petals were included in the final dataset.

Field specimen collection

During the months of January 2018 to August 2018, contemporary flower specimens (of the above families) were collected on an *ad hoc* basis from various global field locations: Great Britain, Portugal, Ecuador, North America, and Iceland (global distribution of all herbarium, field, and published data specimen locations are shown in Fig. 1). A minimum of one flower was randomly sampled per plant, and the location (in digital degrees), date and species were recorded. The curvature of petals in the majority of species we sampled for preliminary photography studies often compromised measures of UVP and its components (UV_{area} and petal area) in UV light; all petals were therefore removed from sampled flowers and pressed at the time of collection. Koski and Ashman (2013) indicated that this method of drying and pressing specimens to score the size of UV bullseyes/quantify the relative area of UV-absorbing pigment does not significantly influence UVP.

Published data

UVP data for several taxa was either taken from published studies, or measured from published UV photography. Published data were only included in the analysis if associated dates and locations were provided with the UVP score. Where only UV photographs were published, those with scales provided were analysed in ImageJ (version Fiji, Schindelin *et al.*, 2012) and the UV_{area}, UVP, and petal area measured (see details below). If a scale was absent, only UVP was calculated per petal, per flower, since a proportion can still be calculated without measures for petal area and UV_{area}. In total, we obtained UVP values of 32 *Potentilla anserina* (Rosaceae) specimens (Koski, 2015; PhD thesis). The UVP was

measured from photographs for two *Rubus* (Rosaceae) specimens (Douglas, 1983), one specimen of *Duchesnea* (*D. indica*; Rosaceae, Naruhashi & Sugimoto, 1996), 16 *Potentilla* specimens (Naruhashi & Ikeda, 1999), and one specimen of *Sibbaldia* (*S. parviflora;* Rosaceae, Naruhashi & Ikeda, 1999). Other datasets did contain UVP data or UV photographs, however due to a lack of both spatial and temporal information, could not be added. Finally in one case where UVP was published for 177 species, the spatial and temporal data for these specimens would not be shared by the author, and so could not be included in the dataset.

Ultraviolet photography

A total of 1723 petals, of 485 pressed flowers from 86 species were photographed in UV light (global distribution of all herbarium, field, and published data specimen locations are shown in Fig. 1). All photographs were taken with a Canon EOS 400D camera, fitted with a Baader UBVRI 1 1/4" Photometric U-filter. The filter permits the transmission of UV light between 320 nm to 390 nm, peaking with 85% transmission at 350nm, whilst removing infrared and daylight wavelengths. The availability of naturally occurring UV light in the daylight spectrum can be limited indoors; the image field was therefore illuminated with a UV torch (Convoy S2+ Nichia UV waterproof LED flashlight) with a peak UV emission at 365 nm. The camera was mounted on tripod and arm (Mantona, Germany) to achieve a plan view of the pressed specimen. All photographs included a standard scale, and the UV torch was also positioned for a plan view approximately 15 cm above the specimen. For all photographs, the exposure was 10 seconds, and the aperture f/5.6.

Image analysis

The UV_{area} (mm^2), total petal area (mm^2), and UVP (%) were measured for 1721 petals where petals had pressed without damage, and focus had not been compromised. All area and UVP measures were made in ImageJ (2012), version Fiji (Schindelin *et al.*, 2012) following Yoshioka *et al*. (2005) and Koski and Ashman (2013; 2016). After each individual flower was scaled, colour channels (blue, green, red) were obtained using the 'split' function. Using the 'threshold' tool, the UV_{area} of each petal was outlined and measured in millimetres squared in the red channel, and similarly total petal area was measured in the blue channel.

The UVP of each petal was calculated as the ultraviolet absorbing area divided by the total area of the petal.

Bioclimatic data

Global climatic data were downloaded from WorldClim at 30 arc-second intervals (Fick & Hijmans, 2017; http://www.worldclim.org/). Initially all 19 bioclimatic variables were selected and extracted (O'Donnell & Ignizio, 2012; http://worldclim.org/bioclim). Three variables for temperature (Bio 9: mean temperature of the driest quarter, Bio 10: mean temperature of the warmest quarter, Bio 11: mean temperature of the coldest quarter) and three equivalent variables for precipitation (Bio 17: mean precipitation of the driest quarter, Bio 18: mean precipitation of the warmest quarter, Bio 19: mean precipitation of the coldest quarter) were included in the models to reduce multi-collinearity (Varga & Soulsbury, manuscript under review). These variables were selected because previous studies have indicated that they are strong abiotic predictors of UVP variation (Koski & Ashman, 2015a; 2016). Altitude was also included in our models and either obtained from the metadata associated with the pressed specimens (accessed via the associated herbarium's online database), or calculated from http://www.twcc.fr/ using published/reported coordinates. Finally, we included global UV-B exposure (mean UV-B radiation during the highest quarter and mean UV-B radiation during the lowest quarter) at 30 arc-second resolution, in the models. UV-B irradiance was extracted from glUV: http://www.ufz.de/gluv/ (Beckmann *et al.*, 2014).

Data analysis

UV floral pattern

To determine whether UVP is influenced by underlying differences at the family-level, a Kruskal-Wallis test was run in R (R Development Core Team & R Core Team, 2017). We also performed multiple pairwise-comparisons between families to identify those families that differed significantly in the UVP of their petals.

Spatial and temporal variation in UV proportion

To assess whether floral UVP varies on a geographical scale, and furthermore whether it has changed over time, we carried out LMMs using lmer in R version 3.4.4 (R Development Core Team & R Core Team, 2017). Before analysis, UVP data were mean-centred at the species level to account for inherent differences in UVP. The specimen collection dates constituted a

substantially long time period (1750-2018), and therefore detecting temporal effects on UVP may be lost in broad-scale analysis. We therefore sub-set the data into two time periods: 1750-1950 (period A) and 1951-2018 (period B). These two time periods represent episodes where anthropogenic induced reductions in the stratospheric ozone layer (via release of chlorofluorocarbons into the atmosphere) led to changes in global surface-level UV-B (compared to an estimated 1850–1859 baseline annual UV-B average, Watanabe *et al.*, 2012). In period A, increase in ozone UV-B transmittance is gradual, and in period B, is markedly increased. We therefore ran two LMM models on both sets of time period data, with the same fixed and random effects as before. Latitude and year of collection were entered into the model as fixed effects, and species and specimen were included as random effects. Plant family was also added into the model as a fixed effect to account for family-specific variance in the UVP. Data for latitude and year of specimen collection were non-linear, therefore the polynomial (second order) of both variables was included in the model. UV-B radiation varies globally along latitudinal clines, increasing in average annual transmission over the past 50 years (Herman, 2010). Amongst other factors such as aerosol and cloud coverage, the extent of annual changes in UV-B are in themselves dependent upon latitude; we suspect that latitude and year of collection interact to influence UVP due to the underlying relationship between these factors and UV-B radiation. A latitude and year of specimen collection interaction was therefore also included in each of the models.

Variation in UV proportion and with abiotic factors

To assess the effect of abiotic factors on the UVP of flowers, we carried out both a linear-mixed model (LMM) and a phylogenetic mixed model (PMM) in R version 3.4.4 (R Development Core Team & R Core Team, 2017). We created a global model for both phylogenetic and non-phylogenetic analysis whereby the bioclimatic variables were all entered individually as fixed effects, and both species and specimen entered as random effects to account for non-independence of data. Although previous studies have identified abiotic variables as significant predictors of UV patterning (Koski & Ashman, 2015a,b), we felt that AIC-IT (Akaike's Information Criterion-Information Theoretic, Guthery *et al.*, 2003) modelling of our selected variables was necessary to assess the relative importance of individual parameters; our dataset comprised of a broader range of taxa, across greater geographical ranges (where bioclimatic factors are known to correlate or co-vary with latitude) than previous studies.

Before analysis, the bioclimatic variables were standardised (mean ± SD, 0 ± 1) and run individually without interactions using the 'arm' R package (Gelman *et al.*, 2015). We standardised the independent variables in order to relativize parameter estimates for comparison after model averaging. To check for multi-collinearity between bioclimatic variables, we used variance inflation factors (VIF) using a custom function for variance inflation, prior to analysis. All of the parameters were VIF<10, and were therefore retained in our global model. We carried out a AIC-IT model selection (Grueber *et al.*, 2011) using the 'dredge' function from R package MuMIn (Bartón, 2018) to run lmer models (Kuznetsova *et al.*, 2015) on all combinations of our parameters. We retrieved a top model of ΔAIC<10 and carried out model-averaging to obtain conditional model-averaged parameter estimates and relative importance values for each parameter.

We used a PMM to account for non-independence in our dataset using the MCMCglmm package in R (Hadfield, 2010). We used the same lmer model as above for analysis, with the addition of a phylogenetic covariance matrix. Both specimen and species were retained in the model as random effects. We set parameter expanded uninformative priors with independent chains of 500,000 iterations, and sampling occurring every 50 iterations after a 2000 burn in. Model estimates and the relative importance for each parameter were not returned from the global model, however P-values (pMCMC) were obtained.

Results

UV floral pattern

Variation in the area of the petal that absorbed UV represented the full possible range from 0 absorption (fully UV-reflective petals) to 100% absorption (fully UV-absorbing petals) in our dataset (Fig. 2). UVP differed significantly at the family level ($\chi^2 = 545.65$, $P \leq 0.001$), and pairwise comparison tests indicated UVP did not significantly differ between Brassicaceae and Geraniaceae ($P = 0.641$). UVP however differed significantly between all other families: Brassicaceae and Ranunculaceae ($P \leq 0.001$), Rosaceae and Brassicaceae ($P \leq 0.001$), Geraniaceae and Ranunculaceae ($P \leq 0.001$), Geraniaceae and Rosaceae ($P \leq 0.001$), and Ranunculaceae and Rosaceae ($P = 0.027$).

UVP, latitude, and time

For period A, plant family ($F_{4,40.52} = 0.658$, P = 0.624), latitude (polynomial) ($F_{2,245.48} = 1.725$, P = 0.180) and year (polynomial) ($F_{2,217.30} = 0.490$, P = 0.613) did not significantly affect UVP. Furthermore, the latitude x year interaction (both polynomial) ($F_{4,221.98} = 0.923$, P = 0.452) did not significantly affect UVP.

Similarly, between the years 1951 and 2018, family, latitude, and year of specimen collection did not significantly affect UVP. For period B, family ($F_{4,77.13} = 1.068$, P = 0.378), latitude (polynomial) ($F_{2,220.61} = 1.919$, P = 0.149) and year (polynomial) ($F_{2,217.21} = 0.197$, P = 0.821) did not have a significant effect on UVP, however the latitude x year interaction (both polynomial) did significantly affect UVP ($F_{4,180.26} = 2.617$, P = 0.037). In the southernmost latitudes (40°S to 60°S; Fig. 3C) and northernmost latitudes (40°N+; Fig. 3A), there were sharp increases in floral UVP post 1950.

UVP and climate

The best LMM explaining UVP included mean temperatures of the warmest and coldest quarters, and mean UV-B in the lowest and highest quarters as the most important significant bioclimatic variables (Table 1; Fig. 4). Of these variables, mean temperature in the coldest quarter and UV-B in the highest quarter were positively correlated with UVP, and mean temperature in the warmest Q and UV-B in the lowest quarter were negatively correlated with UVP (Table 1; Fig. 4). These results were replicated in the PMM, with the addition of mean temperature in the driest quarter and mean precipitation in the warmest quarter significantly negatively correlating with UVP (Table 1).

Discussion

In the present study, we identified geographic, temporal, and bioclimatic factors associated with variation in floral UV pigmentation in four angiosperm families, supporting the hypothesis that abiotic factors may drive floral diversification on a global scale. We observed significant differences in the UVP between families, however ultimately such differences did not significantly affect spatio-temporal changes in UV pattern.

Our findings suggest that the UVP of UV bullseyes has increased over time, and overall are suggestive of evolved floral phenotypic responses to UV-B. The fact that latitude and time interact to significantly affect UVP in period B indicates that for latitude-specific increases in UV-B, UV-B radiation has potentially selected upon floral phenotypes to

produce petals with increased UV-protective, UV-absorbing pigments. Our results indicate that UVP has increased significantly from 1950 onwards, potentially in the southernmost and northernmost latitudes. Because surface-level UV-B radiation has markedly increased over time as a function of global anthropogenic-induced ozone reduction (namely the release of CFC's into the atmosphere) (Watanabe *et al.*, 2012), and because the extent of this UV-B change depends on latitude (Herman, 2010), we expected that the latitude and temporal interaction would indicate increases in the UVP of flowers over time.

Long-term changes in ozone have been monitored since the 1970s when the ozone hole over the Antarctic was first discovered, however, inherent global variations in UV-B also exist, regardless of anthropogenic action (WMO, 2010). In general, UV-B irradiance has been found to decrease with increasing latitude outside of the equatorial zone. Equatorial zones also tend to experience greater UV-B irradiance specifically because maximum daily noon solar elevation angles are greatest, and this also corresponds with high summer clear-sky UV-B irradiances (Herman, 2010). Since the advent of global industrialisation in the late 19th century, ozone-reducing CFCs have been produced, however aerosols and pollutants being released into the atmosphere have also determined the extent to which UV-B radiation has changed. Highly polluted regions can for example reduce the local amount of UV-B reaching the Earth's surface by scattering or absorbing pollutants, to some extent mediating UV-B transmission via reduced ozone (Herman *et al.*, 2009). The challenge of determining the exact effect of latitudinal and temporal changes in UV-B on plants, and in this case UV bullseyes therefore, is that percentage increases and decreases in surface-level UV-B over the last 30 years (at least) is extremely variable, often reflecting multiple co-occurring determinants of UV-B transmission, that are in themselves variable depending on latitudinal (and within latitudinal) clines. Overall however, we detect significant changes in UVP over time, particularly in the nothern and southernmost latitudes; for flowers collected post 1950 and between 40°S and 60°S, this may reflect the fact the largest zonal average increases in UV irradiance has occurred in the Southern Hemisphere (despite increased cloud and aerosol reflectivity, Herman *et al.*, 2009). The level of UV-B irradiance in the Northern Hemisphere has levelled-off over recent years with indications of recovery, however current ozone values are still below the amounts measured in 1979–1980 at high latitudes, and therefore may account for apparent increase in UVP at 40°N+ (Herman, 2010).

After correcting for phylogeny, temperature and precipitation significantly predicted the extent of UVP in petals, and as predicted, UV-B also emerged as a significant abiotic

predictor of UV pigmentation. Specifically, we observed a strong positive effect of UV-B in the highest quarter, and a strong negative effect of UV-B in the lowest quarter on UVP. Our results corroborate previous findings whereby UV-B was thought to be an important selective agent for the UV pattern of 177 *Potentilla* species (Koski & Ashman, 2016), however we expand on this to suggest that it is also important for species of Ranunculaceae, Geraniaceae, and Brassicaceae.

Temperature also accounted for the extent of UV pigmentation. Given that previously, increasing altitude (which is associated with both lower temperatures and increased UV-B) and lower temperatures, have predicted greater UV-absorbing areas in flowers (Koski & Ashman, 2016), increasing UVP as a result of decreasing temperature alone is difficult to explain in the present study. Altitude was not highlighted as a significant predictor of UVP, and has similarly failed to explain the frequency of red-coloured phenotypes limited to high elevation habitats of *Mimulus* (Ogutcen *et al.*, 2014). This strong negative effect of temperature on the UVP of UV bullseyes, however, has been thought to function as a means of reducing the negative effects of cold-stress (Bharti & Khurana, 1997). In tomato (*Lycopersicon esculentum L*) and watermelon (*Citrullus lanatus)* plants for example, soluble phenolic compounds (flavonoids and phenylpropanoids) found in the plants tissues accumulated in response to thermal stress during growth; for tomato plants in particular, this was perhaps as result of an acclimation mechanism to overcome cold stress (Rivero *et al.*, 2001). Flavonoids however can also ameliorate heat-stress too; watermelon plants in the same experiment produce more soluble phenolic compounds in the high temperature (35°C) growth treatment, and for plants of *Ipomoea purpurea* pigmented morphs (compared to non-pigmented morphs) experienced significantly greater seed maturation and fertilization success under heat-stress as result of floral tissue flavonoids (Coberly & Rausher, 2003). Although these examples explain why thermal stress may select for greater UVP within species, or even large populations of a species, they are unable to suggest a mechanism by which floral phenotypic variation is structured at a macroevolutionary level in response to thermal stress. It is perhaps the case that pigmentation (particularly in the aforementioned study) arising from enhanced flavonoid production represents a generalized response to abiotic factors such as thermal stress and UV-B stress, not limited to specifically cold or heat-stress (Coberly & Rausher, 2003). Finally, whilst precipitation has been shown to be important for the selection of floral structures in plants that do not produce water-repellent pollen (Sun *et al.*, 2008; Mao & Huang, 2009), the importance of precipitation for UV-

pigmentation in floral tissues is less clear. The fact that UVP declines with increasing precipitation is consistent with previous studies (Koski & Ashman, 2015a; 2016), and has generally been interpreted as being important for the geographic variation of pigmented and non-pigmented morphs of a single species, rather than broad-scale variation in UV floral patterning specifically (Warren & Mackenzie, 2001; Arista et al., 2013). Since precipitation appears to be an important predictor of UV petal pigmentation, and like other bioclimatic variables is a significant correlate of latitude, further works should aim to identify by what underlying mechanism precipitation (as a stand-alone abiotic factor) might be selecting for increasing UV bullseyes.

As mentioned, the fact that altitude did not account for UVP is surprising. Increasing UVP with increasing altitude has been observed in populations of *Argentina anserina*, and for another 176 species of *Potentilla* (Koski & Ashman, 2015b, a; 2016), and in alpine plants, UV-B emerged as an important abiotic agent, selecting for flower structures that protect sensitive pollen from UV-B damage (Zhang *et al.*, 2014). Overall, such studies indicate that UV-B radiation plays an important role in influencing the evolution of floral traits in geographical locations where altitudinal increases in UV-B transmittance are significantly increased. We suggest that the close associations between altitude and UV-B radiation may only be relevant for species within *Potentilla* (Rosaceae) in the present study; several of the species we measure for UVP within *Potentilla* originated from high elevational locations, and large UV bullseyes/complete petal UV-absorbance in Himalayan *Potentilla* species are well-documented (Naruhashi & Ikeda, 1999).

The UV-B data used in the present study are contemporary values, and our finding of a spatial-temporal increase in floral UVP is therefore partly limited. Although we present evidence that UV-B has a positive effect on UV absorption in flowers at a macroevolutionary scale, we propose that hind-casting of UV-B radiation over the timeframe of data points in the present study would reveal enhanced predictions of UVP with changing UV-B over time. Integrating calculations of percentage change (of contemporary UV-B values) in UV-B based upon specific locational degrees of cloud albedo, aerosol and clear-sky UV-B (Watanabe *et al.*, 2012; Beckmann *et al.*, 2014) would enable very small-scale spatio-temporal UV-B effects on UVP to be observed from as early as 1840 to present day. Nevertheless, in our study we do detect the fact that plants respond to increasing UV-B over time by increasing floral UVP, and suggest that this could be indicative of an evolved response.

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Tables and Figures

Table 1. Results from the linear-mixed effects model and the phylogenetic analysis (pMCMC). Significant values are marked in bold.

Model	Variable	RI_{lmm}	Estimate	± SE	z	*P*	pMCMC
	Intercept		1.774	4.505	3.935	**<0.001**	λ = 0.380
UV proportion 86 species 479 flowers 1661 data points	Mean Temp Warmest Q (Bio 10)	1.00	-6.642	1.872	3.547	**<0.001**	**0.014**
	UV-B Lowest Q	1.00	-1.707	4.968	3.434	**<0.001**	**0.001**
	UV-B Highest Q	1.00	1.759	3.694	4.760	**<0.001**	**<0.001**
	Mean Temp Coldest Q (Bio 11)	0.87	4.008	1.812	2.211	**0.027**	**0.012**
	Mean Temp Driest Q (Bio 9)	0.63	-2.194	1.303	1.683	0.092	**0.039**
	Precipitation Warmest Q (Bio 18)	0.43	-5.350	4.337	1.233	0.218	**0.008**
	Precipitation Driest Q (Bio 17)	0.33	-7.027	1.132	0.620	0.535	0.567
	Precipitation Coldest Q (Bio 19)	0.32	-2.829	6.659	0.424	0.671	0.788
	Altitude	0.27	-2.826	6.659	0.424	0.671	0.175

Q: quarter, RI: relative importance.

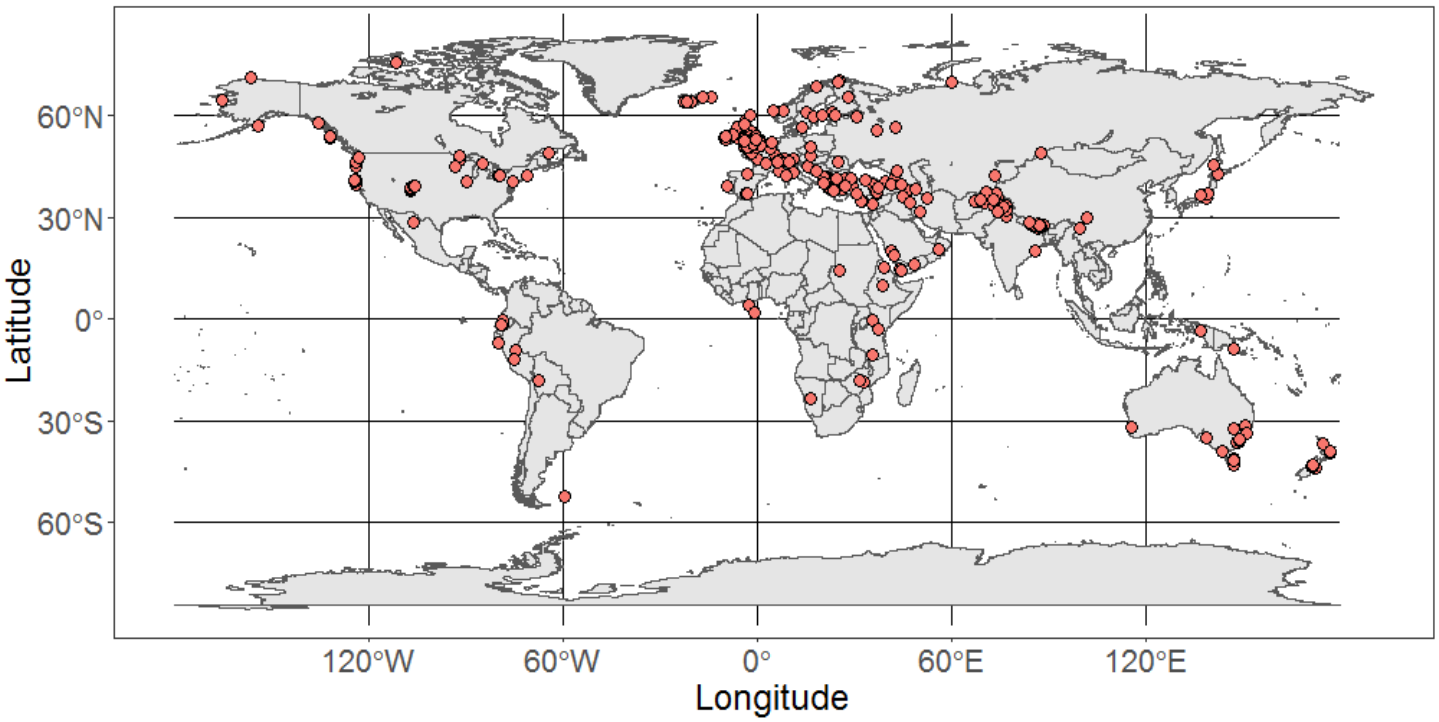

Figure 1. Global distribution of herbarium and field-sourced specimens for which UV floral pattern was quantified. Red circles represent the individual location of each specimen.

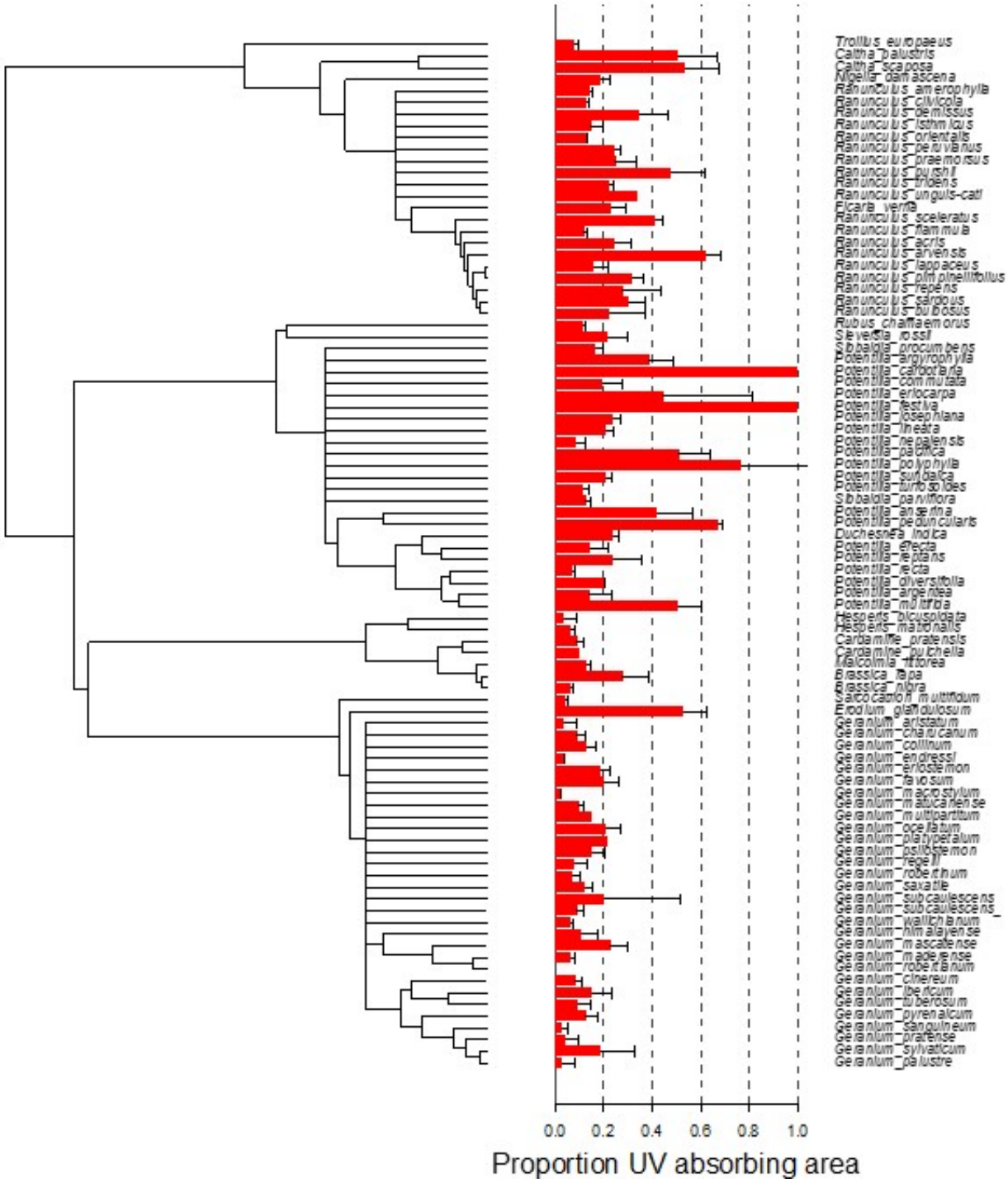

Figure 2. Phylogenetic barplot of mean ± SE UV proportion for each species in: Ranunculaceae, Rosaceae, Brassicaceae, and Geraniaceae. A proportion of 0 corresponds to 100% UV-reflective petals and a proportion of 1 corresponds to 100% UV-absorptive petals.

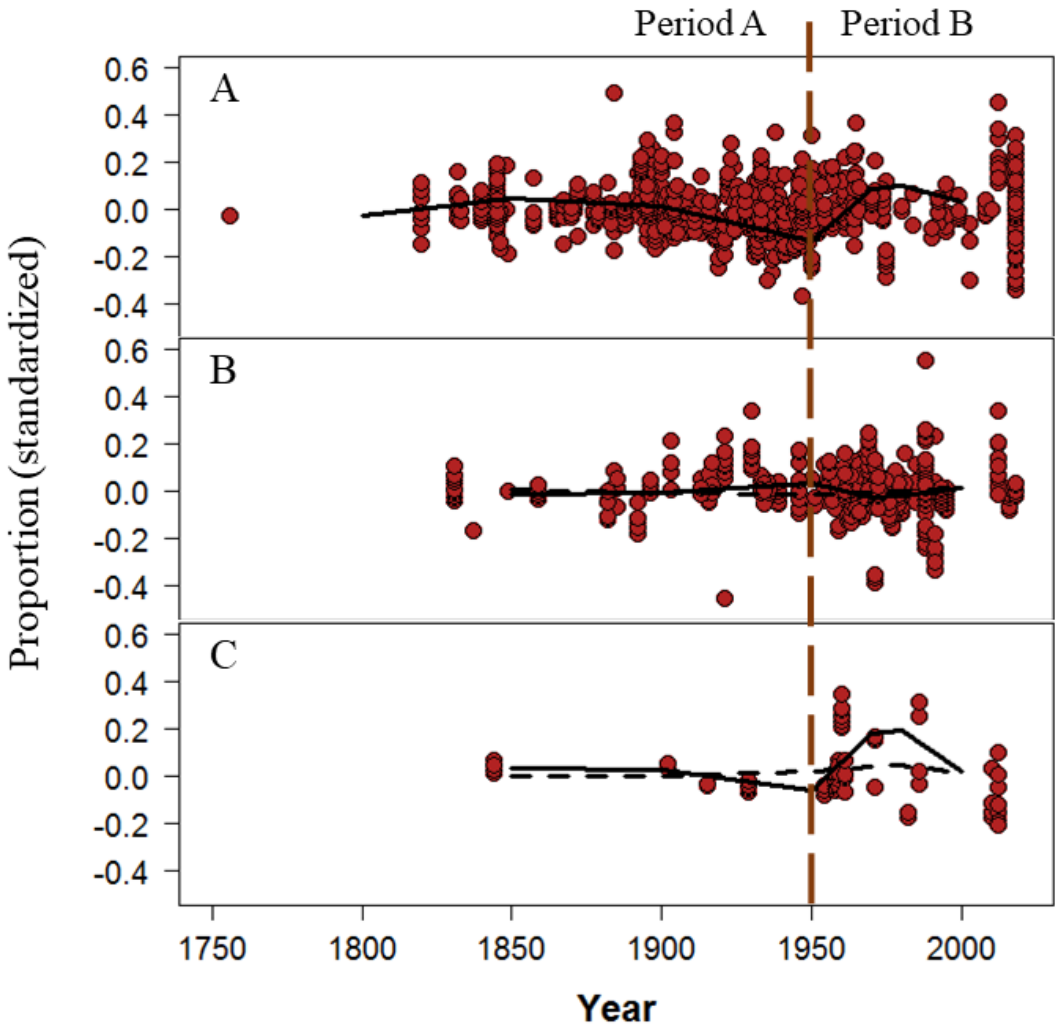

Figure 3. Model effects showing change in UVP over time as a function of temporal and latitude interactions. A: solid line represents 40°N+; B: solid line represents 20°S to 40°N; C: solid line represents 40°S to 60°S, dashed line represents 20°S to 40°S.

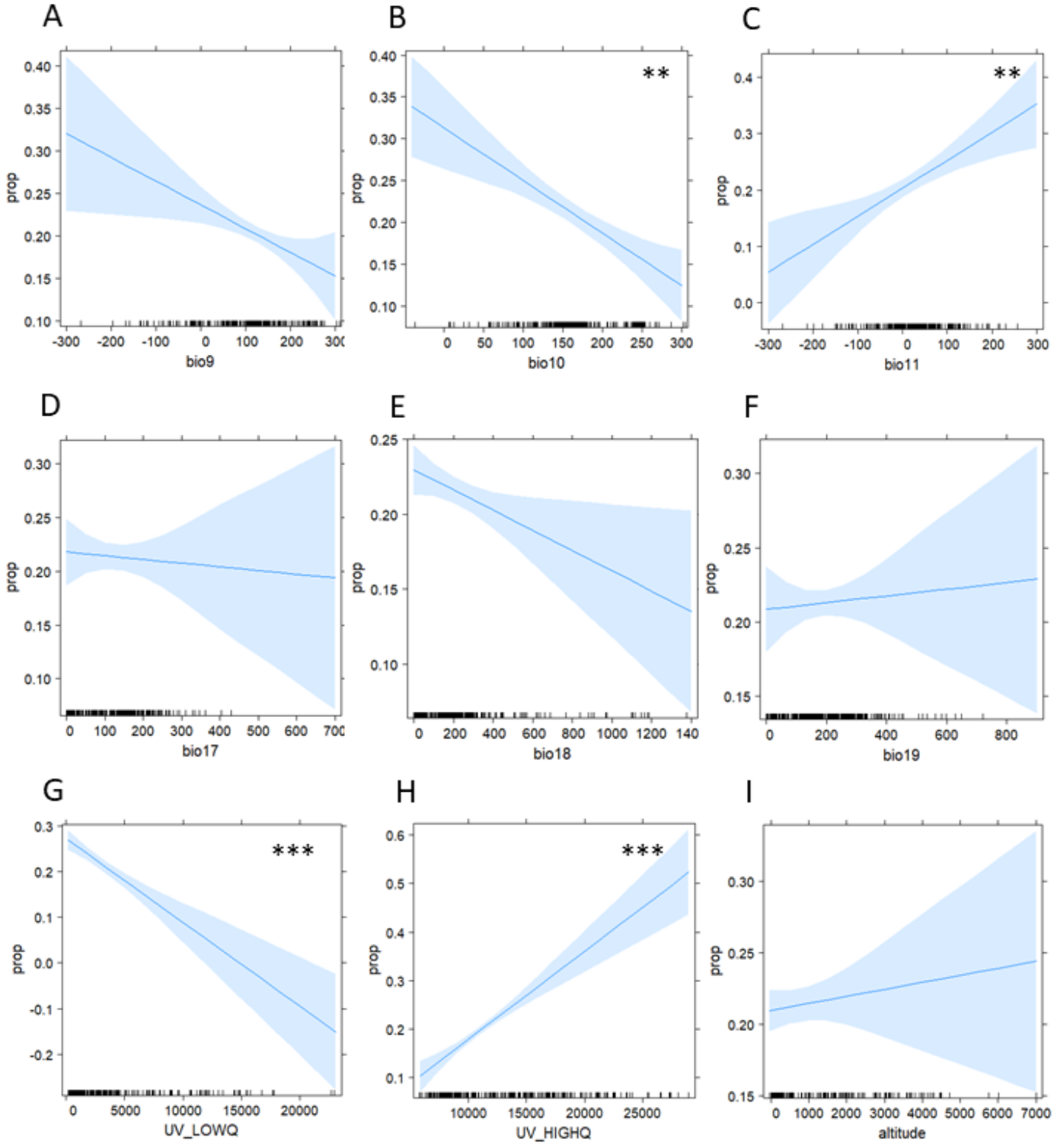

Figure 4. Effect plots for the relationship between the bioclimatic variables and UVP. Mean ± 95% CI are indicated per plot. Asterisks indicate significant correlations between UVP and bioclimatic variable. A = mean temperature of the driest quarter, B = mean temperature of the warmest quarter, C = mean temperature of the coldest quarter, D = mean precipitation of the driest quarter, E = mean precipitation of the warmest quarter, F = mean precipitation of the

coldest quarter, G = mean UV-B radiation during the lowest quarter, H = mean UV-B radiation during the highest quarter, and I = altitude.

III

A MANUSCRIPT WRITTEN IN ACCORDANCE WITH THE GUIDELINES OF THE JOURNAL OF EXPERIMENTAL BOTANY

UV bullseyes respond plastically to elevated UV-B in *Brassica rapa.*

UV-B intensity and duration of exposure during growth induces positive plastic changes in the ultraviolet proportion of *Brassica rapa* petals.

Abstract

Recently, UV-B has emerged as an important driver of macroevolutionary patterns of UV bullseyes in flowers, and genetic studies suggest that abiotic factors (such as UV-B) interact with specific regulatory UV-trait quantitative trait loci to produce such floral UV patterning. It is not known, however, whether UV bullseyes are capable of more dynamic responses to UV-B, and this could be important given that i) average UV irradiance reaching the Earth's surface over the past 30 years has substantially increased and that ii) ambient UV-B can be considerably variable depending on season, latitude, altitude, time of day, cloud albedo, aerosols, and pollutants. Here, we investigate whether UV bullseyes respond plastically to UV-B, quantifying the ultraviolet-absorbing proportion (UVP: the area of UV-absorbing pigment relative to petal area) of *Brassica rapa* petals grown in three intensities of UV-B radiation (control, low, and high). We also determine whether the period of UV-B exposure prior to anthesis is important for the extent of plastic change in UV bullseyes. Our findings indicate that UV bullseyes do respond plastically over the flowering period when exposed to longer periods of UV-B, and with increasing UV-B intensity. Decline in UVP over the flowering period also suggests that UV-absorbing pigments are costly, however overall, plastic UVP responses in the petals of *B. rapa* may be an important immediate response to unpredictable elevations in UV-B while adaptive genetic changes accumulate.

Key words: *Brassica rapa,* UV-B, plasticity, UV exposure, ultraviolet radiation, UV bullseye, maintenance costs.

Introduction

Although most of the ultraviolet (UV) solar radiation that reaches the earth's surface is absorbed by the stratospheric ozone layer (McKenzie *et al.*, 2007), atmospheric ambient UV-B can be variable depending on factors such as season, latitude, altitude, and time of day (Paul and Gwynn-Jones, 2003). Overall, average UV irradiance reaching the Earth's surface over the past 30 years has substantially increased, with particular effects in high and low latitudes (Herman, 2010). Factors that are themselves inherently variable also contribute to sometimes very localised elevated levels of UV-B, such as the degree of cloud cover, surface

reflection, atmospheric aerosols, pollutants, and fluctuations in ozone layer via chlorofluorocarbons (Jenkins, 2009; Andrady *et al.*, 2017). Such variable fluctuations in surface-level UV-B represent a challenge for ecologists examining the implication of UV radiation for evolutionary and plastic changes in plants. Are the UV-B responses of plant species, populations, and communities capable of reacting to and 'keeping pace' with contemporary (and sometimes rapid) global climatic change (Franks *et al.*, 2014), and are we able to explain the mechanisms by which plants respond?

Early studies demonstrated that UV-B is a potentially harmful environmental stress for terrestrial plant species (Caldwell, 1968; Caldwell *et al*., 1989; Tevini and Teramura, 1989), detrimentally affecting biomass accumulation, photosynthesis, large macromolecules such as DNA and other proteins, and inducing significant oxidative stress within the vegetative tissues of plants (Rozema *et al.*, 1997; Jansen *et al.*, 1998; Brosché and Strid, 2003; Frohnmeyer, 2003). More recently, studies have begun to scrutinize the concept of UV-B plant stress, assessing the extent to which the vast examples of UV-B induced plastic responses in both vegetative and reproductive plant tissues truly reflect harmful stress or damage (Llorens *et al.*, 2015). In some cases, plastic responses to enhanced UV-B such as rapid increase in flower production and earlier anthesis (Ziska *et al.*, 1992; Torabinejad *et al.*, 1998; Marshall *et al.*, 2010) are suggestive of a strategic stress response; increasing reproductive output before yielding to the stressor for example (Wada and Takeno, 2010). In other instances, UV-B radiation can be characterised as regulatory or 'eustress', functioning to regulate or maintain plant metabolism and other important processes (Hideg *et al.*, 2013). A good example of these plastic responses includes increased accumulation of UV-absorbing compounds (such as flavonoids) in the pollen walls of plants grown in UV-B treatments that are significantly higher than typical ambient levels (Demchik and Day, 1996). Similarly, UV-B can be a significant regulating factor of petal colour. Enhanced UV-B increased the accumulation of UV-B protecting pigments (anthocyanins and flavonoids) in both *Rosa hybrida* (Hennayake *et al.*, 2006) and *Anigozanthos* flowers (Ben-Tal and King, 1997), overall producing petals with more intense colour than petals of flowers grown in UV-absent conditions. Whether these UV-B induced plastic changes in petal pigmentation influence overall plant reproductive fitness has not been experimentally tested; however it has been suggested that aside from the alleviating properties of increased UV-absorbing pigments, colour change in petals may function to modify floral temperature or increase the saliency of

inflorescences to pollinators (Llorens *et al.*, 2015), both of which may improve reproductive fitness.

Contemporary research into UV floral pigmentation has identified UV-B as a selective agent of the UV bullseye floral pattern (Koski and Ashman, 2015). The bullseye is a pattern formed by the arrangement of UV reflecting petal apices and UV-absorbing petal bases (Guldberg and Atsatt, 1975; Harborne and Nash, 1984). UV bullseyes, and their ecological development, have been traditionally understood as the result of plant-pollinator driven interactions (Rausher, 2008). Much of the floral diversity we see today, and in particular this UV bull's-eye pattern (present in as least 36 angiosperm species), has been well supported by evidence demonstrating that UV bullseyes function to aid pollinator perception (Chittka *et al.*, 1994; Briscoe and Chittka, 2001; Sheehan *et al.*, 2016), orientation (Dinkel and Lunau, 2001), foraging efficiency (Dinkel and Lunau, 2001), visitation (Rae and Vamosi, 2013), and ultimately plant fitness (Morgan, 1992). However, the recent finding that UV bullseyes of a globally widespread plant – *Argentina anserina* – produce flowers with greater petal ultraviolet proportions (UVP; the relative area of ultraviolet absorbing pigment (UV_{area})) with decreasing latitude (where UV-B irradiance is greater), and that UV-B irradiance predicted broad geographic variation in the UVP of a further 177 species of *Potentillae* (Koski and Ashman, 2016), suggests that UV-B could be an important abiotic driver of phenotypic variation (Koski and Ashman, 2015). Flowers producing UV bullseyes with greater UVPs are thought to be the object of selection because they have the potential to significantly improve male fitness in elevated UV-B environments. Flowers with more UV reflectance are thought to experience decreased pollen viability via diffuse reflection of UV onto pollen-bearing anthers; conversely, flowers with larger bull's-eyes protect pollen by absorbing UV radiation (Fig. 1). The extent of protection is such that the germination rate of flowers in UV conditions, but with large bullseyes, is comparable with that of flowers grown in UV absent conditions (Koski and Ashman, 2015). UVP is also highly heritable in at least two species of *Potentillae*, *A. anserina* and *A. pacifica* (Koski and Ashman, 2013). Similarly, intraspecific variation across eight varietal cultivars of *Brassica rapa* was quantified, with underlying genotypic differences potentially accounting for this bullseye variation (Yoshioka *et al*. 2005). It has also been suggested the genes responsible for UV bullseye variation are regulatory and independent from petal morphology genes (Brock *et al.*, 2016).

For species possessing UV bullseyes, it appears that they have developed via interacting environmental forces and underlying genetic architecture over time (Brock *et al.*,

2016), however, UV bullseyes may also be capable of plastic change. Given that surface-level UV radiation can vary from negative to positive levels on a daily cycle alone (Serrano *et al.*, 2006; Hooke *et al.*, 2012), plastic responses in the bullseyes of *B. rapa* may be a more important immediate response to unpredictable elevations in UV-B while adaptive genetic changes accumulate. Since male fitness is likely to be immediately enhanced by such plastic responses when environmental UV-B levels are unfavourable, plasticity may potentially maximise overall reproductive success later in the plant life cycle (Koski and Ashman, 2015).

In this study we aim to identify if the UV bullseye phenotype in *B. rapa* is variable, and if such variation directly reflects changing UV-B intensity and exposure during plant growth. Previous studies have indicated that UV bullseyes are the target of selection, functioning in part to protect pollen from harmful levels of UV-B damage; it now seems pertinent that we examine whether UV bullseyes are capable of more dynamic responses to UV-B. *B. rapa* represents a valuable agro-economic resource (Williams and Hill, 1986) and improved knowledge of its floral response(s) to UV-B may inform appropriate use of genotypes and cultivars on a regional to global scale, where UV radiation varies according to both latitude and season (Herman, 2010). We predict that UV bullseyes are phenotypically plastic, and expect that UVP (and its components) will be greater in the petals of plants exposed to elevated intensities of UV-B.

Methods

Plant material

Extensively cultivated, *B. rapa* is distributed globally, and found on waste ground, roadside, and both river and stream bankside habitats across Europe. A species within the *Brassica* genus, the extensive phenotypic plasticity of diploid *B. rapa* makes it a model candidate for directional selection, artificial selection and domestication experiments (Tang and Lyons, 2012). UV bullseyes in *B. rapa* have also been well documented (Yoshioka *et al.*, 2005). To test if UV-B is a driver of phenotypic plasticity in a specific floral trait (UV bullseye), we chose the rapid-cycling *B. rapa*, developed by Williams and Hill (1986) via several cycles of selection. Rapid-cycling *B. rapa* plants flower early (18 ± 5 days from planting) and produce ~3-4 buds daily, on inflorescences that produce 20-25 flowers. Rapid-cycling *B. rapa* also produces flowers with four symmetrically arranged yellow petals (in the human visible spectrum) and 6 stamens. The benefits of such flowering characteristics afford substantial morphological and floral data to be collected from relatively few plant individuals. In

addition, *B. rapa* represents a valuable agro-economic resource (Williams and Hill, 1986) and improved knowledge of its floral response(s) to UV-B may inform appropriate use of genotypes and cultivars on a regional to global scale, where UV radiation varies according to both latitude and season (Herman, 2010).

Plant chambers and simulation of UV-B

Three UV intensity treatments were simulated across two (SANYO MLR-351 H) plant chambers: a) control (no UV-B exposure), b) low UV-B exposure, and c) high UV-B exposure. Low UV-B intensity in one chamber was achieved using 15 x36W 6% UV-B bulbs (Fig. 2A) that are typically used in reptile enclosures, and similarly for the high UV-B intensity treatment, a second chamber was fitted with 15 x36W 12% UV-B bulbs (Fig. 2B). Both chambers had a control treatment; custom-built VE grade UV-protecting perspex boxes (L 45 cm x W 40 cm x H 40 cm) with an open top that permitted air flow but restricted UV light transmission (Fig. 2C).

Individual *B. rapa* plants were also exposed to one of two periods of UV-B exposure prior to anthesis: a) long-term UV-B exposure and b) short-term UV-B exposure (Fig. 3). Fifty-four plants were grown from seed within the plant chambers (eighteen plants per UV-B intensity treatment) for the long-term duration of UV-B exposure experiment. The duration of exposure was set at 75 days (Martínez-Lüscher *et al.*, 2013). The seeds were potted in compost and vermiculite (both Verve, UK) in square pots (7 cm x 7 cm x 7 cm), and were randomly assigned to UV-B treatments. All plants were exposed to 12 hr light/12 hr dark cycle, 23°C light/20°C dark cycle and 60% relative humidity within their chambers, and were watered every second day for the duration of the experiment. The control boxes were rotated between chambers fortnightly and plants in the low and high UV-B treatments were rotated and assorted within and across shelves within their assigned chambers.

For the short-term duration of UV-B exposure experiment (Fig. 3), *B. rapa* seeds were potted and grown (soil and pots used as above) in greenhouse conditions prior to chamber growth conditions. Daily Solarmeter® (Model 6.2 Sensitive UV-B Meter) measures indicated the greenhouse UV level at 0 μW/cm² for the duration of growth prior to transfer to the plant chambers. The short duration of UV-B exposure was defined as 25% of UV-B radiation (in days) received by the long exposure plants prior to flowering. Mean day of first flowering in the long-term UV-B exposure experiment was day 24, therefore we estimated that the plants

should receive approximately 6 days of UV-B radiation in the growth chambers before flowering would begin. On day 18 of growth, 51 surviving plants were randomly assigned UV-B treatment groups and transferred to the growth chamber. The chamber conditions and growth parameters were as the above long-term UV-B exposure experiment, and the plants were grown for a further 75 days. The start dates and duration of flowering for all plants were monitored and recorded.

UV radiation was measured in the central point of each treatment shelf weekly. Mean UV-B for the control treatments was 0.27 ± 0.27 μW/cm², mean UV-B for the low intensity chamber was 20.72 ± 1.98 μW/cm², and mean UV-B for the high intensity chamber was 32.38 ± 1.9 μW/cm². UV-B treatment intensities were significantly different ($F_{2,30} = 377.8$, $P < 0.001$), and Tukey multiple pairwise comparisons indicated that all three UV-B intensity treatments significantly differed from one another (all P = 0.000). Tinytag data loggers (Plus 2 TGP-4500) recorded chamber temperature and humidity for the 75 days periods of both UV-B duration experiments.

Flower collection and pressing

All flowers were removed from plants upon opening (pollen was also collected for viability testing, see Chapter 4). The curvature of *B. rapa* petals made it difficult to obtain accurate measures of UVP in preliminary photographic studies. We therefore removed the petals from each flower and pressed them in a flower-press, ensuring that all petals were adaxially presented and evenly flattened. All petals were individually pressed along with a record of the plant they were removed from, UV-B treatment, duration of UV-B exposure, and date of pressing. Koski and Ashman (2013) indicated that this method of drying and pressing specimens to score the size of UV bullseyes does not significantly influence UVP. Several studies have also successfully used pressed herbarium specimens to either categorise or quantify UV floral patterns across several plant families (Horowitz and Cohen, 1972; Eisner *et al*., 1973; Parker, 1975; Scogin *et al*., 1977; Koski and Ashman, 2013; 2016).

UV photography

A total of 726 pressed flowers were photographed in UV light. All photographs were taken with a Canon EOS 400D camera, fitted with a Baader UBVRI 1 1/4" Photometric U-filter. The filter permits the transmission of UV light between 320 nm to 390 nm, peaking with 85% transmission at 350nm, whilst removing infrared and daylight wavelengths. The availability of naturally occurring UV light in the daylight spectrum can be limited indoors; the image

field was therefore illuminated with a UV torch (Convoy S2+ Nichia UV waterproof LED flashlight) with a peak UV emission at 365 nm.

The camera was mounted on a tripod and arm (Mantona, Germany) to achieve a plan view of the pressed specimen. All photographs included a standard scale, and the UV torch was also positioned for a plan view approximately 15 cm above the specimen. For all photographs, the exposure was 10 seconds, and the aperture f/5.6.

Image analysis

The total UV area (UV_{area}, mm^2), total petal area (mm^2), and UVP (%) were measured for 1890 petals where petals had pressed without damage, and focus had not been compromised (Fig. 4). Focus, precision, and clarity often become compromised due to the inherent nature of short UV wave lengths in small floral specimens (Primack, 1982; Arribas, 2012). All area and UVP measures were made in ImageJ (2012, version Fiji, Schindelin *et al.*, 2012) following Yoshioka *et al*. (2005) and Koski and Ashman (2013; 2016). After each individual flower was scaled, colour channels (blue, green, red) were obtained using the 'split' function. Using the 'threshold' tool, the UV_{area} of each petal was outlined and measured in millimetres squared in the red channel, and similarly total petal area was measured in the blue channel. The UVP of each petal was calculated as the UV absorbing area divided by the total area of the petal.

Statistical analysis

Using the statistical package lme4 (Bates *et al.*, 2015) variation in UVP, UV_{area}, and petal area was modelled using a linear mixed-effects model (LMM). To observe the relationship between UVP and its components, UV-B intensity, duration of UV-B exposure, and flowering days (days flowering since the onset of anthesis) were entered as fixed effects into the model. Lilliefors test for normality indicated that the UVP, UV_{area}, and petal area data were not normally distributed, therefore the data was ranked. Data for flowering time were also non-linear, however when the polynomial (second order) of flowering time was entered into the model, variation in UVP, UV_{area}, and petal area was only explained by the linear of flowering time; the polynomial of flowering time was therefore removed from the model. As random effects, intercepts were included for both plant individual and flower individual. A likelihood ratio test compared our model with a model of greater complexity, however the parameters of this model fit our dataset significantly better. LMM modelling was run in R

version 3.4.2 (R Development Core Team and R Core Team, 2017). Predicted changes in UVP and its components as a function of UV-B intensity, UV-B exposure period, and flowering days interactions were also estimated using the "effects" function in R (Fox, 2003).

Results

Ultraviolet proportion

The interaction between UV-B intensity, duration of UV-B exposure, and flowering time indicates that UVP was significantly greater for all plants exposed to long-term periods of growth in UV-B conditions as compared with short-term UV-B exposure (Table 1a; Fig. 5A, B). In the short period of UV-B exposure, and with increasing flowering days, UVP is significantly greater for plants grown in low and high UV-B intensity conditions than control plants (Table 1a; Fig. 5B). The interaction between a long duration of UV-B exposure, UV-B intensity, and flowering days indicates that UVP is greatest in plants grown in high UV-B conditions, however plants from all UV-B intensity treatments decline with time (Fig. 5A). Plants grown in the short-term UV-B exposure experiment follow a different trend however; UVP declines over time in plants grown in high UV-B intensity, whilst control and low UV-B plants maintain a consistent level of UVP in their petals (Fig. 5B). Overall, it is the key differences in the responses of both high and low UV-B intensity plants when grown in either long or short-term UV-B exposure protocols that indicates that UVP is highly plastic.

Ultraviolet-absorbing area

Whilst the interaction between UV-B intensity, duration of UV-B exposure and flowering time did not significantly impact upon UV_{area} in *B. rapa* plants (Table 1b), the interaction between flowering time and UV-B intensity significantly impacts on UV_{area}. Specifically, with increasing flowering time, UV_{area} increased for plants grown in the low UV-B intensity treatment (Table 1b; Fig 6). The inverse of this relationship was true for control and high intensity UV-B plants, despite producing petals with greater UV_{area} than low UV-B intensity plants at the beginning of the flowering period (Fig. 6).

Petal area

The interaction between flowering time and duration of UV-B exposure period was significant for *B. rapa* petals, such that when exposed to UV-B for a short period over increasing flowering days, petal area decreased (Table 1c). Petal area was also significantly affected by UV-B intensity and flowering time interactions (Table 1c). Petal area in high UV-B intensity treatments, although greater than petal area in low UV-B intensity plants, declined over the flowering period (Fig. 7). In contrast, petal area in plants grown in the low UV-B intensity treatment increased over time as a result of UV-B intensity and flowering time interactions (Fig. 7).

Discussion

Our results indicate, for the first time, that UVP in *B. rapa* plants is capable of plastic change in response to UV-B radiation. At the beginning of the flowering period, petal UVP of plants grown in high UV-B intensity conditions is greater than UVP of low and control UV-B intensity plants. Similarly, at the beginning of the flowering period, plants from the high UV-B intensity treatment produce larger petals, and petals with a greater UV_{area}. The fact that length of UV-B exposure, UV-B intensity and flowering days interactions were not significant for UV_{area} and petal area suggests that UVP is more flexible (plastically) by comparison. Critically, the fact that there is a difference between short-term and long-term UV-B exposure for UVP only, highlights that UVP is highly plastic. Although UV-B induced plastic changes in UVP are evident, the UVP declines over the flowering period. For high UV-B intensity plants in particular, declines in UVP perhaps indicate that increases in UV-absorbing pigments are costly, and furthermore costly to maintain with continued UV-B exposure over the flowering period.

Plastic responses of UVP

The findings of the present study support the hypothesis that plants grown in increasingly intense UV-B environments respond plastically by producing petals with greater UVPs. This is not surprising given that in several studies, it has been shown that one of the most effective defensive mechanisms against UV-B-induced stress is the accumulation of a wide range of UV-absorbing phenolic metabolites in the petals of flowers and in other structures such as pollen (Demchik and Day, 1996; Rozema *et al.*, 1997; Jenkins, 2009). Furthermore, our

results in part corroborate previous evidence that UV-B as an abiotic agent acts to select for bullseye size, that in turn modifies the floral UV environment (Koski and Ashman, 2015). Larger bullseyes in artificial flowers have been shown to provide enhanced protection to UV sensitive pollen, as compared with flowers with smaller UV bullseyes (Koski and Ashman, 2015). Our findings, however, suggest that the UVP of *B. rapa* plants is capable of much more dynamic, plastic change, with the potential to ensure maximum reproductive fitness within one generation.

In our study, plants that were exposed to UV-B for a longer duration also produced greater ultraviolet proportions in their petals. It is possible that increased duration of UV-B exposure in general produces greater petal UVP; it may also be the case that exposure early in plant growth (i.e. from seedling stage) may be important for the production of flavonoids that are responsible for the UV-absorbing pigments in floral tissues. Whilst UV-B exposure early in plant development may increase flavonoid accumulation in both vegetative tissues of *B. rapa*, and later in floral tissues, (Berli *et al.*, 2010), it is at present unclear by what mechanism exposure of vegetative tissues to UV-B affects later floral development (Musil and Wand, 1993).

UV_{area} and petal area also appear to be capable of plastic change, however only as the result of UV-B intensity and flowering days interaction. Overall, this suggests that i) UV_{area} area and petal area are less flexible in propensity for plastic change, and ii) plastic changes in UVP were not driven by all parts of the flower changing. Differences in the extent of plastic responses between these three floral components may be due to underlying differences in genetic architecture (Brock *et al.*, 2016). In recombinant inbred lines of *B. rapa*, strong genetic correlations have been found within petal morphological traits (floral size, blade area, blade length : width ratio etc.) and within measures of UV patterning (UV_{area}, UVP, UV length : width ratio etc.), however correlations between these traits are weak or non-significant (Brock *et al.*, 2016). A regulatory gene (QTL 8-2) that determines the proportion of the petal blade that absorbs UV has also been identified (Brock *et al.*, 2016), suggesting that overall, differences in UVP, UV_{area} and petal area in our study probably reflect by individual gene x UV-B intensity interactions associated with these individual components.

Decline in UVP and its components

Significant trends for decline in UVP and UV_{area} over the course of the flowering period suggest there are likely costs associated with the production and continued maintenance of the UV-absorptive pigments underlying UV bullseye patterns. In the long UV-B exposure experiment all plants decline in UVP, and the same is true of high UV-B intensity plants in the short UV-B exposure experiment treatment. Few studies have explored plastic responses of floral tissues to UV-B, and none have measured the phenotypically plastic responses of petal UVP under UV-B radiation. The findings of previous studies investigating the maintenance costs of phenotypic plasticity, however, indicate that production of plant secondary metabolites responsible for UV-screening are costly, leading to subsequent reduced vegetative growth and/or reproduction (Brown, 1988; Briggs, 1990). In *Mimulus aurantiacus* (Phrymaceae) for example, individual plants allocating substantial amounts of carbon to leaf resin-production (UV-screening function on the epidermis of leaves) resulted in slow growth, and reduced carbon allocation to leaf and stem tissues (Han and Lincoln, 1994). Understanding the pattern of resource allocation among several growth/developmental parameters within plants has been a challenge for plant ecologists. Our findings are however consistent with general patterns for decline in resource allocation and furthermore reproductive investment over the flowering period that are frequently observed in floral structures, gametes, and seeds (Ashman and Hitchens, 2000; Kliber and Eckert, 2004). Specifically, the availability of nutrients, light, and water for floral tissues may decline over the flowering period due to increased resource consumption early in the flowering period (Diggle, 1995), or declines in a floral/reproductive trait may reflect direct resource limitation regardless of earlier resource consumption (Wolfe, 1992). For example in plants of *Aquilegia canadensis* (Ranunculaceae), reducing the availability of photosynthate via experimental defoliation strongly decreased overall fruit set and seeds per fruit, and caused a more severe decline in fruit set as compared with control plants (Kliber and Eckert, 2004). Aside from protective physiological processes associated with UV-B radiation, other floral physiological processes may account declines in UVP over the flowering period. For example, in *Trillium grandiflorum* (Melanthiaceae), a substantial 32.7% of floral maintenance cost is due to support of corolla respiration alone (Ashman and Schoen, 1994), and when water is a limiting factor, significant energetic costs have been associated with water and carbohydrate reallocation from vegetative tissues to floral tissues to maintain inflorescences (Nobel, 1977). Respiration and transpiration are clearly costly for both vegetative and floral tissues, and

therefore regulation of these processes under UV-B radiation may account for low nutritional support of UVP, ultimately leading to its decline.

Finally, overall predicted decline in UVP may be magnified by our experimental design. The nature of UV photography and quantification of UVP, UV_{area} and petal area required the removal of flowers upon flower opening. Repeated removal of flowers perhaps introduced an element of simulated herbivory. In addition to UV-B radiation, herbivory typically induces defensive metabolites (Strauss *et al.*, 2004; Valladares *et al.*, 2007). Flower removal in our experiments therefore may be costly for both the degree of continued flavonoid synthesis (i.e. it cannot be sustained), and costly for the carbon, nitrogen, and other nutrients required for repeated floral construction over the flowering period (Charlesworth and Morgan, 1991).

To conclude, the plastic responses of floral UVP demonstrated in this study indicate the potential for plants (of *B. rapa* at least) to persist when faced with environmental UV-B change by enhancing petal UVP. UV-B intensity and UV-B exposure period both significantly increase the ultraviolet absorbing proportion of petals, however this degree of plasticity appears to be costly to support. Nevertheless, these responses may be important for the hypothesised reduction of the fitness consequences associated with global UV-B increase (Koski and Ashman, 2015).

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Tables & Figures

Table 1. LMM model outputs for the relationship between UV-B intensity (control, low, high), UV-B exposure duration (long vs short), flowering days, and a) UVP (%), b) UV_{area} (mm^2), and c) petal area (mm^2) in *B. rapa*. Significant results are marked in bold.

	Effect	Estimate	± SE	t	*P*
a) UVP	Intercept	1584.56	98.81	16.04	**<0.001**
	Short UV-B duration	-589.37	139.40	-4.23	**<0.001**
	Days flowering	-14.75	2.14	-6.89	**<0.001**
	High UV-B	87.55	129.40	0.68	0.500
	Low UV-B	-171.49	123.91	-1.38	0.169
	Short UV-B duration x days flowering	14.82	3.31	4.47	**<0.001**
	Short UV-B duration x high UV-B	83.00	197.88	0.42	0.676
	Short UV-B x low UV-B	248.63	173.41	1.43	0.153
	Days flowering x high UV-B	7.73	3.14	2.46	**0.014**
	Days flowering x low UV-B	10.31	2.89	3.56	**<0.001**
	Short UV-B x days flowering x high UV-B	-24.89	8.22	-3.03	**0.002**
	Short UV-B x days flowering x low UV-B	-10.39	4.67	-2.23	**0.026**
b) UVA	Intercept	1325.42	110.90	11.95	**<0.001**
	Short UV-B duration	-141.56	156.66	-0.90	0.368
	Days flowering	-7.43	2.55	-2.92	**0.004**
	High UV-B	220.81	145.18	1.52	0.131
	Low UV-B	-80.43	140.55	-0.57	0.568
	Short UV-B duration x days flowering	1.00	3.91	0.26	0.799
	Short UV-B duration x high UV-B	-32.66	221.67	-0.15	0.883
	Short UV-B x low UV-B	62.64	196.10	0.32	0.750
	Days flowering x high UV-B	-3.37	3.73	-0.90	0.366
	Days flowering x low UV-B	11.11	3.46	3.21	**0.001**
	Short UV-B x days flowering x high UV-B	-15.67	9.58	-1.64	0.103
	Short UV-B x days flowering x low UV-B	-4.33	5.51	-0.79	0.432
c) PA	Intercept	833.76	117.10	7.12	**<0.001**
	Short UV-B duration	298.49	164.22	1.82	0.072
	Days flowering	5.14	2.55	2.02	**0.044**
	High UV-B	-48.18	153.06	-0.32	0.754

Low UV-B	-116.35	147.59	-0.79	0.432
Short UV-B duration x days flowering	-13.23	3.91	-3.38	**<0.001**
Short UV-B duration x high UV-B	141.75	232.24	0.61	0.543
Short UV-B x low UV-B	-16.23	203.97	-0.08	0.937
Days flowering x high UV-B	-9.15	3.72	-2.46	**0.014**
Days flowering x low UV-B	7.29	3.47	2.10	**0.036**
Short UV-B x days flowering x high UV-B	-2.75	9.64	-0.29	0.775
Short UV-B x days flowering x low UV-B	0.61	5.50	0.11	0.912

PA: petal area

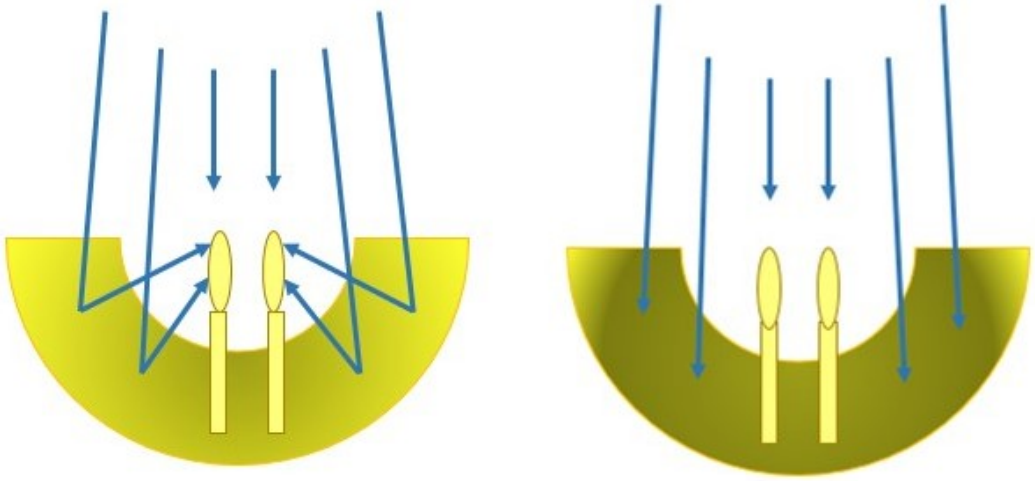

Figure 1. Koski and Ashman (2015) hypothesis for the effect of UV-absorbing bullseye variation on floral micro-environment. Left: flowers with smaller UV-absorbing bullseyes are hypothesised to reflect UV light from the petal tips to the pollen bearing anthers. Right: flowers with larger UV-absorbing bullseyes are thought to absorb UV light over larger areas, decreasing the effect of diffuse reflection onto the pollen-bearing anthers.

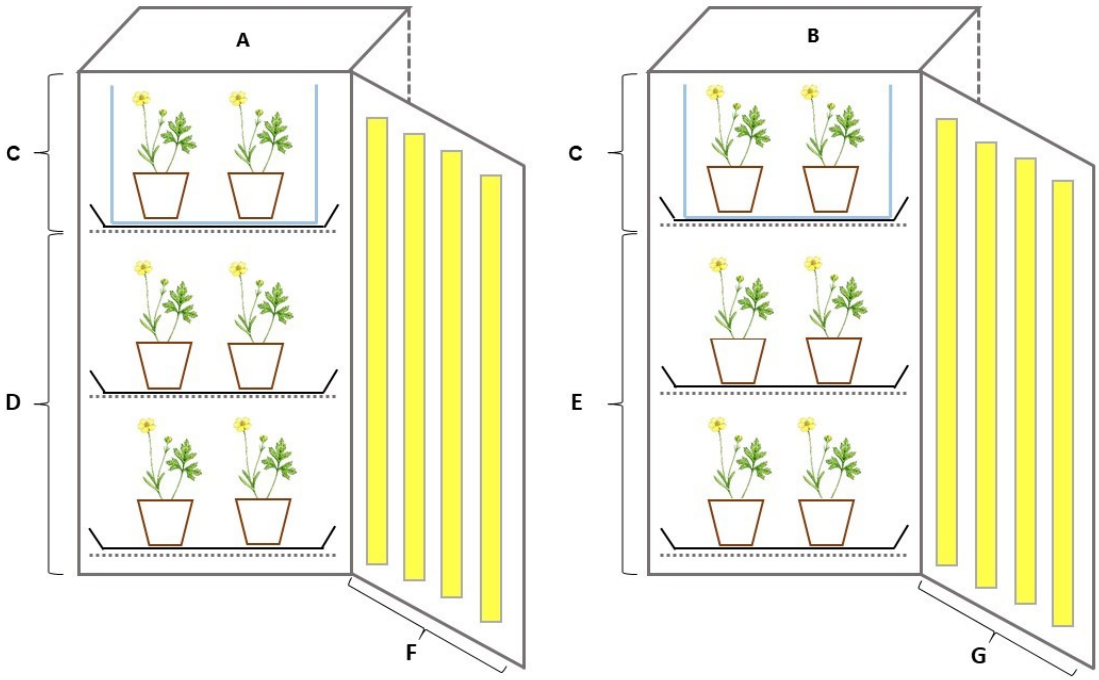

Figure 2. Plant chambers used to simulate UV-B radiation treatments for both long and short exposure experiments. A: low intensity of UV-B chamber; B: high intensity of UV-B chamber; C: control treatments with custom-built VE grade Perspex boxes restricting the transmission of UV-B; D: low UV-B intensity treatment; E: high UV-B intensity treatment; F: 15 36W 6% UV-B bulbs fitted into the front and side panels of the chamber; G: 15 36W 12% UV-B fitted into the front and side panels of the chamber.

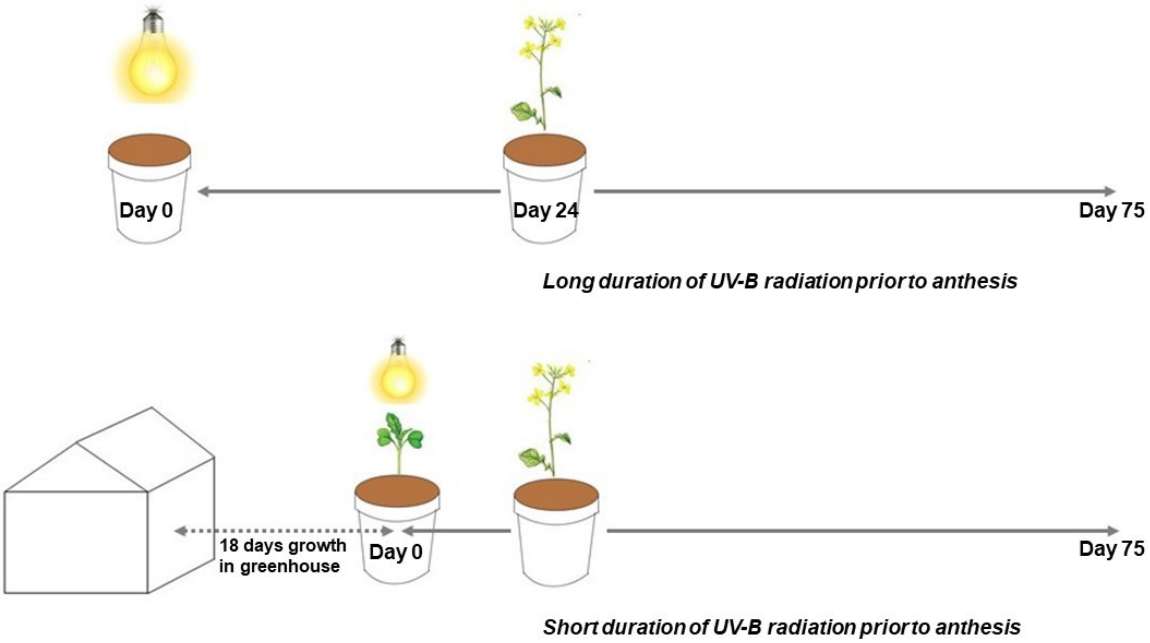

Figure 3. Long and short-term UV-B exposure periods of *Brassica rapa* plants.

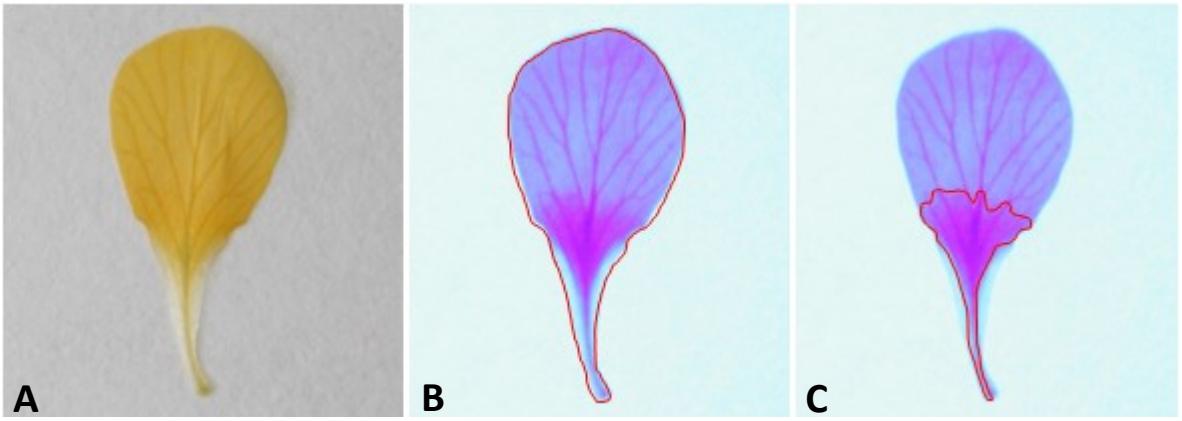

Figure 4. Pressed *Brassica rapa* petal in visible light (A) and UV light (B, C). The UVP (%) of petals is the measure of C) UV_{area} (mm^2) relative to B) total petal area (mm^2).

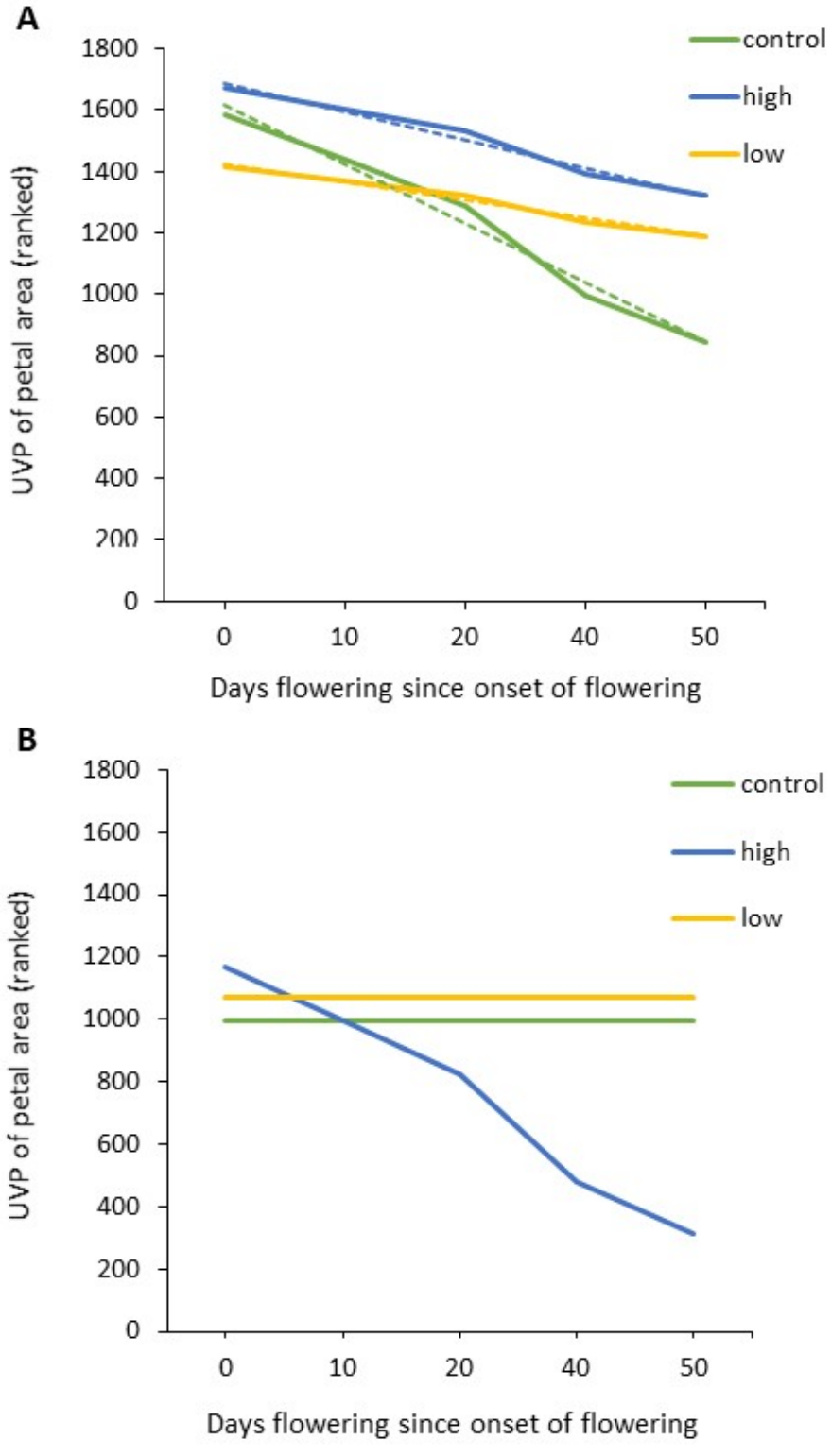

Figure 5. Model effects showing changes in UVP (ranked) over time as a function of the flowering days, UV-B intensity and UV-B exposure period interaction. A: changes in UVP in

the long-term UV-B exposure experiment; B: changes in UVP in the short-term UV-B exposure experiment.

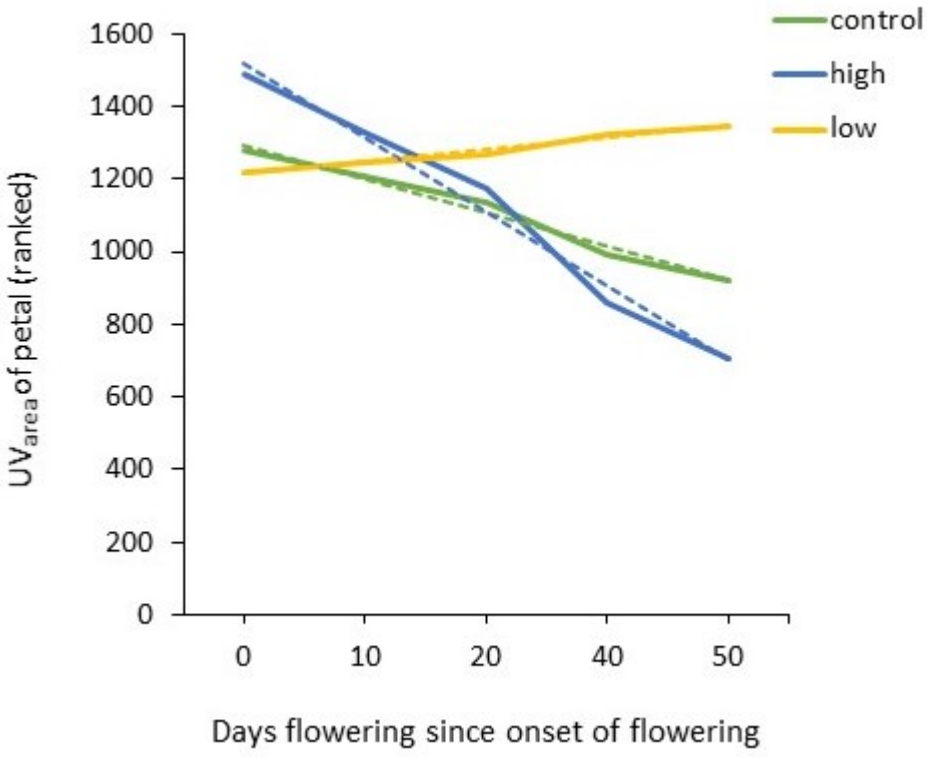

Figure 6. Model effects showing changes in UV_{area} (ranked) as a function of UV-B intensity and flowering days interaction.

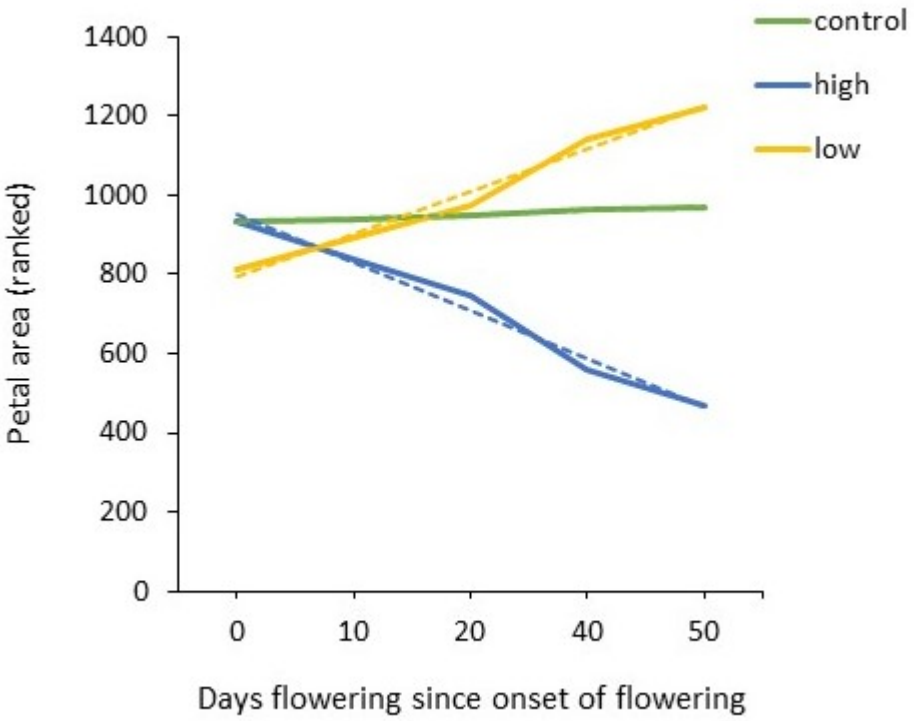

Figure 7. Model effects showing changes in petal area (ranked) over time as a function of UV-B intensity and flowering days interaction.

IV

A MANUSCRIPT WRITTEN IN ACCORDANCE WITH THE GUIDELINES OF THE *JOURNAL OF EXPERIMENTAL BOTANY*.

The impact of ultraviolet radiation on flowering phenology and pollen viability in *Brassica rapa*

UV-B radiation intensity, not the duration of exposure, detrimentally affects pollen viability in *Brassica rapa*.

Abstract

The amount of UV-B reaching the earth's surface has increased over the past 30 years due to ozone reduction, and therefore represents a significant environmental stressor for plants. Many studies have tested the effect of UV-B dose, or intensity, on plant phenological traits such as the timing of flowering, or reproductive traits such as pollen viability. The interactive effect of UV-B intensity and UV-B exposure period on plants grown in UV-B conditions, however, has not yet been tested. We exposed *Brassica rapa* plants to either high, low, or control levels of UV-B radiation, under two different exposure protocols: short-term or long-term exposure, and subsequently measured phenological responses and pollen viability. As predicted, increasing UV-B intensity had a detrimental effect on pollen viability, and observed trends for decline in pollen viability over the flowering period may reflect plant-age effects. High UV-B intensity significantly delayed flowering in *B. rapa* and reduced overall flowering duration. Plants grown in the shorter period of UV-B exposure flowered significantly later, for a reduced number of days, and produced ~ 80% less flowers than plants of the long UV-B exposure experiment. Our findings indicate that plant-age and the degree of UV-B exposure prior to anthesis are significant determinants of male fitness and suggest that further experiments should aim to understand how UV-B exposure at seed-stage growth influences both the timing of phenological events and reproductive success.

Key words: *Brassica rapa,* flowering, growth, male fitness, pollen, pollen viability, ultraviolet, UV-B exposure.

Introduction

Over the past 50 years, increased stratospheric ozone depletion has led to increase levels of UV-B (280-315 nm) radiation reaching the earth's surface (Lubin and Jensen, 1995). An extensive body of research into the effects of raised UV-B on plant physiological and developmental processes has followed. Early studies quantified UV-B as a significant environmental stressor for higher plants (Caldwell, 1968; Caldwell *et al.*, 1989; Tevini and Teramura, 1989), influencing plant morphology, photosynthesis, metabolism, and physiology

of both wild and cultivated plants (Teramura, 1983; Tevini and Teramura, 1989). Although mainly focussed on vegetative aspects of plant growth and responses to heightened UV-B, some studies were important for understanding the potential of plants to adapt to continued increases in surface-level UV-B radiation. In Ziska *et al*. (1992) for example, plants grown from seeds collected along an elevational UV-B gradient differed in their growth and metabolic responses when subjected to laboratory simulated levels of UV-B. Usually, plants grown from seeds originating from higher elevations, and therefore subject to greater UV radiation, reproduce earlier, have increased photosynthetic rates, and overall biomass accumulation than plants sourced from low elevations, when grown in high UV-B conditions (Blumthaler and Ambach, 1990; Herman, 2010). High elevation plants appear to be already adapted to high UV-B conditions therefore, and no increases in flavonoid compounds (UV-absorbing compound that accumulates in plants tissues in response to UV radiation) were detected for these plants grown in high UV-B conditions. In contrast, overall reduced growth (i.e. reduced plant and floral dry biomass) and substantial increases in flavonoids present in plants from low elevations suggested a lack of adaptation to high UV-B exposure. These results, although only true for eight species sampled, were highly suggestive of ecotypic differentiation, demonstrating the ability of naturally occurring plant populations to adapt to altitudinal variations in UV-B radiation.

The impact of UV-B on reproductive plant parts, and in particular, flowering time and pollen viability has been studied for a long time after realising that pollen walls may transmit up to 20% of UV-B (Sadler and Uber, 1942), with UV-B significantly reducing *in vitro* pollen germination (Chang and Campbell, 1976; Pfahler, 1981; Flint and Caldwell, 1984), in addition to the negative effect of UV-B on flower production (Caldwell, 1968; Ziska *et al.*, 1992). In contrast, the female reproductive systems of plants have been hypothesised to be well-protected against UV radiation (Caldwell *et al.*, 1983; Flint and Caldwell, 1984) and to protect pollen once it has penetrated the stigmatal surface. In *Hesperis matronalis* (Brassicaceae) for example, only 2% of UV-B radiation passes though the epidermis of the stigma, considerably lower than UV-B transmittance through foliage of the same species (Flint and Caldwell, 1986; Day and Demchik, 1996). In contrast, male plant fitness stands to be more vulnerable, given the varying extents to which pollen is protected against UV radiation. For example the walls of pollen have been found to attenuate only 80% of UV-B radiation in *Zea mays* (Uber, 1939) and are therefore considered to be much more vulnerable to UV radiation than the gynoecium.

UV-B can also indirectly compromise pollen viability when plants have been exposed to UV-B *prior* to anthesis (Musil and Wand, 1993), although the exact mechanism for this reduction in pollen performance is not comprehensively understood. The extent to which pollen grains are vulnerable to UV-B damage *post* anthesis however, is well documented. Highly variable depending on pollen type (binucleate or trinucleate; Flint and Caldwell, 1986; Torabinejad *et al.*, 1998) and species, the time between anther dehiscence and penetration of the pollen tube into the stigmatal surface exposes pollen grains to direct, naturally occurring UV-B (Feng *et al.*, 2000). Torabinejad *et al*. (1998) found that for more than 50% of 34 taxa examined, UV-B negatively impacted pollen tube growth, furthermore inhibiting pollen germination in five species.

Highlighted also by this study, was the important distinction between pollen type, and its relationship between pollen viability, pollen tube growth and germination success. Binucleate pollen is thought to be physiologically and developmentally less advanced than trinucleate pollen (Flint and Caldwell, 1986), however in Torabinejad *et al*. (1998) 70% of trinucleate species tested experienced detrimental pollen damage. By comparison, 60% of the binucleate pollen species indicated signs of significant UV-B damage, i.e. pollen tube length reduction or low germination success. Whilst the suggestion that slower pollen-tube development exposes binucleate pollen to UV-B radiation for longer than pollen of trinucleate species, some studies have found that binucleate pollen grains can remain viable for longer (reviewed in Brewbaker, 1967; Lora *et al.*, 2009). This is potentially due to the presence of more flavonoids in the pollen exine (Wiermann and Vieth, 1983).

UV-B-induced changes in flowering phenology have been noted in several studies; for example in Ziska *et al*., (1992) plants grown in simulated high UVB treatments, from wild seeds growing at high elevations, were found to have greater overall biomass invested in flowers. This was true for *Oenothera* (Onagraceae)*, Plantago* (Plantaginaceae) and *Hypocheris* (Asteraceae) plants, and reflected earlier reproductive development, earlier flowering (*Oenothera* and *Plantago* flowered 11 and 13 days earlier than control plants), increased flower production per plant, and overall increased reproductive effort. In contrast, plants from low elevations and grown in high UVB conditions produced less flowers, and were unable to shift flowering time in response to perceived changes in the light environment. Earlier anthesis might be advantageous to avoid increasing insolation as the flowering season progresses (Torabinejad *et al.*, 1998; Marshall *et al.*, 2010*b*), and conversely, some plants species may also delay bloom in direct response to increased levels

of UV-B. For example in Sampson and Cane (1999) increased UV-B dosage delayed flowering time of *Phacelia campanularia* (Boraginaceae) by 1.5 days for every 1 $kJ \cdot m^{-2} \cdot d^{-1}$ incremental increase in UV-B dosage. In addition, the total lifetime flower production per plant was reduced by 18 flowers. Whilst this may be a dynamic and short-term strategy to avoid damage during the reproductive stage of the life-cycle, the authors highlighted the detrimental significance of these delayed blooms for potential desynchronisation between pollen and nectar production, and therefore between plants and plant pollinators such as bees. More specifically, delayed or reduced flower production may impact the carrying capacity and nest-cell prevision of bee pollinators, and on a more general scale interrupt important synchronized plant-pollinator services (Sampson and Cane, 1999). Ultimately, such trends between raised UV-B and decreased flower production logically lead to reduced whole-plant pollen production, and therefore overall lower reproductive success (Demchik and Day, 1996).

Finally, pollen viability and longevity can also change as flowers age. For example, in *Gentiana pneumanthe* (Gentianaceae), pollen viability (measured as a percentage of pollen grains germinated) was greatest at two days following anthesis, however sharply declined 4-5 days after this (Petiandou *et al.*, 2001). By day nine post-anthesis, 0% of the pollen was measured as viable. Similar results have also been obtained from other species whereby pollen viability has appeared to be time-dependent (Smith-Huerta and Vasek, 1984; Aizen and Rovere, 1995; Proctor, 1998). The challenge this presents when assessing the degree to which elevated UV-B is responsible for either positive or negative changes in pollen viability and floral phenology, is that there are likely several interacting factors contributing to the physiological and architectural changes of a plant throughout its life cycle (Marshall *et al.*, 2010*b*). At any given point abiotic factors may influence short term changes in plant physiology and phenology, and equally, longer-term changes in floral characteristics, such as the quality of pollen, may also occur as a plant ages over a flowering season. In both cases, UV-B-induced variations in pollen production and viability on both a whole-plant and individual-flower basis implicate reproductive success (Demchik and Day, 1996).

To our knowledge, the simultaneous effects of UV radiation intensity and length of exposure prior to anthesis on pollen viability have been never explored before. Ultimately, to predict how plant populations will respond to further ozone depletion, and therefore fluctuations in atmospheric UV-B, we need to understand the effects of these interacting factors on plant fitness. In this study, we examine the influence of simulated UV-B on the i)

flowering characteristics and ii) pollen viability of *Brassica rapa* plants. We grew *B. rapa* plants under three UV-B treatments (control, low, and high), and over two UV exposure protocols (long exposure vs short exposure). Specifically, we examined whether UV-B level during growth influenced *in vitro* pollen viability, flower production, flowering duration, and onset of flowering.

Methods

Plant material

Extensively cultivated, *B. rapa* is distributed globally, and found on waste ground, roadside, and both river and stream bankside habitats across Europe. A species within the *Brassica* genus, *B. rapa* (in addition to several other species) is known as a "morphotype" because it has an incredible capacity for leaf and floral diversity (Tang and Lyons, 2012). Furthermore, the extensive phenotypic plasticity of diploid *B. rapa* makes it a model candidate for directional selection, artificial selection and domestication experiments (Tang and Lyons, 2012). UV bullseyes (floral patterns visible in UV light when petal bases are UV-absorbing and petal apices are UV-reflecting (Guldberg and Atsatt, 1975; Harborne and Nash, 1984)) in *B. rapa* have also been well documented. Yoshioka *et al*. (2005) photographed cultivars across 8 varietal groups of *B. rapa* in UV light, demonstrating that they exhibit significant intraspecific variation in the ultraviolet proportion of their flowers. In this study, we chose the rapid-cycling *B. rapa* (Fig. 1) developed by Williams and Hill (1986) via several cycles of selection. Rapid-cycling *B. rapa* plants flower early (18 ± 5 days from planting) and produce ~3-4 buds daily, on inflorescences that produce 20-25 flowers. The benefits of such flowering characteristics afford substantial morphological and floral data to be collected from relatively few plant individuals. In addition, *B. rapa* represents a valuable agro-economic resource (Williams and Hill, 1986) and improved knowledge of its floral response(s) to UV-B may inform appropriate use of genotypes and cultivars on a regional to global scale, where UV radiation varies according to both latitude and season (Herman, 2010).

Plant chambers and simulation of UV-B

Three UV intensity treatments were simulated across two (SANYO MLR-351 H) plant chambers: a) control (no UV-B exposure), b) low UV-B exposure, and c) high UV-B exposure. Low UV-B intensity in one chamber was achieved using 15 x36W 6% UV-B bulbs

(Fig. 2A) that are typically used in reptile enclosures, and similarly for the high UV-B intensity treatment, a second chamber was fitted with 15 x36W 12% UV-B bulbs (Fig. 2B). Both chambers had a control treatment; custom-built VE grade UV-protecting perspex boxes (L 45 cm x W 40 cm x H 40 cm) with an open top that permitted air flow but restricted UV light transmission (Fig. 2C).

Individual *B.rapa* plants were also exposed to one of two periods of UV-B exposure prior to anthesis: a) long-term UV-B exposure and b) short-term UV-B exposure (Fig. 3). Fifty-four plants were grown from seed within the plant chambers (eighteen plants per UV-B intensity treatment) for the long-term duration of UV-B exposure experiment. The duration of exposure was set at 75 days (Martínez-Lüscher *et al*., 2013). The seeds were potted in compost and vermiculite (both Verve) in square pots (L 7 cm x W 7 cm), and were randomly assigned to UV-B treatments. All plants were exposed to 12 hr light/12 hr dark cycle, 23°C light/20°C dark cycle and 60% humidity within their chambers, and were watered every second day for the duration of the experiment. The control boxes were rotated between chambers fortnightly and plants in the low and high UV-B treatments were rotated and assorted within and across shelves within their assigned chambers.

For the short-term duration of UV-B exposure experiment (Fig. 3), *B. rapa* seeds were potted and grown (soil and pots used as above) in greenhouse conditions prior to chamber growth conditions. Daily Solarmeter® (Model 6.2 Sensitive UV-B Meter) measures indicated the greenhouse UV level at 0 μW/cm² for the duration of growth prior to transfer to the plant chambers. The short duration of UV-B exposure was defined as 25% of UV-B radiation (in days) received by the long exposure plants prior to flowering. Mean day of first flowering in the long-term UV-B exposure experiment was day 24, therefore we estimated that the plants should receive approximately 6 days of UV-B radiation in the growth chambers before flowering would begin. On day 18 of growth, 51 surviving plants were randomly assigned UV-B treatment groups and transferred to the growth chamber. The chamber conditions and growth parameters were as the above long-term UV-B exposure experiment, and the plants were grown for a further 75 days. The start dates and duration of flowering for all plants were monitored and recorded.

UV radiation was measured in the central point of each treatment shelf weekly. Mean UV-B for the control treatments was 0.27 ± 0.27 μW/cm², mean UV-B for the low intensity chamber was 20.72 ± 1.98 μW/cm², and mean UV-B for the high intensity chamber was

32.38 ± 1.9 μW/cm². UV-B treatment intensities were significantly different ($F_{2,30}$= 377.8, P < 0.001, and Tukey multiple pairwise comparisons indicated that all three UV-B intensity treatments significantly differed from one another (all P = 0.000). Tinytag data loggers (Plus 2 TGP-4500) recorded chamber temperature and humidity for the 75 day periods of both UV-B duration experiments.

Pollen collection

Pollen was collected and tested for viability from all flowering *B. rapa* plants grown in the plant chamber experiments. Pollen staining for viability began from the onset of anthesis in each plant, until the end of flowering (or until the plant reached the end of the experiment at day 75, if still flowering). Pollen was tested shortly following anther dehiscence because both pollen collection and storage method can detrimentally affect pollen viability, and viability rapidly deteriorates following anthesis in most plant species (Kearns and Inouye, 1993).

A maximum of three flowers per plant were indiscriminately selected, and two anthers removed per flower for staining (the corresponding flower petals were pressed for the measure of ultraviolet colour proportion, see Chapter 3). Pollen grains from control plants were also tested for viability to account for differences over the length of the study period, i.e. viability can be plant age-dependent (Wang *et al.*, 2010).

Pollen viability test

Aniline blue lactophenol stain (Sigma-Aldrich; 5 mL 1% aqueous aniline blue, 20 mL phenol, 20 mL lactic acid, 40 mL glycerine, 20 mL water) was used to stain fresh pollen grains. Aniline blue is commonly used to detect callose in pollen walls and tubes, staining viable pollen grains blue whilst sterile grains stain faintly or not at all (Kearns and Inouye, 1993). The pollen grains of each anther were added to microscope slides containing one drop of water. One drop of aniline blue solution was then added to the pollen grains on the slide. A coverslip was placed over the stained pollen grains and samples were observed under a light microscope. At x100 magnification, 100 pollen grains were counted per anther. Pollen viability (%) was calculated as:

$$\frac{\text{No. of stained pollen grains}}{\text{No. of counted pollen grains}} \text{ X } 100$$

Statistical analysis

UV and flowering characteristics

To gain an understanding of how UV impacts phenology, flowering pattern measures were taken. Two-way ANOVA was used to determine i) onset of flowering following exposure to UV-B (day of first flowering since planting), ii) the mean number of flowers produced per plant, and iii) flowering duration (days). In each model, the categorical variables were UV-B treatment (control, low, high), duration of UV-B exposure (long vs short) and their interaction. For each treatment and exposure period, the proportion (%) of flowers that did not produce pollen was also calculated. ANOVAs were run in R version 3.4.2 (R Development Core Team and R Core Team, 2018).

UV and male fitness

Pollen viability following exposure to experimentally modified levels of UV was used as a component of male fitness. Using the statistical package lme4 (Bates *et al.*, 2015) variation in pollen viability was modelled using a linear mixed-effect model (LMM). To observe the relationship between UV exposure and male fitness, UV treatment, UV exposure length, and flowering time (days flowering since onset of anthesis) were entered as fixed effects into the model. Lilliefors test for normality highlighted that the viability data was not normally distributed, therefore the data was ranked. Non-linear flowering responses have been reported in many studies, potentially highlighting a threshold in the degree to which plants can adjust their flowering times in accordance with abiotic factors (Inouye, 2008; Iler *et al*., 2013). Flowering time in our dataset followed a non-linear trend; the polynomial (second order) of flowering time was therefore also entered into the model. As random effects, intercepts were included for both plant individual and flower individual, as well as a by-plant individual random slope for flowering time. LMM modelling was run in R version 3.4.2 (R Development Core Team and R Core Team, 2018). The rationale for including both plant individual and flower as random effects was to account for possible differences in pollen

viability among individuals. In Oni (1990) for example, the collection of 60 flowers from three *Triplochiton scleroxylon* trees demonstrated significant variation in pollen viability between trees, and additionally between flowers from all three trees. We used the "effects" function in R (Fox, 2003) to predict significant changes or differences in pollen viability as a function of factor interactions that were highlighted as significant in the LMM.

Results

UV-B and flowering characteristics

Onset of flowering

In the long-term UV-B exposure experiment, plants from the control treatment began flowering significantly earlier than plants from the low and high intensity UV-B treatments (Fig. 3; Table 1a). All plants in the short UV-B exposure experiments began flowering significantly later than plants exposed to a long period of UV-B radiation prior to anthesis (Fig. 3; Table 1a). Whilst both UV-B intensity and duration of UV-B exposure significantly affected the timing of flowering, an interaction between these factors did not significantly impact the onset of flowering (Table 1a). Post hoc Tukey tests indicated that differences in flowering time were significant between the high and control treatment ($P = 0.029$).

Flowering duration

Flowering duration for the plants from the high treatment was considerably shorter than plants of the low and control treatments, with a mean flowering duration of 8 days in the short exposure period (Fig. 4). Control plants flowered for approximately 6 days longer and 11 days longer than plants of the low and high treatment, respectively (Fig. 4). A two-way ANOVA indicated that both the length of UV exposure and UV treatment significantly affected flowering duration, however their interaction did not affect flowering duration (Table 1b.). A post hoc Tukey test showed that flowering duration differed significantly between high and control UV treatments ($P = 0.032$).

Number of flowers produced

Plants grown in the long period of UV-B exposure produced the greatest number of flowers per plant (Table 1c; Table 2), overall producing ~ 80% more flowers than plants from the short duration of UV-B exposure experiment. Differences in the numbers of flowers between

UV-B intensity treatments did not prove statistically significant, and the interaction between UV-B intensity and duration of UV-B exposure did not significantly affect the number of flowers produced per plant (Table 1c). The highest proportion of flowers that did not produce pollen were from plants grown in the low UV-B intensity treatments (Table 2), whilst plants from all treatments in the short duration of UV-B exposure experiment produced a greater proportion of flowers with no pollen than plants exposed to UV-B for a longer period.

UV and male fitness

Mean pollen viability was greatest in control plants across both UV-B exposure periods (Fig. 5). Plants from the high intensity UV-B treatment produced pollen with the lowest mean viability, with pollen viability being lowest in the short duration of UV-B exposure experiment (Fig. 5). LMM results indicated that exposure to both high and low UV-B intensities had a significantly negative effect on pollen viability (Table 3).

The polynomial of days flowering since the onset of anthesis also had a significantly negative effect on pollen viability (i.e. pollen viability decreased with increasing flowering time of the plants, Table 3; Fig. 6). Whilst the period of exposure to UV-B prior to anthesis did not have a significant effect on pollen viability, the interaction between the high UV-B intensity treatment and the polynomial of days flowering since anthesis significantly affected pollen viability (Table 3). Specifically, the trend for increasing pollen viability over flowering time in high UV-B intensity plants was the inverse of the relationship between flowering days and UV-B intensity in low and control plants (Table 3; Fig. 6).

Overall, flowers from the control treatments consistently produced pollen with the greatest mean viability across all but the last (50 days following anthesis) time intervals following the onset of anthesis (Fig. 6). Mean ranked pollen viability was greatest for control and low UV-B intensity plants 20 days after the beginning of anthesis, before declining thereafter (Fig. 6). Plants from the high intensity of UV-B treatment however peaked in pollen viability 50 days following the onset of flowering (Fig.6)

Discussion

Flowering characteristics

UV-B radiation proved to be a significant determinant of all flowering parameters measured. Such results are consistent with previous findings, where simulated levels of UV-B radiation have had detrimental effects on flowering phenology, but also fitness components such as the number of flowers, seeds, and fruit, and overall reproductive output (Ziska *et al.*, 1992; Feldheim and Conner, 1996; Mark and Tevini, 1997; Sampson and Cane, 1999; T. *et al.*, 2001; Koti *et al.*, 2004; Wang *et al.*, 2008).

In our study UV-B intensity consistently delayed the onset of flowering post-UV-B exposure, with mean first day of flowering occurring the latest in the high intensity of UV-B treatment. Similar results were found in two close species (*B. rapa* and *B. nigra*) by Feldheim and Conner (1996). In two treatments (12 kJ m^{-2} UV-$B_{be\ 300}$ and 17 kJ m^{-2} UV-$B_{be\ 300}$) representing 30% and 45% ozone layer depletion, onset of flowering was significantly increased in both species when grown in UV-B conditions above ambient-level. Whilst alternative hypotheses for shifts in floral phenology suggest that earlier anthesis may be a strategic avoidance of perceived changes in the light environment, and therefore of UV-B damage earlier in the reproductive process (Torabinejad *et al.*, 1998; Marshall *et al.*, 2010*a*), results of delayed bloom do not necessarily signal overall reduced plant reproductive performance. For example in Feldheim and Conner (1996) and Sampson and Cane (1999), total flower production was found to significantly decrease for plants grown in a high UV-B environment, following delayed bloom. One interpretation of delayed flowering could be that UV-B induced a direct stress response, however, further measures of plant fitness in Sampson and Cane (1999) found that seed production increased in both *Brassica* species in high UV-B treatments. Similarly in Feldheim and Conner (1996), delayed bloom and reduced total flower production in *B. rapa* was offset, or compensated for, by increased seed production, and in *B. nigra* flower production increased in the later stages of high UV-B exposure, despite an overall reduced flowering duration. Measures of seed production in our study would have perhaps indicated the extent to which UV-B influences long-term floral phenology and overall reproductive fitness. I.e. if overall seed production between control, low and high UV-B intensity plants were comparable, then UV-B prompted delays in the onset of flowering could function as a regulatory mechanism as opposed to merely signalling UV-B induced stress (Nord and Lynch, 2008; Comont *et al*., 2012).

Although not statistically significant in this experiment, differences in overall flower production between treatments can reflect variations in UV-B that can either provide beneficial or harmful levels of UV-B. In other studies, flower production increased following

exposure to UV-B levels of above-ambient UV-B (Musil and Wand, 1994; Demchik and Day, 1996; Phoenix *et al.*, 2001), and potentially represents an approach toward an optimal or beneficial level of UV-B for flower production. Whilst evidence of a beneficial UV-B radiation threshold for flowering phenology – and specifically flower production – is lacking, from a broad regulatory perspective, plant growth in natural light containing UV-B does not just entail macromolecular damage and tissue damage. For examples in Ziska (1992) low elevation plants, when grown in high UV-B conditions, increased up- regulation of flavonoids, whereas high elevation plants consistently maintained higher levels of flavonoid compounds when grown in high UV-B-absent conditions. The authors suggest that the maintenance of such high UV-absorbing compounds (even in UV-B absent environments) is indicative of beneficial mechanisms developed to maximise overall productivity when naturally occurring variations of surface-level UV-B increase. In support, UV-B is known to stimulate the expression of genes responsible for: UV protection and repair (increasing survival in environments where light intensity, and therefore the UV-B proportion of light, is naturally variable, Jenkins, 2009), to modify plant biochemical composition (Brosché and Strid, 2003; Frohnmeyer, 2003), and influence plant morphology, such that it deters pathogens and pests (Caldwell *et al.*, 2007; McKenzie *et al.*, 2007). Thus, UV-B promotes the synthesis of secondary metabolites (UV-protective flavonoids, Rozema *et al.*, 1997), and these are thought to deter herbivory (Izaguirre *et al.*, 2007). Overall, this "induced acclimation" – responses to non-damaging levels of UV-B - is key for the regulation of developmental responses in higher plants (Jenkins, 2009).

As with flowering time, flowering duration was also shifted significantly by UV-B treatment. In high UV-B conditions, and in particular in the short UV-B exposure experiment, *B. rapa* flowered on average 8 days less than plants of the control treatment. Comparable reductions in flowering duration can be found in Feldheim and Conner (1996) and Wang (2008). Perhaps most notable however, and evident for all phenological measures in this study, is the impact of UV-B exposure period prior to anthesis. Few studies have investigated the effect of differing UV-B exposure period regimes on plants, and none have investigated its impact on the floral or reproductive characteristics of plants. In Martinez-Luscher *et al*. (2009) for example, grapevine (*Vitis vinifera,* Vitaceae) plants were exposed to two levels of UV-B (5.98 and 9.66 kJ m^{-2} d^{-1}), and two lengths of exposure period (20 days of growth under UV-B radiation, and 75 days of growth), and their photosynthetic and biochemical responses were monitored. Comparable with the present study, the short-term" UV-B

treatment exposed grapevine plants to UV-B later in development at mid grape-ripening or "veraison". The "long-term" UV-B treatment irradiated grapevine plants from the fruit set through to ripening. The study found that the most significant effects on photosynthesis and biochemical responses (such as oxidative stress in the vegetative parts of the plants) occurred after short-term exposure to both doses of UV-B. Antioxidant enzyme production and activity increased, and net photosynthesis, stomatal conductance, sub-stomatal CO_2 concentration, and photosystem II efficiency decreased. By comparison, plants in the 75 day UV-B experiment experienced no photosynthetic inefficiency, no oxidative damage in leaves, and deviations in biochemical activity. It was suggested that such marked differences in responses under differing exposure periods were due to the ability of long-term plants to develop efficient protective mechanisms; we suggest that this may also account for the marked difference in flowering time between our two UV-B exposure experiments.

Despite the potential for chamber experiments to overestimate the effect of UV-B on the phenological parameters we measured, longer flowering times, earlier onset of flowering, and greater flower production in the long exposure period suggest a long-term acclimation capacity of *B. rapa* to both high and low UV-B levels. For example, long-term UV-B exposure from seed-stage growth may have been important for the production of secondary metabolites such as flavonoids, which accumulate in the vegetative tissues of plants, enabling overall long-term UV-B-acclimation (Kakani *et al.*, 2003; Berli *et al.*, 2010; Wargent and Jordan, 2013). Whilst we currently lack an understanding of how early exposure of vegetative plant tissues during development to UV-B affects later floral/reproductive phenology and fitness, and indeed what the most significant stages of growth development might be, it is evident that UV-B exposure prior to anthesis impacts upon the outcome of flowering time, duration, flower production, and even in some cases pollen viability (Musil and Wand, 1993).

Pollen viability

UV-B treatment and flowering days since initial onset of anthesis appear to be significant factors determining the viability of pollen in *B. rapa*. Several studies have reported similar findings whereby exposure to simulated above ambient-level UV-B has reduced *in vitro* pollen viability specifically in the *Brassica* genus (Demchik and Day, 1996; Feldheim and Conner, 1996; Torabinejad *et al.*, 1998; Feng *et al.*, 2000; Marshall *et al.*, 2010*a*). As expected, in our experiments pollen viability in both low and high UV-B treatments was significantly decreased in comparison with pollen collected from plants grown in the control

treatments. Despite suggestions that species such as *B. rapa* -with trinucleate pollen- suffer decreased UV-B-induced pollen damage as compared with binucleate species, decreased viability in our study is consistent with Torabinejad *et al*. (1998), who found for 34 taxa investigated, 70% of trinucleate pollen species experienced significant UV-B-induced reductions in pollen viability. Interestingly, whereas UV-B exposure period prior to anthesis determined all phenological flowering characteristics of *B. rapa* in our experiments, UV-B exposure length was not a significant predictor of variations in pollen viability. Musil and Wand (1993) found that in species of *Erica* (Ericaceae), UV-B radiation detrimentally affected pollen germination and tube length growth *prior* to anthesis although the mechanism for such results were not fully understood. The vegetative and floral tissues (e.g. the closed perianth just prior to flower opening) of *B. rapa* are perhaps better at screening the reproductive organs from harmful UV-B than species of *Erica,* and the biosynthesis of UV-B-screening flavonoids and phenolic compounds may have been upregulated regardless of UV-B exposure period before anthesis. In *B. rapa*, for example, the potential prolonged flavonoid upregulation and production in the long UV-B exposure period (as compared with the short UV-B exposure period) might not have been costly for pollen production and viability, but rather costly for phenological characteristics such as the above flowering duration or total flower production.

Flowering days, or more broadly, "plant age" was also a significant predictor of pollen viability in our experiments. With increasing flowering days over both the long and short UV-B exposure periods, pollen viability decreased. The reasons for declines in pollen viability in *B. rapa* are unclear. Previous studies have neglected this aspect when investigating longevity and pollen viability; pollen grains are typically measured *in vitro,* and their decline (or lack of) in viability is measured with respect to time spent exposed to a given abiotic condition once removed from the anther. Other studies measure pollen both *in vivo* or *in vitro*, but again focus rather on the change in viability of pollen from a given flower or plant, in incremental time periods (i.e. hours or days) following anthesis (Smith-Huerta and Vasek, 1984; Proctor, 1998; Marshall *et al.*, 2010). In the present study, we removed pollen throughout the flowering period and measured viability upon removal. In both control and low UVB treatments, predicted mean viability (as a function of UV-B treatment and flowering time interaction) indicated that viability increased in the early stages of plant age/flowering age, before declining after a peak between days 15 to 20. Similar declines in viability, specifically pollen abortion rates, have been observed in *Austrocedrus chilensis*

(Cupressaceae), a wind-pollinated tree native to the temperate forests of South America (Aizen and Rovere, 1995). The authors found that the proportion of aborted pollen grains steadily increased with tree age, and size, and suggested that such declines were the result of accumulating somatic mutations with age. Petiandou *et al*. (2001) similarly found declines in pollen viability in flowers of *Gentiana pneumonanthe* (Gentianaceae). Grown in greenhouse environments, collected pollen of *G. pneumonanthe* plants indicated that pollen viability was greatest approximately within 2 days following anthesis, after which viability rapidly declined (by approximately days 4-5 post-anthesis). The explanation for such a rapid fall in viability suggested that declines in viability may be an adaptation to increase the level of outcrossing within a population. They argued that maximising fertilization by freshly transferred pollen from flowers in the male stage of other plants (when it is at its most germinable) will discourage self-fertilisation. Pollen that remains in the flower until the stigma becomes receptive would probably compete less successfully with the fresh pollen imported from flowers in the male stage. Although possible, the success of this process would rely on the ability of pollinators to effectively transfer pollen within a very narrow timeframe where pollen is at its most viable.

Unusually in our experiment, predicted mean viability for plants in the high UV-B treatment increased with increasing flowering time. The reason for this trend is unclear. It is unlikely that over the course of both long and short UV-B exposure periods, plants in the high treatment would be able to achieve acclimation to high UV-B levels without prior genotypic acclimation. In such cases, prior acclimation would typically function to decrease the magnitude of stress/damage response, as opposed to directly benefitting a floral characteristic that typically declines under UV-B-induced stress. Specifically, we would expect acclimation to high UV-B to reduce the extent of UV damage to pollen, as opposed to promoting increased viability.

In sum, the findings of this study highlight the importance of UV-B exposure period prior to anthesis as a key determinant of phenological and floral characteristics such as flowering duration, total flower production, and timing of anthesis. As expected, UV-B had a detrimental effect on pollen viability, however the extent to which viability declines may depend on several other interacting factors such as plant age. The precise relationship between UV-B radiation and plant-age is at present unknown, however further research in this area should aim to determine if plant age and UV-B radiation interact to cumulatively deteriorate pollen viability, or simply occur simultaneously.

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Tables and Figures

Table 1. ANOVA results indicating the effects of UV-B exposure period prior to anthesis (UV-B duration) and UV-B intensity on patterns of flowering for *B. rapa* in growth chamber experiments. Results in bold mark significant effects.

		Onset of flowering following exposure to UV-B (in days)		**Flowering duration (days)**		**Number of flowers produced**	
Effect	***df***	***F***	***P***	***F***	***P***	***F***	***P***
UV-B duration	1	12.81	**<0.001**	9.99	**0.002**	9.37	**0.003**
UV-B intensity	2	3.89	**0.026**	3.65	**0.032**	2.72	0.073
UV-B duration x UV-B intensity	2	1.31	0.276	0.78	0.463	0.25	0.779

Type III ANOVA tests. Onset of flowering n = 67 plants; flowering duration n = 70 plants; number of flowers produced n = 76 plants.

Table 2. The mean ± SE number of flowers produced per plant in the short and long periods of UV-B exposure, and the proportion of flowers (%) that did not produce pollen.

	Mean no. of flowers produced per plant		**Proportion (%) of flowers that produced no pollen**	
	Duration of UV-B exposure		**Duration of UV-B exposure**	
UV Treatment	**short**	**long**	**short**	**long**
Control	13.58 ± 6.89 (n=12)	20.77 ± 14.26 (n=13)	1.9	0.9
Low	12.24 ± 4.21 (n=16)	25.08 ± 7.90 (n=12)	4.3	3.2
High	3.64 ± 1.59 (n=11)	13.92 ± 5.59 (n=12)	3.7	2.0
Total	411	738		

The values in brackets indicate the number of plants per UV-B intensity treatment.

Table 3. LMM model outputs for the relationship between UV-B intensity (control, low, high), UV-B exposure period (long vs short), flowering days since onset of anthesis, and pollen viability in *B. rapa.* Significant results are marked in bold.

Effect	Estimate	± SE	t	*P*
Intercept	703.38	58.95	11.933	**<0.001**
High treatment	-169.85	82.48	-2.059	**0.046**
Low treatment	-158.57	67.70	-2.342	**0.025**
Days flowering since anthesis (linear)	1208.34	1389.17	0.870	0.392
Days flowering since anthesis (polynomial)	-2797.41	667.13	-4.193	**<0.001**
Short UV exposure period	-25.81	54.43	-0.474	0.637
High treatment x days flowering since anthesis (linear)	-57.30	2474.59	-0.023	0.982
Low treatment x days flowering since anthesis (linear)	-1547.90	1898.35	-0.815	0.421
High treatment x days flowering since anthesis (polynomial)	3532.90	1646.69	2.145	**0.033**
Low treatment x days flowering since anthesis (polynomial)	116.94	1044.67	0.112	0.911

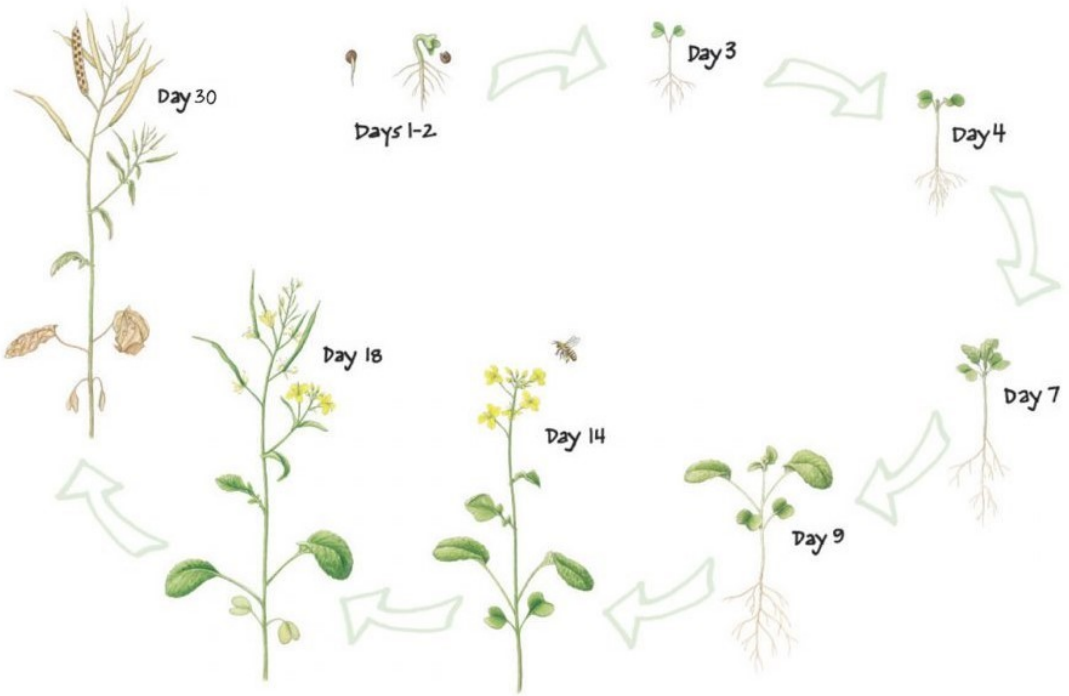

Figure 1. Life cycle of Rapid Cycling *Brassica rapa,* Wisconsin Fast Plants®.

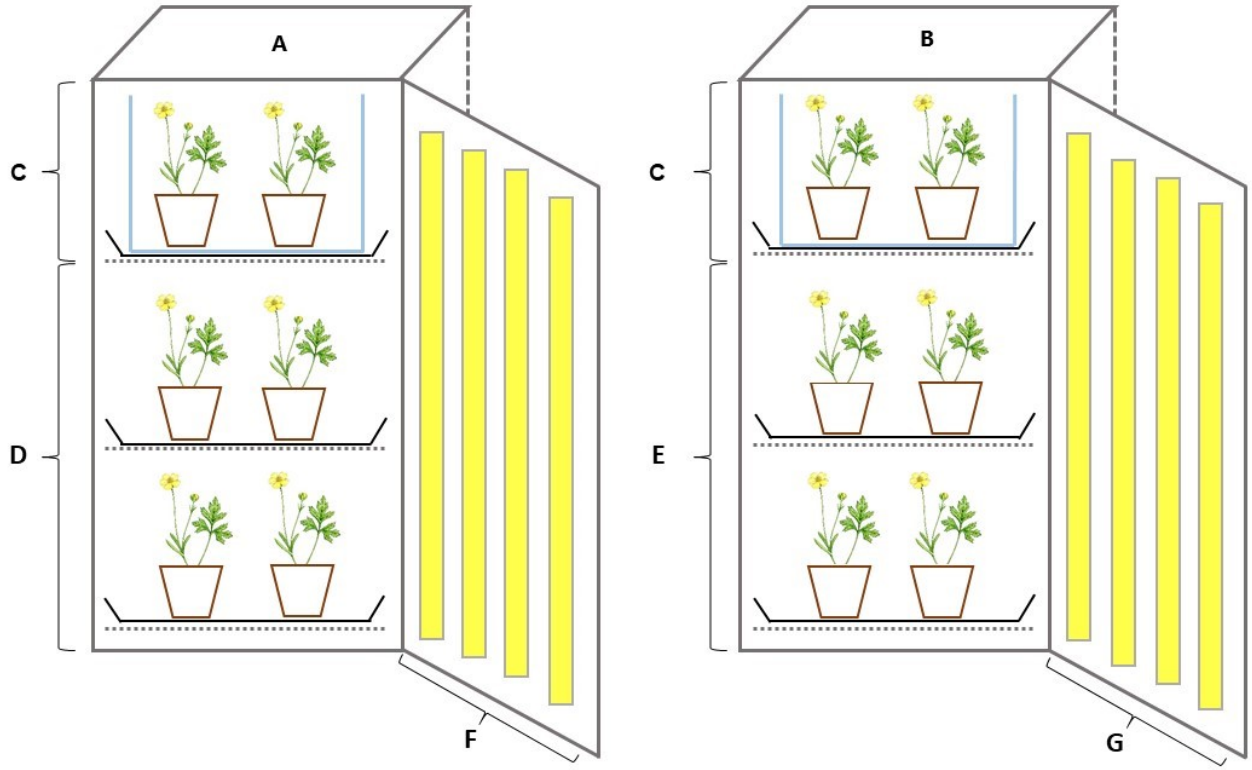

Figure 2. Plant chambers used to simulate UV-B radiation treatments for both long and short duration experiments. A: low intensity of UV-B chamber; B: high intensity of UV-B chamber; C: control treatments with custom-built VE grade Perspex boxes restricting the transmission of UV-B; D: low UV-B intensity treatment; E: high UV-B intensity treatment; F: 15 36W 6% UV-B bulbs fitted into the front and side panels of the chamber; G: 15 36W 12% UV-B fitted into the front and side panels of the chamber.

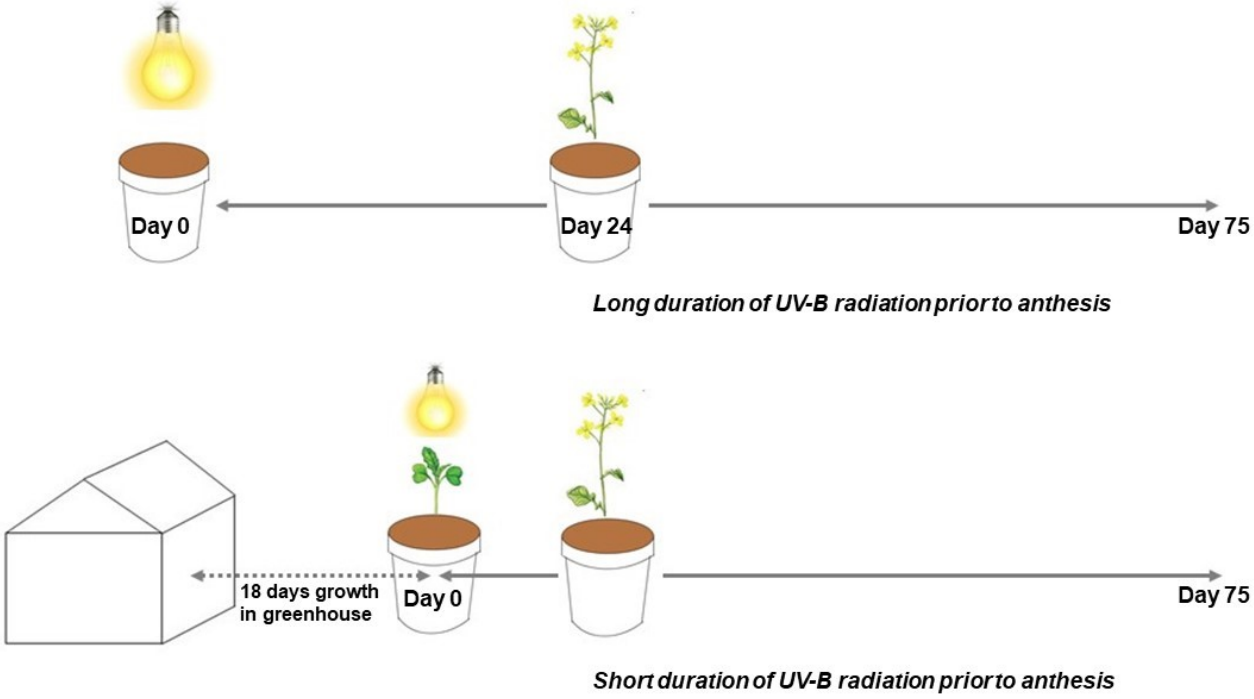

Figure 3. UV-B exposure periods. Long-term UV-B exposure plants were grown from seed within the plant chambers and exposed to either control (no UV-B), low, or high intensity UV-B for 75 days. Short-term UV-B exposure plants were grown from seed in greenhouse conditions (UV-B absent) for 18 days prior to transfer to the plant chambers. Short-term plants were exposed to UV-B radiation in the plant chambers for approximately 6 days before anthesis (equating to 25% of the UV-B exposure time long-term *B. rapa* plants received before flowering). Short-term plants were also grown in the plant chambers for a total of 75 days.

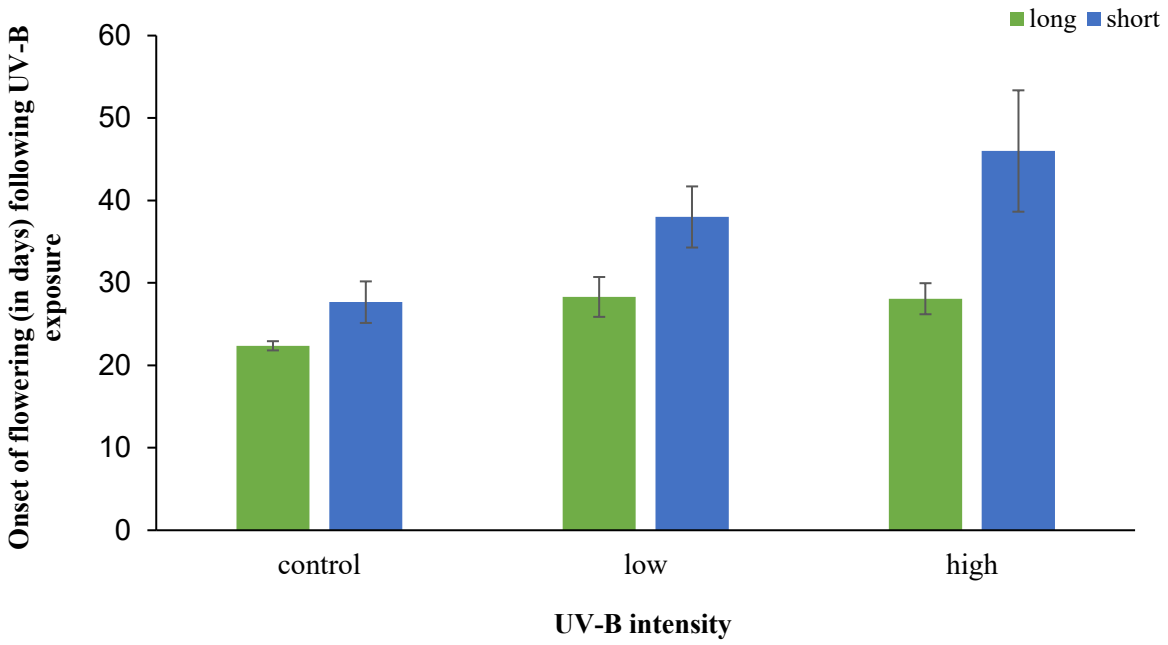

Figure 3. Mean flowering times (days) ± SE of *B. rapa* for three UV-B intensity treatments following exposure to UV-B in the plant chambers.

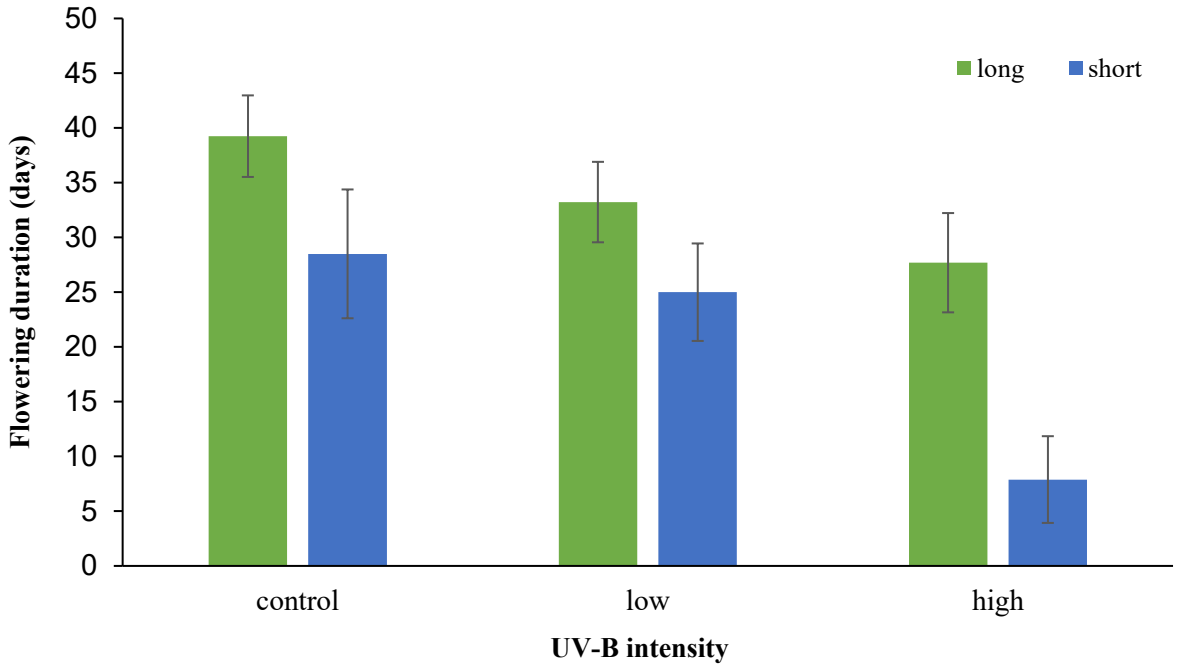

Figure 4. Mean flowering duration (days) ± SE for *B. rapa* plants for three UV-B intensity treatments across two UV-B exposure periods.

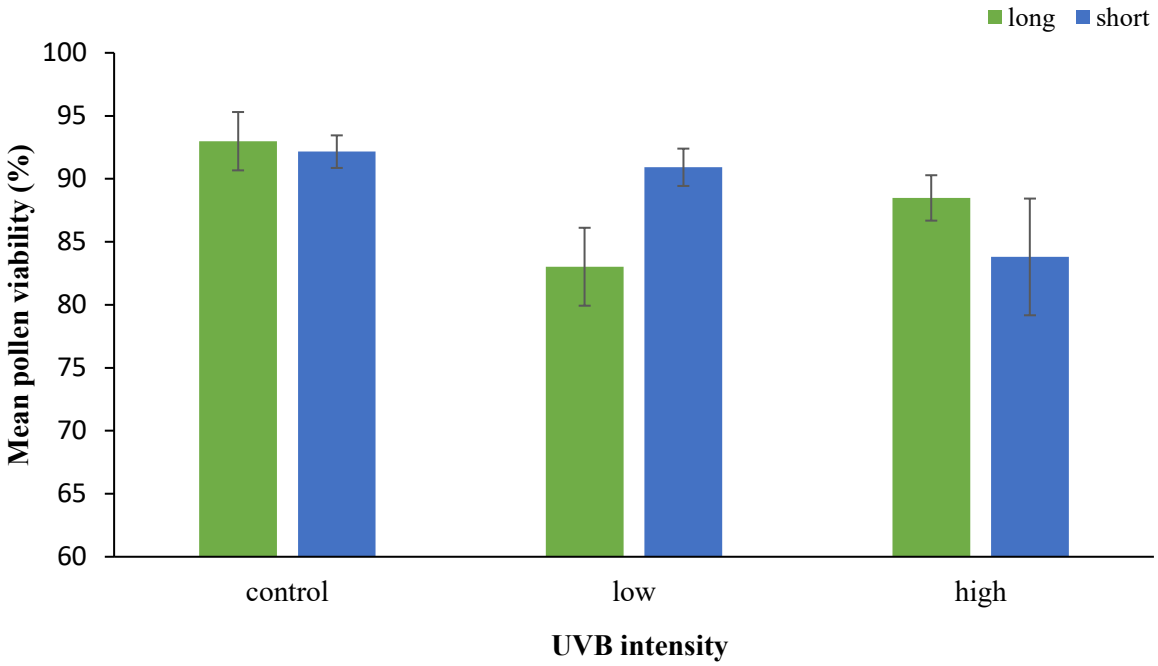

Figure 5. Mean pollen viability (%) ± SE for *B. rapa* plants in simulated UV-B intensity treatments.

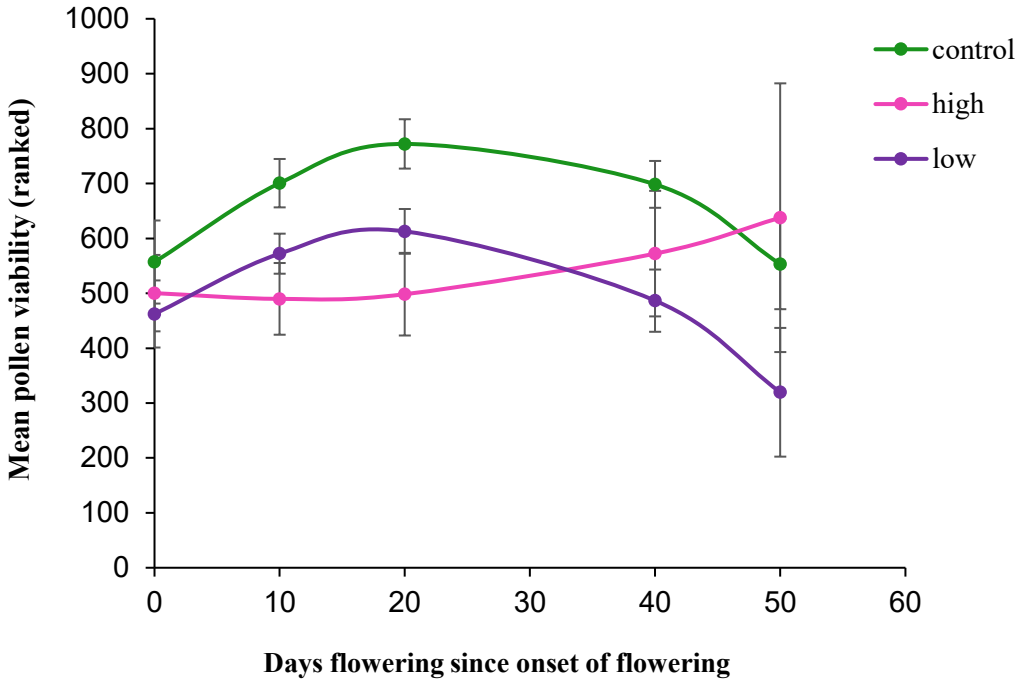

Figure 6. Predicted LMM changes in mean pollen viability over time as a function of UV-B intensity x flowering days since flowering interaction. Error bars indicate 95% upper and lower confidence limits.

Conclusion

In this thesis, I set out to investigate the underlying mechanisms by which UV-B could account for UV bullseyes in flowers from four focal angiosperm families. In Chapter 2, I collected UV floral phenotype data to create a dataset from specimens that were taxonomically widespread, temporally widespread, and geographically covered all latitudes. Using these data, I was first able to establish that UV-B (and other abiotic factors) predicted UV-absorbing area. I was able to test if temporal increases in UV-B radiation, that have occurred across all latitudes, but particularly in southernmost and northernmost latitudes (Herman, 2010), accounted for changes in the UV-absorbing area of UV bullseyes. I found that plants have responded to temporal increases in UV-B radiation over time, with increased UV-absorbing pigment area in the petals of their flowers. In the context of global human history, these results were expected; global anthropogenic-induced ozone-depletion has resulted in increased UV-B radiation reaching the earth's surface (Thompson, 2011). Previous work by Koski and Ashman (2015) detailed a hypothesis by which UV-B radiation could directly select for increased UV-pigmentation in the petals of flowers, by protecting UV-B sensitive pollen, enhancing pollen viability and therefore reproductive success when prevailing UV-B conditions were unfavourable. The findings of this study to a certain extent support this hypothesis, and I suggest that UV bullseye size may be an evolved response to both global and very spatially refined increases in UV-B over the last century.

I also set out to determine if plants responded dynamically to UV-B intensity in Chapter 3, testing specifically if UV-B could induce plastic increase in the ultraviolet absorbing area of *Brassica rapa* petals. Given that UV-B can vary annually, seasonally, monthly, and even daily (Herman, 2010; Beckman, 2014), the ability to respond plastically to fluctuating degrees of UV-B would offer a fitness advantage to plants that could reduce pollen-damage during adverse UV-B conditions. I provide clear evidence of phenotypic plasticity in the UV bullseyes of *B. rapa*, indicating that in higher UV-B intensities and with increased UV-B exposure time, plants produced petals with dramatic increase in UV-absorbing pigmentation. I aimed to test whether Koski and Ashman's (2015) mechanism for selection on bullseyes via UV-B was evident in flowers of *B. rapa* by measuring pollen viability in Chapter 4, however, the plasticity experiments indicated that the ultraviolet absorbing proportion (UVP) of petals declines over the flowering period. I propose that the nutrient and metabolic demands required for the regulation and production on flavonoids responsible for UV absorbing pigmentation could not be met over sustained UV-B exposure.

In Chapter 4 therefore, I did not obtain evidence of enhanced pollen viability in plants hypothesised to arise as result of UV-B-induced plastic increase in UVP (Koski and Ashman, 2015). I did however produce results that were consistent with both contemporary and older studies. Generally, pollen viability decreased with increasing UV-B intensity, also reflecting plant-age associated decline in pollen viability. Duration of UV-B exposure was not a significant determinant of pollen viability, however proved to be significant for flowering duration, time of flowering, and flower production.

Overall, the findings of this study suggest that both plastic and evolved responses to UV-B may account for UV floral phenotypes. Phenotypic plasticity may be one of the main, and arguably most important responses to global change in the short-term, such that when faced with variable and potentially rapid change in UV-B, bullseye plasticity may promote population persistence (Matesanz, 2010). In addition, it is also possible that plasticity may be facilitating the evolution of increased UV bulleyes, allowing populations to persist long enough for selection to act on genetic variation, and until genetic changes can accumulate (Matesanz, 2010). As is the case for all studies that aim to identify the underlying mechanisms that explain plant traits, without genetic sampling it is difficult to isolate evolutionary and plastic responses from one another. What is clear however, is that the increased ultraviolet proportion of UV bullseyes reflects UV-B as a driver of UV floral phenotype, and that both phenotypic plasticity and evolution over time are probably accounting for these changes.

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