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Transcontinental patterns in floral pigment abundance among animal-pollinated species

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Flower color arises primarily from pigments that serve dual functions: attracting pollinators and mitigating environmental stresses. Among major pigment types, anthocyanins and UV-absorbing phenylpropanoids (UAPs) fulfill one or both roles and should be widespread. Our review of the UV-vis absorption profiles of major floral pigments demonstrates that UAPs are the primary UV protectants. Next, we analyzed the floral pigment composition of 926 animal-pollinated species from California, Southern Spain, and Southeastern Brazil. UAPs were ubiquitous (the "dark matter" of the flower). Among the remaining pigment types, ~ 56% of species had anthocyanins, ~ 37% had carotenoids, and ~ 17% had chlorophylls (some species had > 1 pigment type). Pigment abundance varied in response to abiotic and biotic factors, particularly with pollinator type in California. Despite regional differences in environmental filtering, pollination guilds, and relatedness, UAPs are omnipresent and there is a transcontinental stable distribution of flower colors and their underlying floral pigments.

Keywords Anthocyanins, Betalains, Carotenoids, Chlorophylls, Flower color, Flower pigments, UV-absorbing phenylpropanoids, UV-vis absorption capacity

The vast majority of flowering plants depend on pollinators for reproduction (~ 90%[1]), , yet plants produce different types of floral signals to attract different pollinators and ensure seed production. Flower color is one of the most important visual cues perceived by pollinators[2] and is conferred by floral pigments[3,4]. Pollinators can affect flower colors at two scales (1) as important selective agents in populations[5] and (2) as ecological filters in communities[6–8]. Therefore, we expect floral pigment composition to be tightly linked to the region, its pollinators and the local environmental factors that may be selective agents or ecological filters of floral pigments[9,10]. Nonetheless, the relative frequency of different types of pigments in relation to region, pollinators and environmental factors remains largely unexplored at a community and floristic scale.

In addition to their primary role in attracting pollinators, floral pigments may also confer stress tolerance[11–14]. All major types of pigments and their functional intermediates and side branches, such as flavonoids, carotenoids, chlorophylls and betalains, possess antioxidant properties to some extent[15–18]. The UV-absorbing phenylpropanoids (UAPs hereafter) are noteworthy for their energy diffusing, UV protective, and ROS scavenging capabilities[19–21]. This category encompasses UV-absorbing flavonoids, primarily flavonols and flavones, as well as hydroxycinnamic acids, a subgroup of phenylpropanoids. UAPs and anthocyanins in vegetative tissues mitigate many plant stresses including extreme temperatures, drought, pathogens, and herbivores, as demonstrated[19,22]. UAPs are also recognized for their ability to prevent UV-A and UV-B damage[20,21]. In vegetative organs, UAPs play a critical role in protecting against UV radiation during plants' transition from water to land[21,23]. However, the presence, amount and function of UAPs in flowers has received limited attention[3,24] and then, only investigated in a few lineages (e.g., Brassicaceae and Petunieae[25,26]). On the contrary, the abundance of anthocyanins at community or regional scales has received more attention, but is most often deduced from the reflected light or even more generally based on human perceived flower color categories, rather than directly through pigment

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analysis (e.g., British Isles, Australia[7,27]). The concurrent accumulation of anthocyanins and UAPs in flowers may not only enhance the antioxidant capacity of these pigment groups but also provide a biochemical toolkit for pollinator attraction as a main effect or a byproduct of pigment production elsewhere[16,28]. A critical challenge in this context is determining how prevalent UAPs and anthocyanins are in flowers across a diversity of plant lineages and communities from different regions of the Earth.

Given that UV irradiance is a significant stressor for terrestrial plants[29], and pigments can mitigate this stress[16,19], we first review the UV-Vis absorption spectra of the most common floral pigment types. In particular, we compare each pigment's ability to absorb damaging UV-A/B light (280–400 nm). We then analyse floral pigments in 936 animal-pollinated species spanning 115 families and 486 genera from three geographic regions: California, southern Spain, and southeastern Brazil. We predict that if the accumulation of floral UAPs is essential for coping with many environmental stresses[21,29,30] then they should be the most common pigments in angiosperm flowers where they might "double dip" as UV-absorptive guides for pollinators[31,32]. In comparing floral pigments across these regions, we also examined each pigment's abundance with regards to light environments (forest shade vs. open) and primary pollinator (insect vs. bird). Pollinator attraction is influenced by the wavelengths of light present in a given environment, which can vary across different habitats[33,34]. We predict that flowers in forest shade should be yellow or yellow-green to maximize brightness and/or achromatic contrast when that is the primary wavelength of light penetrating a dense canopy[33]. These flower colors are usually conferred by carotenoids, aurone-chalcones, and/or chlorophylls. Finally, bird-pollinated flowers are usually red (as perceived by humans) and strongly UV-absorptive to simultaneously attract birds and be less conspicuous to bees[35–37]. These bird pollinated flowers should be more likely to combine UAPs with red anthocyanins (often complemented with carotenoids) than insect pollinated flowers[9,37]. We reveal that regardless of the flower color and irrespective of which pigments are present in the flower, UAPs are ubiquitous (the "dark matter" of the flower). Floral pigment composition was surprisingly consistent across the three geographic regions studied (a transcontinental stable distribution). Regional variation was primarily influenced by differences in pollination systems and, to a lesser extent, by light environment.

Results and discussion

Absorption properties of major groups of floral pigments

We reviewed the literature to find out which parts of the light spectrum is absorbed by the four main classes of floral pigments, namely phenylpropanoids, carotenoids, chlorophylls and betalains, and their derivatives (Fig. 1). Within the phenylpropanoids, hydroxycinnamates (aka hydroxycinnamic acids), flavones and flavonols exclusively absorb in the UV range. Specifically, hydroxycinnamates absorb mainly in the UV-B region of the spectrum (peaks ≈ 280–330 nm) and flavones and flavonols in the UV-A region (≈ 310–390 nm[38,39]). Aurones and chalcones (treated together herein) absorb in the UV-blue region (peaks ≈ 350–430 nm), whereas anthocyanins mainly absorb in the green-blue region (≈ 475–560 nm)[39–41]. Flowers may contain other groups of flavonoids such as flavanones, isoflavones, catechins or epicatechins, but they are relatively rare compared to the aforementioned groups[41,42]. Alternatively, carotenoids absorb mainly in the blue-green spectral region of the visible light (peaks ≈ 400–530 nm) and chlorophylls absorb in the blue and red regions (≈ 440 and 660 nm, respectively); although chlorophyll *a* has a relatively moderate absorption ability in the UV-A region[39,43,44]. Lastly, betalains show absorption spectra similar to anthocyanins[16,39], but they are restricted to 20 families within the order Caryophyllales[45]. In summary, the only pigments with a considerable capacity to absorb light in the UV region of the spectrum are the UAPs (i.e., hydroxycinnamates, flavonols and flavones) and aurones-chalcones. This would explain why in vegetative tissues UV-B exposure generally promotes the biosynthesis of hydroxycinnamates, while UV-A exposure stimulates the production of flavonols and flavones[20,46,47] (see also[48]). Although evidence in flowers is more limited, available studies suggest a similar trend[14,49].

UV-absorbing phenylpropanoids are ubiquitous in flowers

We performed a biochemical analysis of flowers of 926 animal-pollinated species from diverse habitats of California (442 species), southern Spain (381), and south-eastern Brazil (103) (Supplementary Data 1). Using a differential extraction method followed by an analysis of absorbance spectra (see details in Methods), we were able to identify six major groups of pigments: UAPs (hydroxycinnamic acids, flavones and flavonols), aurones-chalcones, anthocyanins, chlorophylls, carotenoids, and betalains (Fig. 2A). Notably, the presence of UAPs was almost ubiquitous in species from the three regions (> 99.8%; Fig. 2B). Only one species from California, *Geum macrophyllum* (Rosaceae), lacked UAPs in the flowers, but it still exhibited an absorbance peak below 280 nm, however this was below our cut-off for defining UAPs. Anthocyanins were the second most abundant pigment group, present in 55.9% of species across the three study areas, while carotenoids appeared in 37.2% of species (some species contain multiple pigments). Chlorophylls ranged from 12 to 21% (mean 17.2% across the three regions), while aurone-chalcone pigments were infrequent, found in less than 4% of all species. We also confirm that betalains are uncommon pigments, present in only four sampled species from California (0.9%; Fig. 2B). Recently, separate studies in the Brassicaceae, Orchidaceae, and Solanaceae suggest that UAPs accumulate in flowers regardless of visible color and irrespective of the presence or absence of floral guides[25,26,50]. Our results clearly show that floral UAPs are omnipresent in the species studied and, presumably, most angiosperms. Our results align with the ubiquity of UAPs reported from vegetative tissues where they serve multiple protective functions[19,21]. For instance, floral UAPs may counteract oxidative damage induced by UV radiation or drought in petals[51,52] or help to maintain cellular turgor through sugar signaling[53]. However, the exact function(s) of UAPs in petal tissues is largely unknown and therefore, we refer to it as the "dark matter" of the flower, for now.

Although we found that flowers can accumulate up to four types of pigment groups, most species contained only two pigment types (57% species with two types in California & Brazil and 68% of species in Spain) (Fig. 2C and Supplementary Fig. 1). Because of the omnipresence of UAPs in flowers, the most common combination

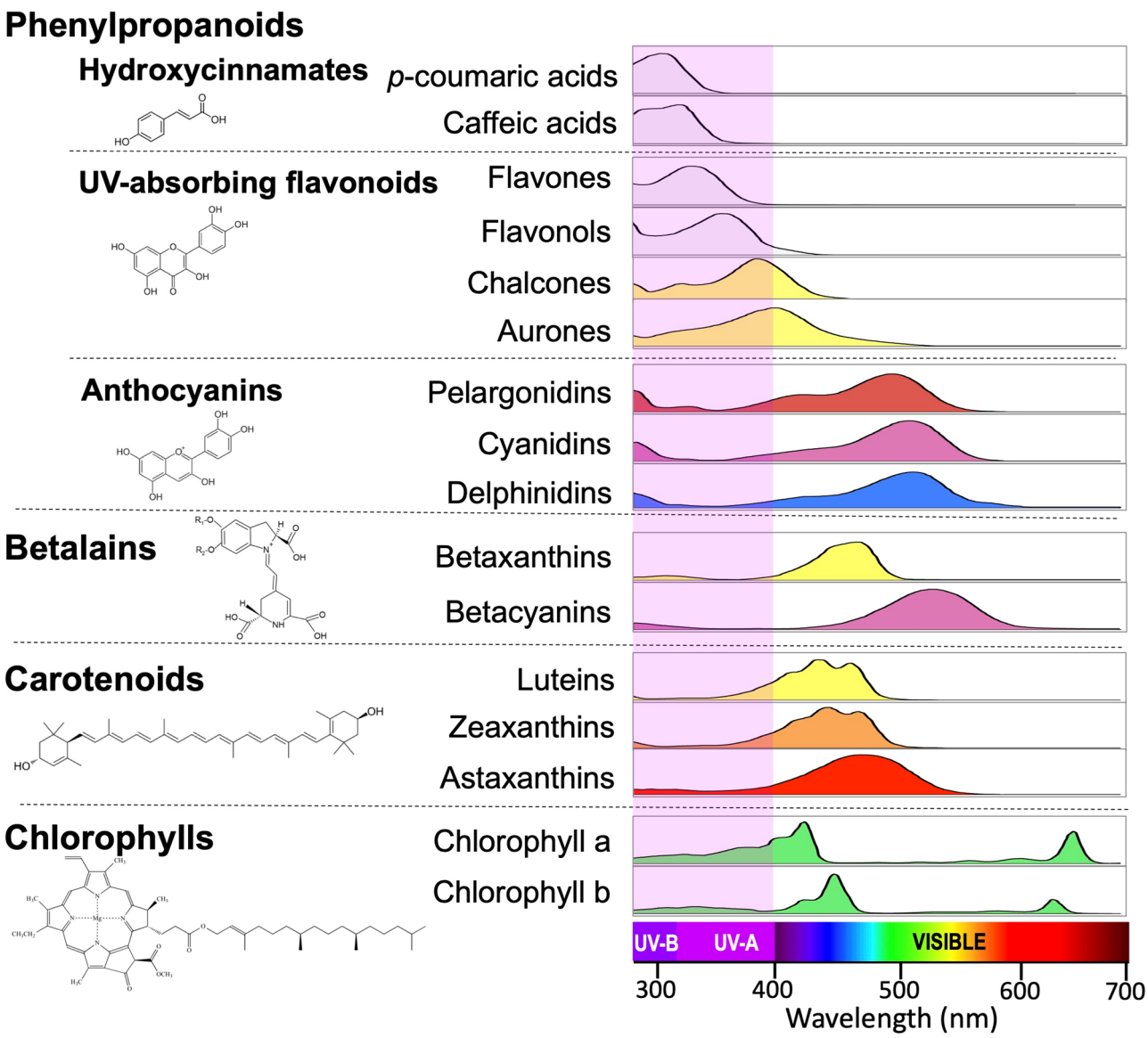
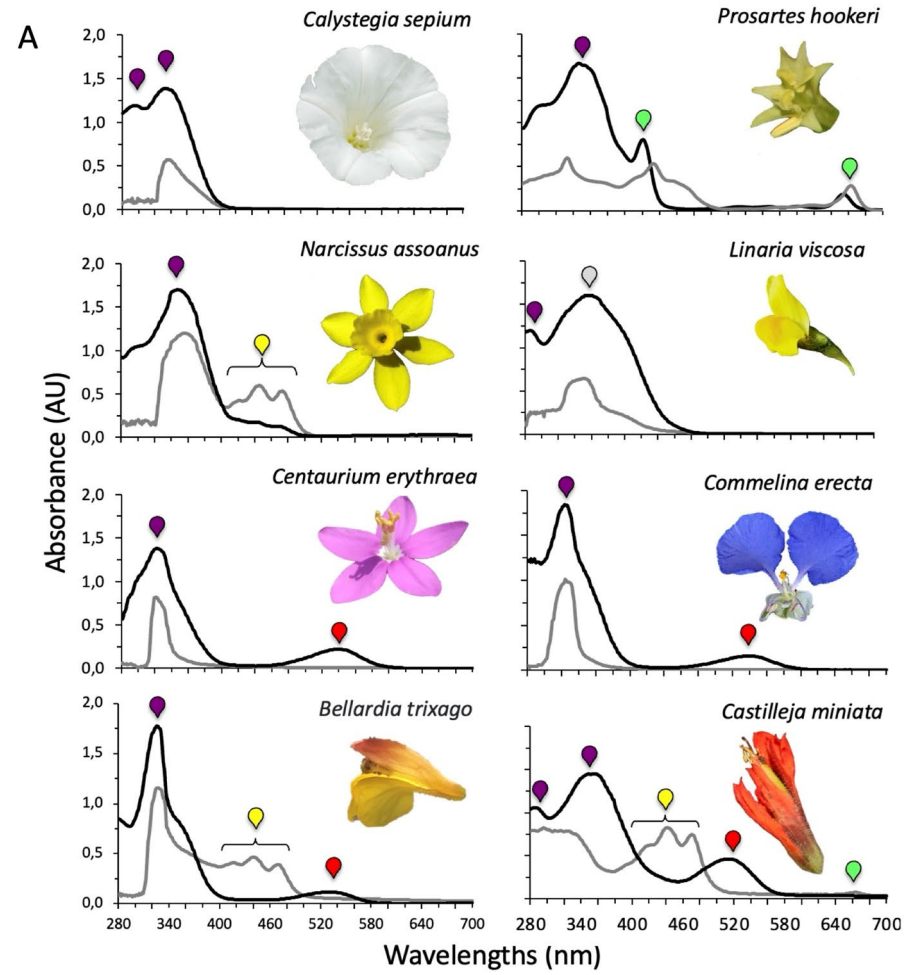
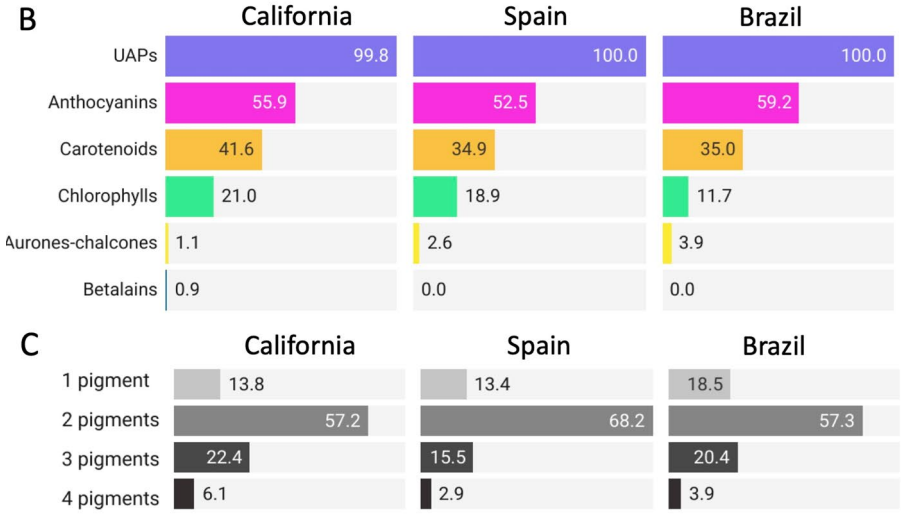


Fig. 1. Ultraviolet (UV) and visible absorption spectra of major pigment groups contributing to flower coloration. Due to their structural diversity and distinct contributions to floral coloration, phenylpropanoids were categorized into hydroxycinnamates, UV-absorbing flavonoids, aurones-chalcones, and anthocyanins. The spectral region corresponding to each pigment type is color-coded based on the human perception of reflected light from petals. The violet-shaded area highlights the UV-A and UV-B regions of the light spectrum (400–315 nm and 315–280 nm, respectively) and shows that hydroxycinnamates and UV-absorbing flavonoids are the pigments with the highest light absorption capacity in this range. For each group of pigments, a normalized absorption spectrum of an example compound is shown (see Methods). The structures of p-coumaric acid (hydroxycinnamate), quercetin (UV-absorbing flavonoid), cyanidin (anthocyanin), betacyanin (betalain), lutein (carotenoid), and chlorophyll a (chlorophyll) are depicted.

of the two pigments was UAPs + anthocyanins (overall 58–66% of species; Supplementary Fig. S2). In petal cells, anthocyanins typically accumulate in vacuoles[54], where they can be found alone or bonded to UAPs by copigmentation or other molecular interactions to stabilize and/or intensify the color[41,55]. Flower color can also appear more intense when pigments are concentrated on the visible side or when light scattered by the underlying unpigmented layer passes through the pigmented layer twice[56]. The association of UAPs with anthocyanin derivatives is one factor contributing to the enormous range of colors and color patterns in angiosperm flowers[41,42,57]. In addition to the effect on floral color perceptible to humans and pollinators, the association between UAPs and anthocyanins results in anthocyanins gaining a substantial light absorption capacity in the UV-A and/or UV-B region[20,58,59]. Thus, accumulation of UAPs and anthocyanins in flowers may be advantageous to the plant and can be shaped by both biotic and abiotic factors[16,60].





UV patterning in petals is rare

Most species accumulated UAPs relatively evenly throughout their petals (80.7, 86.9 and 92.3% in California, S Spain and SE Brazil, respectively). The remaining species had UV–patterning within their flowers including bullseyes, veins, rays, spots, and/or contrasting petals/tepals (e.g., flowers of Fabaceae or Orchidaceae, Fig. 3, Supplementary Data 1; see also[61]). From our UV digital images, a few species appeared have UV-reflective corollas (see other examples in[32,62,63]); however, our absorbance analysis confirmed the presence of UAPs, even in these mostly UV reflective species. This apparent contradiction may be explained by: (1) the higher accuracy of absorbance analysis, which detects UAPs even at low concentrations; (2) the limitations of UV digital images, which do not capture the full UV-A and UV-B range used in the absorbance analysis; and (3) the possibility that UAPs accumulate exclusively on the outer sides of petals[63,64]. Notably, UAPs may protect floral tissues even when

◀**Fig. 2**. Pigment identification and frequency in the studied regions. (**A**) Examples of absorbance spectra for acidified methanol and acetone extracts (black and gray lines, respectively) from flowers of different colors, caused by a combination of pigment classes. The differential solvent extraction and the characteristic absorption peaks of each pigment type allow identification of the pigment types present in the crude extract. Colored drops are used to show the identifiable peaks of the pigment types. In the methanol extracts, violet drops are used for UV-absorbing phenylpropanoids (UAPs), grey drops for aurones-chalcones, red drops for anthocyanins, and green drops for chlorophylls. Betalains not included due to their rarity. In flowers that accumulate several kinds of pigments, some pigment peaks may overlap, but the characteristic types of each pigment may allow them to be differentiated (see methods). Acetone extracts are used to identify distinctive three-peaked carotenoids and are marked by a yellow drop. Betalains show a similar behavior to those of anthocyanins but are not represented because of their rarity. An example of a species showing a single pigment class (*Calystegia sepium*, Convolvulaceae, Spain; white flowers with UAPs). Examples of species with two pigments classes: *Prosartes hookeri* (Liliaceae, California; green flowers with UAPs and chlorophylls), *Narcissus assoanus* (Amaryllidaceae, Spain; yellow flowers with UAPs and carotenoids), *Linaria viscosa* (Plantaginaceae, Spain; yellow flowers with UAPs and aurones-chacoles), *Centaurium erythraea* (Gentianaceae, Spain; pink flowers with UAPs and anthocyanins), *Commelina erecta* (Commelinaceae, Brazil; blue flowers with UAPs and anthocyanins). An example of species with three pigments (*Bellardia trixago*, Orobanchaceae, Spain; yellow-red flowers with UAPs, anthocyanins and carotenoids), and with four pigments (*Castilleja miniata*, Orobanchaceae, California; red flowers with UAPs, anthocyanins, chlorophylls and carotenoids). (**B**) Percentage of species containing each type of floral pigment in California, S Spain, and SE Brazil ($N = 442$, 381 and 103, respectively). Note that in a flower each pigment type may appear alone or coexist with other pigment types. (**C**) Frequency of the number of floral pigment types present in species from California, S Spain, and SE Brazil ($N = 442$, 381 and 103, respectively).

confined to floral guides. For instance, UAPs accumulation in bullseyes may protect reproductive structures from UV radiation, contribute to warming the gynoecium, or enhance petal resistance to desiccation[14,65–67].

Abundance of flower pigments are consistent across regions

We observed striking similarities in the abundance of floral pigment across the three continents (Fig. 2B and Supplementary Fig. 3). A survey of the British flora using 1249 species, which categorized pigments into broad categories based on human perceived color—such as pink, red, blue, violet, and purple (anthocyanins) and yellow (carotenoids)—similarly reported a higher frequency of anthocyanins compared to carotenoids (36 and 26%, respectively)[27], which is lower than what we report herein. This discrepancy likely stems from their method, which considered only the predominant pigment responsible for the main flower color, whereas our approach accounted for all pigments present in the flowers. Using the same flower color categorization as Warren and MacKenzie[27], a higher frequency of anthocyanins compared to carotenoids has been consistently observed across various regions, including France, Scandinavia, Canada, tropical Africa, Australia, and Java[68,69], as well as on a global scale[70]. Phylogenetic similarity can be ruled out as possible explanation of the consistent abundance of pigment worldwide given the distinct floras of these regions. In fact, the frequency of shared families in our study was low: 30.6% (38 of 124 families) between California and Spain, 15.9% (14 of 88) between Spain and Brazil, 14.0% (14 of 100) between California and Brazil, and only 7.6% (12 of 157) of families are found in all three regions. However, the consistent frequencies observed across regions worldwide are likely driven by multiple factors, such as pollinator preferences, evolutionary constrains and/or environmental filtering[6,8,71–74]. Pollinators are considered important selective agents on flower color[5–7]. Hymenopterans show differences in naive preferences among species and have complex learning mechanisms[75], but in general they prefer blue-violet and yellow over other flower colors, with a bias towards blue-violet[76]. Thus, the higher abundance of anthocyanins compared to carotenoids observed across three study regions may be explained by the floral color preferences of hymenopterans, which are the most frequent pollinators in each region. Evolutionary constraints can also shape pigment abundance, as the biochemical pathways responsible for the production of major floral pigment groups (e.g., anthocyanins, carotenoids, and chlorophylls) are highly conserved across angiosperms[16,17,22,23]. Lastly, environmental filtering may contribute to the higher frequency of anthocyanins compared to carotenoids if environmental stressors are similar across regions. It is well-documented that abiotic factors such as temperature, precipitation, and solar radiation are globally prevalent and can increase the frequency of species that accumulate floral anthocyanins and/or UAPs[30,77–79].

Pigment composition is influenced by both abiotic and biotic factors

We tested whether the abundance of major floral pigment groups is influenced by different light environments—shaded (forest, riparian) vs. exposed (grasslands, coastal, rocky, etc.)—in California and S Spain (sample sizes per light environment were too small for SE Brazil). In both regions, most sampled species belong to the exposed light environment (82% and 88.8%, respectively). We found higher frequency of chlorophylls in shaded environments compared to exposed environments in S Spain (Fig. 4 and Supplementary Table 1). However, only 13% of these species showed entirely green flowers, while the remaining species accumulated chlorophylls alongside other pigments (mainly anthocyanins), resulting in diverse color patterns. This interaction between chlorophylls and other pigments has been shown to enhance attraction to insect pollinators[80,81]. Thus, our findings do not support Endler's prediction of a higher frequency of yellow-green flowers in shaded environments[33]. Similarly, a comparative study conducted in German grasslands, which analyzed both chromatic and achromatic

Fig. 3. Visible and UV digital images reveal the homogeneous accumulation of UAPs in petals or their spatial distribution creating patterns. Visible (left) and UV (right) digital images indicate UV-absorbing areas in the corolla caused by the accumulation of UAPs. Scale bars = 10 mm. (**A**) *Aquilegia formosa* (Rannunculaceae, California) showing homogeneous UAPs accumulation in both petals (spur) and sepals (petal like) are UV absorbing. (**B**) *Symphyotrichum spathulatum* (Asteraceae, California) homogeneous UAPs accumulation in both disk and ray florets. (**C**) *Ononis pubescens* (Fabaceae, Spain) showing visible and UV veins, and keel and wings with high UV absorption. (**D**) *Madia elegans* (Asteraceae, California) showing a highly UV-absorbing bullseye in disk florets and base of ray florets. (**E**) *Triteleia ixioides* (Asparagaceae, California) showing UV-absorbing rays on the tepals and corona, in this case is accompanied by chlorophylls causing greenish purple rays in visible photography. (**F**) *Malva sylvestris* (Malvaceae, Spain) showing UAPs nectar guides in addition to anthocyanins in the remainder of the petal. (**G**) *Parkinsonia florida* (Fabaceae, California) showing visible dots and banner with high UV absorption. (**H**) *Adelinia grandis* (Boraginaceae, California) showing a UV-absorbing bullseye in the corolla.

components of flower colors using the honeybee color vision model, found no significant differences in flower colors between closed forest and open light environments[82].

We also examined if insect- and hummingbird-pollinated species differed of floral pigment distribution. Our database includes 9.7% of species categorized as hummingbird-pollinated in California and 12.8% in southeastern SE Brazil (hummingbirds are not present in S Spain). In California, the abundance of anthocyanins and carotenoids in bird-pollinated flowers was nearly double that of insect-pollinated flowers, while chlorophyll showed the opposite trend (Fig. 4 and Supplementary Table 2). The red color is a defining characteristic of the

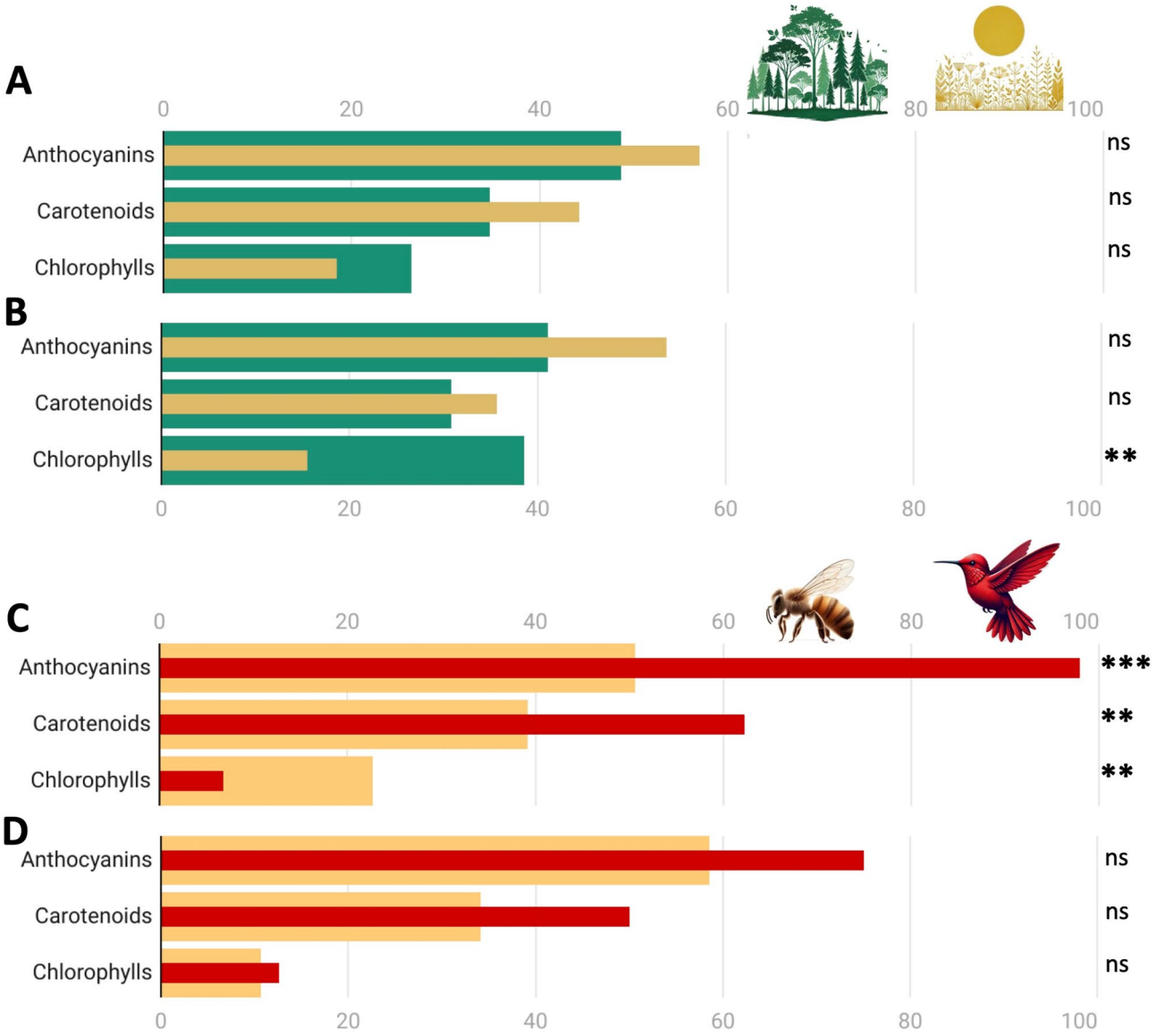


Fig. 4. Percentage of species accumulating floral pigment types across different light environments and pollination systems. (**A**) Comparison of the percentage of species with the three main pigment groups between exposed (light brown) and shaded (green) light environments in California ($N = 328$ and 72, respectively). (**B**) Comparison of the percentage of species with three main pigment groups between exposed (light brown) and shaded (green) light environments in S Spain ($N = 309$ and 39). (**C**) Comparison of the percentage of species with the three main pigment groups between insect (light orange) and hummingbird (red) pollination systems in California ($N = 393$ and 45, respectively). (**D**) Comparison of the percentage of species with three main pigment groups between insect (light orange) and hummingbird (red) pollination systems in SE Brazil ($N = 94$ and 8). Symbols on the right show the results of permutation tests to compare the frequency of each pigment group between light environments and pollination systems (see statistical results in Supplementary Tables 1 and 2). n.s. not significant, $^{**}p < 0.01$, $^{***}p < 0.001$. Images of light environments and pollination systems were generated by AI image generator DALL-E.

hummingbird pollination syndrome[83]. This coloration results from the accumulation of pink-red anthocyanins, specifically cyanidin and pelargonidin derivatives, or, more commonly, from a combination of these anthocyanins with yellow carotenoids[9,37]. Several studies have demonstrated that the presence of these pigments, along with UAPs, enhances flower conspicuousness for hummingbirds while reducing detectability to bees[35,37,84]. Our findings confirm the ubiquity of these pigments across 16 plant families in California (Supplementary Data 1) and suggest that hummingbirds are driving the evolution of floral pigmentation in this region. A higher abundance of anthocyanins and carotenoids was observed in hummingbird-pollinated flowers from SE Brazil, but differences were not statistically significant likely due to the limited sample size. Notably, the number of plant

species exhibiting the hummingbird pollination syndrome in the Brazilian Cerrado is relatively low compared to other regions of the Americas[85–87].

Conclusions

Our floral biochemical results highlight the ubiquitous nature of UAP compounds in animal-pollinated flowers across three continents, diverse habitats and pollination syndromes. We argue that UAPs may have a dual role in flowers, attracting pollinators and protecting against environmental stress[16]. UAPs are present in early land plants, allowing them to cope with UV radiation and thermal stress[23,29], which were likely co-opted by angiosperm petal-specific regulatory modules to produce the diversity of flower colors we know today. Thus, we conclude that the ancestral (and primary) function of UAPs in floral tissues is to protect cells from environmental stressors and the role of pollinator attraction could then be an exaptation[88]. The omnipresence of floral UAPs may be explained by their origin during the terrestrialization of plants, having been retained in all plants due to essential nature for survival and favored due to their versatility as environmental protectors[89]. Nevertheless, UAPs can be regarded as the "dark matter" of flowers, as their presence is demonstrable, yet their precise functions remain uncertain.

We found that abundance of floral pigments are consistent across the three studied regions, a pattern that likely extends globally. Our results offer a more comprehensive understanding of floral pigment abundance and further strengthen previous studies based on flower color or theoretical models[71,77,78]. The causes of these stable distributions are not well understood but likely reflect the flower color preferences of insects—the most frequent pollinators—as well as underlying biochemical and molecular factors, warranting further investigation. The variation in presence of pigments between exposed and shaded environments, as well as between insect- and hummingbird-pollinated flowers, suggests that eco-evolutionary processes are acting at regional scales amidst a relatively stable global distribution of flower color[7,77,90].

Methods

UV-vis absorption spectra of main flower pigment groups

We reviewed references presenting spectral data of pigments extracted in methanol, as it is one of the most common solvents used for plant pigments[31,91]. For each main pigment group, we selected the most representative or frequent subgroups found in flowers[41,42,57,92,93] and present a normalized absorption spectrum of an example compound diluted in methanol in (Fig. 1). The sources used in drawing these average absorption spectra are shown in (Supplementary Table 3). Although there may be variation in the shape of the curves and maximum absorbance wavelength between different pigment types within the same biochemical group, this variation is usually quite small compared to the differences between pigment groups[31,39,92].

Sampling for the floral pigment composition analyses

In California (California Floristic Province), we collected flowers from a total of 442 native species belonging to 249 genera and 69 families, and in southern Spain, we collected a total of 381 native species belonging to 198 genera and 56 families (Supplementary Data 1). In southeastern Brazil (National Park of Serra do Cipó, Minas Gerais, Brazil), we collected flowers from 103 species belonging to 72 genera and 32 families. Samples were collected from 2019 to 2023 in California and Spain and in 2019 in Brazil.

Collection of plant material complied with all relevant national and international guidelines and legislation. Necessary permissions were obtained to collect samples in the three studied regions. Voucher specimens were deposited in the herbaria of the University of Seville (Herbario SEV), University Pablo de Olavide (UPOS), Instituto de Biociências at the Universidade Estadual Paulista in Rio Claro (HRCB), and San Jose State University (SJSU). Collections have accession numbers at the SJSU and SEV herbaria, whereas in UPOS and HRCB, they are unnumbered but stored in separate boxes. Plant specimens were identified by Justen B. Whittall (California), Eduardo Narbona, Marisa Buide, Montse Arista, and Pedro L. Ortiz (Spain), and Patricia Morellato (Brazil) using specialized references, including the Jepson Manual eFlora (https://ucjeps.berkeley.edu/eflora) and Flora Iberica (http://www.floraiberica.es), as well as herbarium collections and previous studies conducted at the same study sites[35].

Both California and S Spain have a Mediterranean climate with typically hot, dry summers and cool, wet winters[94]. In SE Brazil (Minas Gerais state), the climate is tropical, with a cool dry season, a warm wet season, and frequent fires and strong winds during the dry-to-wet transition[95]. In California and S Spain, we sampled broadly across habitats by collecting in grasslands, shrublands, forests, riparian, rocky, wetlands, coastal, mountain and deserts communities throughout the year. In SE Brazil, we surveyed the rocky grasslands at high altitudes (> 900 m a.s.l., Campo rupestre *sensu stricto*) and the woody savanna vegetation (Cerrado *sensu stricto*). The main pollinators in the three study regions were insects (primarily hymenopterans, but also dipterans, lepidopterans, coleopterans, among others[35,87,94]). However, in both California and SE Brazil, hummingbirds serve as pollinators, with approximately 5–12% of the flora exhibiting the typical "hummingbird-pollination syndrome"[35,87,96]. We analyzed the pigment content of floral parts with the highest advertisement display, typically petals or tepals, at anthesis.

In all cases, we picked flowers or inflorescences at anthesis for several individuals to account for between-individual variation in pigment composition. Additionally, 56 species were collected in two different populations to test for pigment variation between populations; in all cases, the qualitative pigment profiles were identical and, thus, we used the data of the first population analyzed.

We sampled "attraction units"[97], which in most species coincide with individual flowers but in some species, we considered the entire inflorescence (e.g., spadix of Araceae spp. or compound inflorescences of Asteraceae spp.). We analyzed pigment content of the floral piece that generates the highest advertising display, usually

petals or tepals, but floral bracts were also examined in some species (e.g., Aristolochiaceae spp., Euphorbiaceae spp., *Castilleja* spp., or *Rhynchospora* spp.).

Extraction, identification, and quantification of major pigment groups

Collections were stored at 4 °C until pigment extraction was carried out, typically within 24–36 h. We used two solvents to extract and separate the principal pigment classes in each sample: methanol with 1% HCl (v: v) and pure acetone[39,98]. The acidified methanol solution is particularly effective in extracting UAPs, anthocyanins, betalains, and chlorophylls, whereas acetone mainly extracts carotenoids and chlorophylls[39,91,99]. We used methanol as a solvent instead of ethanol or aqueous methanol solutions due to its superior efficiency to extract polar compounds[100]. Acidification of methanol with HCl contributes to the stabilization of the anthocyanins in particular[99]. We weighed (5–25 mg of fresh weight) and submerged the floral tissue in two microtubes containing 1.5 ml of each solvent, which was stored at -20 °C until further analysis. In California, we used 5–10 silica beads for tissue homogenization for 2–3 min followed by 5 min of centrifugation at maximum speed. In Spain and Brazil, homogenization was employed solely in cases involving thick tepals or floral bracts[101].

We obtained the absorbance spectra of the samples in each solvent by means of two ultraviolet-visible (UV-vis) spectrophotometers. We used a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and SpectraMax M3 (Molecular Devices, San Jose, CA, USA) with acetone-compatible polypropylene 96-well microplates, and a Drawell DU-8800DS double beam UV/Vis spectrophotometer (Chongqing Drawell Instrument Co., Ltd, China) with 1-cm quartz cuvettes, respectively. Previous studies showed that pigment quantification using plastic plates vs. single-sample cuvettes provides nearly identical results[102]. We set the scan mode from 280 to 700 nm with 1–2 nm steps at a constant temperature of 22 °C (no shaking). We selected wavelengths above 280 nm since below that all phenolic compounds are characterized by a UV band II that peaks at 240–275 nm making it useless for differentiation[39]. We used 150 µl and 500 µl per sample in 96-well microplates and 1-cm quartz cuvettes, respectively. Following the technical specifications of the spectrophotometers, concentrated pigment extracts were diluted to obtain absorbance values (optical density) below 2.0 absorbance units (AU) to guarantee reliable measurements.

Since our objective was to identify major pigment classes, we performed spectrophotometric analyses to obtain the absorption spectra of the sample extracts. Although spectrophotometers have less ability to distinguish biochemical variation within a pigment class, this approach is a reliable, fast, and inexpensive alternative to HPLC separation with mass or NMR spectrometry[99,103]. Since the major pigment classes have a characteristic light absorption spectrum with distinctive peaks, we were able to identify the presence of main pigment types from raw floral extracts[28,39,103]. Thus, carotenoids show three distinctive peaks between 400 and 530 nm with a major peak around 450 nm, whereas chlorophylls show two main peaks at 415–460 nm and 650–665 nm[43,44]. All floral samples with chlorophylls showed a peak at ~ 418 nm, indicative of chlorophyll *a*[44]. We distinguish three major groups of phenylpropanoids: anthocyanins, aurones-chalcones, and UV-absorbing phenylpropanoids (UAPs). The flavonoid anthocyanins show a characteristic peak around 475–560 nm, whereas the aurones-chalcones exhibit a peak at 350–430 nm[39,41]. UAPs include non-visibly pigmented flavonoids such as flavanones, flavones, and flavonols with principal peaks between 280 and 360 nm, and some groups of hydroxycinnamates, such as cinnamic, caffeic, ferulic, p-coumaric, and sinapic acids which have a distinguishing peak at 280–330 nm[38–41]. The different groups of UAPs show distinctive peaks, but most of them overlap, which precludes their differentiation using this methodology[104]. Spectrophotometric identification does not allow the detection of other groups of flavonoids such as isoflavones or catechins that show their main peaks below 280 nm[39,104]. With respect to betalains pigments, pink-red betacyanins were distinguished from anthocyanins due to a higher wavelength of absorption peak (532–554 nm), and yellow betaxanthins were distinguished from carotenoids by the extraction in methanol solvent and the presence of only one peak at 450–500 nm[39]. In addition, we confirmed that the sample was in a plant family that was previously described to produce betalains[45]. We have not quantified concentrations of each pigment group because our methods do not allow for the identification of specific compounds (i.e. type of anthocyanins, class of carotenoids, more specific identification of UAPs, etc.) present in each species. In species from Spain with whitish, cream, pale-yellow, and yellow extracts, in which we observe an absorption peak around 350–450 nm, we performed two additional tests to corroborate the presence of aurones-chalcones vs. carotenoids: color reaction in methanolic HCL extracts and differential separation with water and dichloromethane[105].

We found some species showed absorbance maxima that did not fit any of the major pigments previously mentioned. We performed a bibliographic search to find out if there were biochemical data on the floral pigment of these species or their relatives. Quinones are a rare group of pigments that may be found in some flowers, mainly anthraquinones or quinochalcones[106]. In our study, *Dipcadi serotinum* presented a compound with only one peak at 460 nm that was extracted in both methanol and acetone solutions; this was congruent with a quinone[39], yet yellow anthraquinones have been found in other species of Asparagaceae[106]. Similarly, the compound peaking at 500 nm in all species of *Xyris* was also congruent with previously described anthraquinones for this genus[107]. To calculate the frequency of the main types of pigments, quinones were included in the anthocyanin group since they share the early steps of the biosynthetic pathway[106]. Xanthones is a rare group of flavonoids in flowers that show absorbance peaks similar to isoflavones, flavones, and flavonols[39,106]. In flowers of *Iris* spp. and *Hypericum* spp., xanthones have been previously described[108,109], thus, they may be present, along with other UAPs, in our methanol extracts. Finally, *Adonis macrocarpa* showed a compound with a single peak at 480 nm appeared in both methanol and acetone solutions, which agreed with the red carotenoid astaxanthin previously reported in *A. aestivalis*[110].

In species with observed UV or visible color patterns (see UAPs location in petals section), separate samples were analyzed (e.g., different floral pieces of Orchidaceae spp., apical and basal portions of ligulate/ray flowers of Asteraceae). In the Fabaceae family, we separately analyzed the banner, keels, and wings, except for species with

very tiny flowers (e.g., *Trifolium* spp. or *Medicago* spp.). In these species, the total number of pigments present in a flower was the sum of the pigments found in all the different samples of a flower. The same approach was applied to species with blushing (i.e., flower color change) and flower color polymorphic species.

UAPs location in petals

We investigated whether the presence of UAPs was located in the whole petals or produced spatial color patterns or floral guides by using UV photography (maximum sensitivity at ~360 nm, range ~320–380 nm) and/or measuring UV reflectance spectra (300–400 nm) in different petal areas (see details in[9,64,98]). We considered color patterns as a spatial variation in UV or visible color within a flower, which includes UV or visible bullseyes, veins, rays, spots and/or differently colored petals/tepals (e.g., flowers of the Fabaceae or Orchidaceae; see Supplementary Data 1). These patterns have been traditionally considered as floral or nectar guides (e.g[32,61]). The patterns were confirmed, when possible, by reviewing previous studies[3,62].

Abundance of floral pigments in different light environments and pollination systems

In California and Spain, the studied species grow in a variety of habitats with different light environments and solar UV radiation, ranging from very low (e.g. redwood forest, oak forest, riparian) to extremely high (e.g. grasslands, desert, coastal dunes). For habitat assessments in the California Floristic Province, we used the "ecology" descriptions provided in the Jepson eFlora (www.ucjeps.berkeley.edu/eflora) and categorized them into nine main habitat types: riparian, wetland, coastal, desert, forest, grassland, woodland, montane, and rocky. When a species has more than one habitat, we chose the main habitat according to the description in Jepson eFlora, our own habitat knowledge of the species and photos of the species consulted in iNaturalist (www.inaturalist.org). In Spain, we followed the same procedure using Flora Iberica (www.floraiberica.es), categorizing species into the same habitat types as in California, with the exception of the montane habitat. For comparisons, we grouped these habitats in "shaded" and "exposed" light environments[33], considering forest and riparian as "shaded" light environment and the rest of habitats as "exposed". Woodland species were not included in the analysis ($N = 42$ in California and 33 in S Spain) because most of the species grow in both shaded and exposed light environments. In Brazil, species growing on rocky and savanna habitats, and both are considered open light environments[95], undermining any meaningful comparisons between different light environments.

We categorized species from California and Brazil according to their main functional group of pollinators, i.e. "insect", "hummingbird", or "mixed"[90]. To assign these categories, a bibliographic search of the pollinators of all the species was carried out. (see Supplementary Data 1). Only four species from California were found with a mixed pollination syndrome and therefore were not taken into account in the analysis. In Brazil, one species showed pollination by bat and was not considered in this analysis.

We investigated variation in the abundance of the three major pigment categories (anthocyanins, carotenoids and chlorophylls) between shaded and exposed environments and between insect- and hummingbird-pollinated flowers. UAPs were not considered since they were ubiquitous.

Statistical analysis

We performed permutation tests to determine whether the number of species producing each type of flower pigment varied among the three geographic regions. The observed number of species having a pigment type in each study site was compared with the distribution of permuted data (1000 iterations) generated from the whole dataset[111]. Two-tailed p-values were calculated based on the proportion of permutations yielding values more extreme than the observed, considering a significance threshold of $\alpha = 0.05$ ($p < 0.025$). The same statistical analysis was used to determine whether the number of pigments produced per species varied among the geographic regions and whether the frequency of anthocyanins, carotenoids and chlorophylls varied significantly between light environments and between pollination systems. Statistical analyses were performed in R, version 4.3.2[112] using R-studio interface. The bar graphs were created using datawrapper software (www.datawrapper.de).

Data availability

The list of species used in this study, along with their assigned light environment and pollination system, is provided in Supplementary Data 1. Pigment data are available from the corresponding author upon reasonable request.

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Author contributions

EN, MA, PLO, MLB, LPCM and JBW designed the research; all authors collected samples; EN, JCDV, JBW, MLO, MLB, IP, MGGC, NRC, VR, KC, JHM, and EM performed the biochemical analysis; EN, JCDV, MLO and MGGC analyzed the data and prepared the figures; EN, MA, JCDV, JBW, and PLO wrote the paper. All authors discussed the data and contributed to the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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