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RESEARCH ARTICLE

Pollinator response to yellow UV-patterned versus white UV-patternless flower dimorphism in *Anemone palmata*

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Keywords

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ABSTRACT

- Flower colour polymorphisms are uncommon but widespread among angiosperms and can be maintained by a variety of balancing selection mechanisms. *Anemone palmata* is mostly yellow-flowered, but white-flowered plants coexist in some populations.
- We analysed the distribution of colour morphs of *A. palmata* across its range. We also characterised their colours and compared their vegetative and sexual reproductive traits, pollinator attention and fitness.
- The range of *A. palmata* is limited to the Western Mediterranean, while white-flowered plants are restricted to Portugal and SW Spain, where they occur at low proportions. Yellow flowers have a characteristic UV pattern, with a UV-absorbing centre and UV-reflecting periphery, which is absent in the white morph. Colour features of both morphs were highly delineated, making it easy for pollinators to distinguish them. Both morphs were protogynous, with the same duration of sexual stages, and the main floral traits related to pollinator attraction, apart from flower colour, were similar. Hymenoptera and Diptera were the main pollinators, showing preference for the yellow morph, clear partitioning of pollinator groups between the two colour morphs and a marked constancy to flower colour during foraging. Both morphs combined clonal propagation with sexual reproduction, but sexual reproductive potential was lower in white-flowered plants. Finally, female fitness was higher in the yellow morph.
- Pollinator partitioning and colour constancy could maintain this polymorphism, despite the lower visitation rate and fitness of white-flowered plants, which could facilitate their clonal propagation.

INTRODUCTION

The variety in flower colour is one of the most striking features in nature. The colour of flowers is primarily determined by light reflection following backscattering within the petals and selective pigment absorption of petals (van der Kooi *et al.* 2014, 2016). The main reason for this large variation is that pollinators use colour as a signal to locate flowers and search for resources, thus driving flower colour variations through different insect perceptions and preferences (Kevan & Baker 1983; Schoonhoven *et al.* 2005; van der Kooi *et al.* 2019).

Flower colour can vary both between and within species, even within populations. For example, seveeral species have different colours and patterns within a community, allowing each group of pollinators to select those that are more conspicuous and attractive to them (Camargo *et al.* 2019; Ellis *et al.* 2021). Colour shifts between related species are common and often associated with changes in the pollinator guild that contributed to their speciation (Muchhala *et al.* 2014; Wessinger *et al.* 2019). In contrast to the huge interspecific variability in flower colour, intraspecific variation in this trait is not as common (Narbona *et al.* 2018), although there are examples of both continuous (*Silene littorea*, Rodríguez-Castañeda *et al.* 2020) and discrete (*Raphanus sativus*, Stanton 1987; *Lysimachia arvensis*, Ortiz *et al.* 2015; *Anemone coronaria*, Dafni *et al.* 2020; *Gerbera aurantiaca*, Johnson *et al.* 2021) variation. The term polymorphism is restricted to discrete flower colour variation within populations (Narbona *et al.* 2018; Sapir *et al.* 2021). Moreover, species with stable flower colour polymorphisms are uncommon but distributed throughout the angiosperm phylogeny. It is therefore of interest to determine how these polymorphisms persist, as they can easily be lost due to directional selection combined with random genetic drift (Wang *et al.* 2016; Narbona *et al.* 2018; Sapir *et al.* 2021). However, flower colour polymorphism can be maintained through a variety of balancing selection regimes (Jamie & Meier 2020). These include the presence of multiple selective agents acting in different directions, either directly or pleiotropically (Frey 2004; Carlson & Holsinger 2010; de Jager & Ellis 2014), fluctuating selection (Schemske & Bierzychudek 2001; Tang & Huang 2010), heterozygous advantage (Malerba & Nattero 2012; Takahashi *et al.* 2015; Kellenberger *et al.* 2019), negative frequency-dependent selection (Gigord *et al.* 2001), or mosaics of facilitative–competitive interactions with other co-occurring plant species that share pollinators (Coetzee *et al.* 2021). Furthermore, reproductive assurance through

selfing (Subramaniam & Rausher 2000; Jiménez-López *et al.* 2020b) or clonal propagation (Imbert *et al.* 2014; Johnson *et al.* 2021) has been proposed as a mechanism to maintain negatively selected colour morphotypes that would otherwise be eliminated. The selective agents involved in the loss or maintenance of flower colour polymorphism can be abiotic (Strauss & Whittall 2006; Arista *et al.* 2013) or biotic, including herbivores (Irwin *et al.* 2003; Caruso *et al.* 2010), pathogens (Frey 2004) and, most importantly, pollinators (Malerba & Nattero 2012; Kellenberger *et al.* 2019). Apart from selection, pollinators can cause assortative mating of morphotypes, which may maintain polymorphism in the short term, but in the long term could lead to speciation, i.e. the separation of monomorphic species, as mentioned above (Murúa *et al.* 2017; Jiménez-López *et al.* 2020a, 2023). Lastly, flower colour polymorphism might be maintained through neutral or random processes (Wang *et al.* 2016; Sapir *et al.* 2021).

Pollinators must be able to discriminate between colour morphotypes in order to act as selective agents in the maintenance or loss of floral colour polymorphism (Keasar *et al.* 2016; Jiménez-López *et al.* 2020a). Colour has chromatic components, such as hue and saturation, which relate to the shape of the reflectance curve, and achromatic components, such as brightness, which relate to overall reflectance (van der Kooi & Kelber 2022). Insects visually process both components using independent neural mechanisms, the chromatic ones by opposing mechanisms that compare signals from at least two photoreceptor types, and the achromatic ones by non-opposing mechanisms that can use the signal from a single photoreceptor type or the sum of several types (Kelber *et al.* 2003; van der Kooi & Kelber 2022). Hence, Ng *et al.* (2018) found that bees ignore achromatic components when processing chromatic stimuli. It is postulated that insects utilise achromatic vision to detect flowers oveer long distances under small visual angles, and chromatic vision to discriminate flower colours at short distances under large visual angles (Giurfa *et al.* 1996; Chittka & Raine 2006; Hempel de Ibarra *et al.* 2015; van der Kooi & Kelber 2022).

It is widely acknowledged that bees and flies are the dominant pollinators in Mediterranean ecosystems, where they are responsible for the reproduction of a significant proportion of the flowering plants (Petanidou & Lamborn 2005; Ollerton 2017; Ropars *et al.* 2020). These two groups of insects show remarkable differences in chromatic vision. In bees, three types of photoreceptors are involved, making them trichromatic (Peitsch *et al.* 1992; Chittka & Raine 2006). In contrast, the presence of four different photoreceptors involved in colour vision seems to be the norm in the few dipterans studied so far, so that chromatic vision in dipterans, at least in the most common flower visitors, is tetrachromatic (Lunau 2014; van der Kooi *et al.* 2021; van der Kooi & Kelber 2022). They also have similarities, being sensitive to ultraviolet and less sensitive to red (Lunau 2014). Colour perception and discrimination by both bees and flies have been analysed with models based on physiological characteristics and through behavioural experiments (Kelber *et al.* 2003). The main models used to assess colour perception by bees are the colour hexagon (Chittka 1992) and receptor noise (Vorobyev & Osorio 1998) models, and Garcia *et al.* (2017) found that discrimination functions are sigmoidal regardless of the colour model. The fly vision model proposed by Troje (1993) considers that flies perceive only four colour categories, and the graphical representation of this model has become the generalised way of representing the colour space of flies (Lunau 2014). However, it has recently been shown that, at least in some flies such as hoverflies, colour discrimination is mediated by a continuous function (Hannah *et al.* 2019), as in bees. Consequently, Euclidean distances in Troje's vision space model can be used to assess chromatic contrasts between colour loci as perceived by hoverflies (Hannah *et al.* 2019), as routinely used in the bee colour hexagon since the work of Chittka (1992). Chromatic contrast between the flower and its background has been shown to be a more reliable colour descriptor than saturation metrics, such as spectral purity or chroma (van der Kooi & Spaethe 2022). Bees and flies also show remarkable differences in achromatic vision, with the former using only the longest wavelength sensitive photoreceptor (the green receptor) in the perception of achromatic contrasts, and the latter using six similar photoreceptors (R1-6) with the same broadband sensitivity for this task (van der Kooi & Kelber 2022). Chromatic and achromatic contrasts between flowers and their backgrounds have been highlighted as the best predictors of flower visibility to pollinators (van der Kooi & Kelber 2022; van der Kooi & Spaethe 2022).

Different pollinator species have been found to have innate preferences for certain colours, which may condition their choice of flowers, but can be modulated by various factors such as associative learning with floral rewards or the presence of competing pollinators (Reverté *et al.* 2016). It has been reported that some bees display preferences for stimuli of a blue hue, but such preferences may be conditioned by colour aspects other than hue and can be easily overridden by associative learning (Giurfa *et al.* 1995; Dyer *et al.* 2016; Koethe *et al.* 2016). Bees are able to recognise and discriminate flower colour patterns (Hempel de Ibarra *et al.* 2022) and, in the case of yellow flowers, they prefer those with a UV-absorbing centre and a UV-reflecting periphery (Papiorek *et al.* 2016). In contrast, hoverflies have strong innate preferences for yellow flowers (An *et al.* 2018). Naïve hoverflies prefer yellow over other colours for landing, but can learn to visit flowers of different colour, although proboscis reaction cannot be modified by training (An *et al.* 2018; Hannah *et al.* 2019; Garcia *et al.* 2022). On the other hand, *Eristalis* flies prefer brighter yellow flowers over darker yellow flowers for landing response (An *et al.* 2018). In white/yellow dimorphic *Raphanus raphanistrum*, all insect visitors, including bees and syrphid flies, preferred yellow over white flowers (Stanton *et al.* 1986, 1989), and in white/yellow/pink/bronze polymorphic *Raphanus sativus*, bees preferred both white and yellow flowers, and syrphid flies preferred pink over all other flower colours (Stanton 1987).

In the Ranunculaceae, flower colour polymorphisms have been described (Narbona *et al.* 2018). In particular, some species of *Anemone* have individuals with flowers of different colours, such as *A. coronaria* with red, purple–blue and white flowers, and *A. pavonina* with red or purple flowers (Streinzer *et al.* 2019; Dafni *et al.* 2020). In this study, for the first time we analyse in detail a flower colour polymorphism in *Anemone palmata*, a species that inhabits scrublands and holm oak and cork oak forests in the Western Mediterranean, in which individuals are most frequently yellow-flowered but may also be white-flowered (Montserrat 1986).

The main aims of this study were to explore the floral colour dimorphism in *A. palmata* though the following questions: (i) what is the distribution of colour morphs across its range; (ii) are there differences in spectral reflectance, UV–VIS photography and pigments between and within (patterns) colour morphs; (iii) are there differences in reproductive traits between the common yellow morph and the rarer white morph; (iv) do the morphotypes share pollinators, and do the pollinators cause any assortative mating; (v) how are the flower colour loci of the two morphotypes distributed in the colour vision spaces of bees and hoverflies; and (vi) are there fitness differences between floral colour morphs?

MATERIAL AND METHODS

Study system

Anemone palmata L. is a perennial herbaceous plant with a tuberous rhizome that grows in wet grasslands or under trees up to 1,500 m altitude in the western Mediterranean region (Montserrat 1986). *A. palmata* have a basal rosette and few, frequently only one, scape-like flowering stem bearing a terminal solitary flower, sometimes with another late developing lateral flower stem. Flowers have a perianth composed of petaloid sepals, an androecium with multiple stamens, and a gynoecium with a variable number of free carpels, each with a single ovule, fruit being a poly-achene (Montserrat 1986). Plants reproduce both sexually and vegetatively. The species generally exhibits yellow flowers, although a colour polymorphism with both white- and yellow-flowered individuals appears in several populations. The species only offers pollen as a reward to pollinators (Hidalgo & Cabezudo 1995). It seems to be totally dependent on pollinators to set fruit, since when pollinators are excluded by bagging (11 flowers per morph) absolutely no achenes set, and could be mostly allogamous, since after manual self-pollination of flowers (only six), most did not set any achenes and the rest set very few (Rodríguez-Castañeda, unpublished data).

Frequency of flower colour morphs and reproductive trait variation between morphs

To analyse the geographic distribution of the two colour morphs, we downloaded all records of *A. palmata* from iNaturalist (iNaturalist.org, 23/11/2022) and revised photographs to identify the colour morph. A total of 432 records were retained after removing records with <5 km of positional accuracy (N = 19) and records with photographs that do not allow flower colour identification (N = 39). We also checked photographs of white morphs of *A. coronaria, A. hortensis* and *A. pavonina* as well as those of *Anemonastrum narcissiflorum*, *Anemonoides nemorosa* and *A. trifolia*, to detect possible misidentifications.

Field study was carried out in February, March and April from 2018 to 2020 in two polymorphic populations in Aznalcázar (37°14′ 8.46″ N, 6°10′ 17.40″ W; Seville) and Punta Umbría (37°14′ 03.8″ N, 7°03′ 52.6″ W; Huelva), both with a typical Mediterranean climate, characterised by high temperatures and a scarcity of precipitation in summer. To assess the proportions of morphs in both populations, we counted plants of each morph in two consecutive years at the peak of flowering (2018–2019). The population of Punta Umbría is small, thus we recorded the entire population: 542 and 149 flowering plants in the first and second year, respectively. In Aznalcázar, we made a linear transect throughout the population centre, recording 1816 and 438 plants, respectively. The lower number of flowering plants in the second sampling year was probably related to the low rainfall in 2019 (Aznalcázar meteorological station, January–April: 351.4 mm in 2018, 111.2 mm in 2019). At the same time, we characterised some floral traits related to the visual attraction of pollinators. Sepal number was recorded in 37 yellow and 19 white flowers in Aznalcázar, and in 20 yellow and 21 white flowers in Punta Umbría. To estimate floral area, these flowers were photographed, and their diameter measured using the software ImageJ (version 1.52a; National Institute of Health, USA). Moreover, flowering stem length of individual plants was recorded in 43 yellow- and 42 white-flowered plants in Aznalcázar, and 20 yellow- and 24 white-flowered plants in Punta Umbría. We took all the photographs and measured the length of the stem when the flower was fully open and at the beginning of the female phase.

In addition, in Aznalcázar, we collected 15 floral buds of each morph from different plants to determine numbers of carpels and stamens, and to estimate pollen production by means of a particle counter (Coulter Multisizer 3). At the same time, to assess pollen as a reward, we recorded pollen grain diameter, and calculated the volume of pollen produced by each flower. Moreover, to characterise sexual phases and longevity of flowers, we followed the development of 11 yellow and 10 white flowers from bud to senescence. Lastly, we weighed 10 rhizomes of the yellow morph and 13 of the white morph.

Flower colour patterns and pigments

To identify possible UV colour patterns in both yellow and white flowers, we took UV photographs. After which, we measured the UV–VIS spectral reflectance of apices and bases of adaxial sides of sepals in 10 flowers per morph and population, using a portable spectrophotometer 'Jaz' equipped with a deuterium–tungsten light source (200–800 nm; Ocean Optics, Dunedin, FL, USA). The spectral data were processed with PAVO R-package (Maia *et al.* 2019). First, each spectrum was scaled, setting the minimum value to zero using the function 'prospect' to handle negative values. Then, the noise was removed with the same function, using the smooth option with a smoothing parameter of 0.2.

To determine pigments responsible of flower colour, we collected 10 yellow and 10 white flowers in the Aznalcázar population in March 2024. We used 10 mg of apices and bases of the sepals of each flower for pigment extraction in both acidified methanol (1% HCl) and in pure acetone. We then analysed the absorbance spectra (280–700 nm) of each extract with a spectrophotometer (Thermo Fisher Scientific, MA, USA) and identified major pigment classes based on absorbance peaks (see Narbona *et al.* 2021a, for details).

Pollinator visits and their perception of flower colour

To assess pollinator visits to each colour morph, 10-min censuses were conducted on three different sunny days in 2019 between 10:00 h (flowers remain closed until then) and

15:30 h (pollinator activity declines sharply after this time), for a total of 18.66 h. For each of the 113 censuses, we chose patches with yellow and white flowers to test visits to each morph and transitions between morphs; number of open flowers of each morph was recorded, with an average of 52.2 yellow flowers and 9.7 white flowers per census. For each pollinator observed, we recorded the number of flowers of each colour visited and the transitions within and between colour morphs. To discount the effect of differences in abundance between morphs, for each morph and census, the numbers of visits recorded for each visitor group were extrapolated to 100 flowers to calculate standardised visitation rates (number of visits to 100 flowers in a 10-min census). From transition data, we calculated the Bateman constancy index (Bateman 1951).

We then assessed flower colour as perceived by bees and hoverflies, the most important pollinator groups found in the abovementioned censuses (see Results). On the one hand, we plotted the smoothed reflectance spectral functions of the sampled sepals as loci in both the vision space model proposed by Chittka (1992) for bees and that proposed by Troje (1993) for dipterans. For the bee model, we used a spreadsheet following Chittka & Kevan (2005) with photoreceptor spectral sensitivities of *Apis mellifera*. For the dipteran model, we used the spreadsheet provided by An *et al.* (2018) with photoreceptor spectral sensitivities of *Eristalis tenax*. *A. palmata* flowers are presented against green leaves in full sunshine, thus we considered the reflectance spectrum of green foliage provided by Chittka & Kevan (2005) as the dominant background, and the standard daylight D65 (Wyszecki & Stiles 2000) as the illuminant. From each spectral function plotted in the two vision spaces, we calculated chromatic contrast to background and hue as chromatic signals. Chromatic contrast was recorded as the Euclidean distance from the sample locus to the dominant background locus, which is coincident with coordinate origin in the centre of the vision space (Spaethe *et al.* 2001; Chittka & Kevan 2005; An *et al.* 2018). Hue was recorded as an angular value, with 0° on the upper vertical axis and increasing values clockwise in the bee vision space (Dyer *et al.* 2012; Shrestha *et al.* 2014), but with 0° on the right horizontal axis and increasing values counterclockwise in the hoverfly vision space (Shrestha *et al.* 2016). Additionally, for each sampled flower, we calculated the Euclidean distance between the loci of its apex and its base (hereafter apex–base chromatic contrast) in both vision spaces. Finally, considering all sampled flowers, we calculated the distances between the apices of each possible pair of flowers, represented by the Euclidean distances between their loci in both visual spaces. Moreover, for each reflectance spectral function of the sampled sepals, we calculated achromatic contrast to background under both bee and hoverfly vision. This was calculated as the difference between the excitations produced by the stimulus and by the background in the photoreceptor involved in achromatic vision (Spaethe *et al.* 2001; Morawetz *et al.* 2013). In the case of bees, this was the green photoreceptor, while in the case of hoverflies, it was the R1-6 photoreceptor (van der Kooi & Kelber 2022).

The fact that the yellow flowers of *A. palmata* have a UV-absorbing centre and a UV-reflecting periphery on the adaxial side but accumulate UV-absorbing flavonoids throughout the sepal (see Results), raises the question of whether the entire abaxial side is UV-absorbing and whether the centre is similar to the androecium (Lunau *et al.* 2017, 2021). We then measured reflectance in another set of flowers (six per morph), both on the adaxial and abaxial sides of the sepals, and on the stamens, and plotted the corresponding loci in the vision spaces of bees and hoverflies.

Fitness measurements

Female fitness under open pollination was calculated for 60 plants from the Aznalcázar population (N = 42 yellow-flowered, N = 18 white-flowered) and 58 plants from the Punta Umbría population (N = 37 yellow-flowered, N = 21 white-flowered). For each sampled plant, we counted the number of flowers, and the numbers of ripe achenes and non-fertilised carpels per flower. The seedset was calculated as the proportion of ripe achenes relative to the number of carpels per flower (sum of ripe achenes and non-fertilised carpels). Lastly, we calculated seed production per plant as the total number of ripe seeds produced, which is the product of the number of ripe achenes per flower multiplied by the number of flowers per plant.

Data analyses

Differences between colour morphs and populations in floral traits and fitness measurements were analysed using generalised linear models (GLM) with different link functions and error distributions depending on the type of variable response modelled. Poisson's distribution was used to analyse sepals per flower, flowers per plant and stem length. Quasi-Poisson's distribution was used to analyse seeds per flower, seeds per plant and floral size. Quasi-binomial's distribution was used to analyse seedset.

Traits related to reproductive potential were compared between colour morphs using a *t*-test for ovules per flower, anthers per flower, pollen grains per flower and rhizome weight, and a Wilcoxon rank-sum test for pollen grain diameter, pollen volume per flower and pollen/ovule ratio. To analyse floral stages, the Wilcoxon rank-sum test was used to compare the medians of each floral stage between colour morphs. In addition, we used the *t*-test to compare flower longevity.

Differences between morphs in pollinator visitation rates were compared using GLM with Poisson error distribution and log link function. To test whether pollinator transitions between colour morphs were those expected from the morph proportions, we used the Chi-square test. Differences between morphs and sepal positions (apex versus base) in chromatic and achromatic signals as perceived by pollinators, as well as differences in distances between apices of flower pairs according to pair type (white–white, yellow–yellow, yellow–white), were analysed by GLM with normal error distribution and identity link function.

RESULTS

Frequency of flower colour morphs and associated trait variations

When analysing data from iNaturalist, most of the records of *A. palmata* were from Portugal (331), followed by Spain (52), Algeria (45) and France (1), and white morphs appeared only

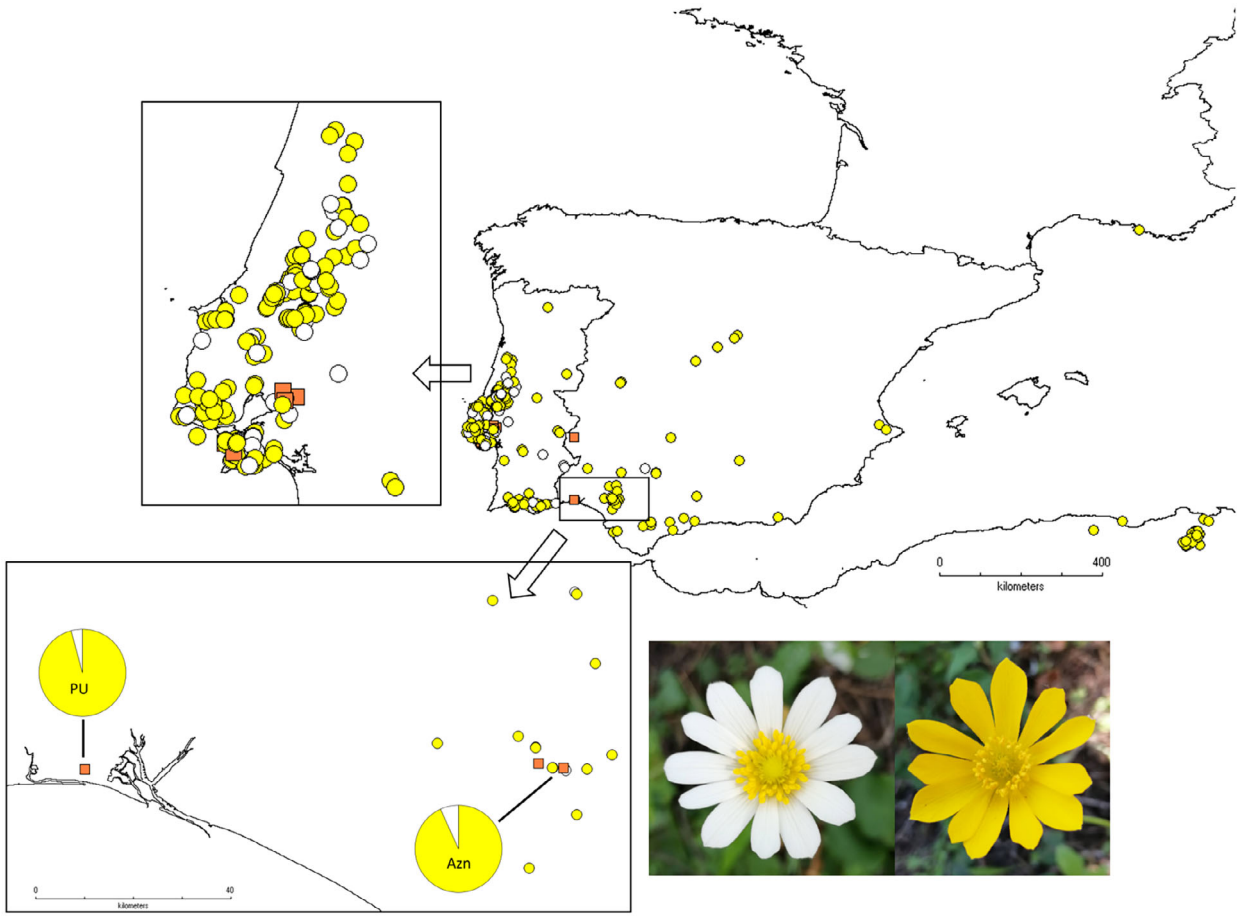

Fig. 1. Geographic distribution of flower colour morphs of *Anemone palmata* collected from iNaturalist and the two populations of this study. White and yellow circles represent records with photographs of either morph, whereas orange squares represent records with both morphs. Pie charts represent the mean proportion of yellow and white morphs in 2 years in the populations studied.

in Portugal (58) and Spain (6). White records were mainly found in the west centre (Leiria, Santarem, Lisboa, and Setubal districts) and in the south (Faro) of Portugal and in the south-west of Spain (Andalusia and Extremadura). There were six records in Portugal with both white and yellow flowers and three records in Spain (Fig. 1).

In the two sampled populations, white-flowered plants were much less frequent than yellow-flowered plants, the relative frequency of morphs slightly varying between populations and years. In Aznalcázar, the frequency of white-flowered plants was 6.2% and 7.4% in 2017 and 2018, respectively, and in Punta Umbría was 4.2% for both years.

Both floral size and flower number per plant tended to be higher, but not significantly, for yellow-flowered plants in both populations (Table 1 and Table S1). In contrast, differences between colour morphs and populations, and their interaction for stem length were significant (Table 1). Plants from Punta Umbría had longer stems than those from Aznalcázar; moreover, white-flowered plants had longer stems than yellow-flowered plants in Punta Umbría but the reverse pattern in Aznalcázar (Table S1).

We found significant differences between colour morphs for some of the traits related to reproductive potential: both number of ovules and pollen grains per flower were higher for yellow-flowered plants (Fig. 2). In contrast, we did not find significant differences between morphs for the number of anthers per flower, pollen/ovule ratio or rhizome weight (Fig. 2, Figure S1). Regarding pollen as reward, we found that pollen grain diameter was significantly higher for white-flowered

Table 1. Summary of GLM results of different factors: colour morphotype (yellow/white), population (Aznalcázar/Punta Umbría) and their interaction on visual attraction traits of *Anemone palmata*.

traits	factors	Wald chi-square	df	*P*
Sepals per flower	Colour	0.01	1	0.93
	Population	0.16	1	0.69
	Colour × population	0.07	1	0.8
Floral size (cm^2)	Colour	0.28	1	0.6
	Population	0.06	1	0.8
	Colour × population	0.19	1	0.66
Flowers per plant	Colour	0.29	1	0.59
	Population	0.03	1	0.87
	Colour × population	0	1	0.95
Stem length (cm)	Colour	9.77	1	**<0.01**
	Population	179.85	1	**<0.001**
	Colour × population	7.73	1	**<0.01**

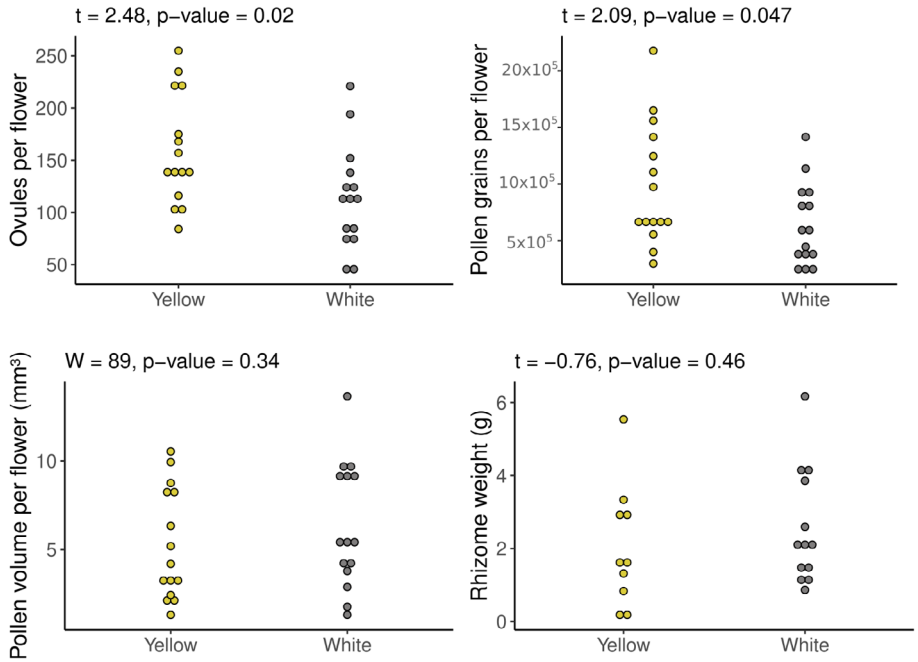

Fig. 2. Dot plots for ovules per flower, pollen grains per flower, pollen volume per flower and rhizome weight of colour morphotypes of *Anemone palmata* from the Aznalcázar population. *t* = *t*-test and W = Wilcoxon rank-sum test.

plants (Figure S1), although pollen volume per flower was not significantly different (Fig. 2).

We recognised three phases in flower anthesis: opening, female phase and male phase, where both morphs showing a marked protogyny (see Table S2, Figure S2). There were no significant differences between colour morphs in the length of the female phase, male phase, or total flower lifespan (Table S2). Female phase lasted for 1–8 days and male phase for 4–24 days; total flower longevity was 14–33 days.

Flower colour patterns and pigments

The yellow flowers showed a 'bull's eye' pattern with a UV-absorbing centre, which included the bases of the sepals and the stamens in the centre, and a UV-reflecting periphery (Fig. 3a, Figure S5a). White flowers were UV-absorbing over their entire surface but had a striking colour pattern because of the chromatic contrast between the yellow androecium and the white sepals (Fig. 3f). In yellow sepals, the reflectance curve of the apex showed a sharp step at 500 nm and reflected uniformly until 700 nm, with a peak of lower reflectance at around 350 nm (Fig. 3b), while the base reflected only from 500 to 700 nm (Fig. 3c). White sepals had an apex that reflected uniformly from 400 to 700 nm (Fig. 3g); some of their bases also reflected uniformly from 400 to 700 nm, but many of them had a lower reflectance from 400 to 500 nm (Fig. 3h), appearing yellowish to the human eye (Figure S2). In both morphs, the reflectance was higher in the apices than in the bases.

For yellow flowers, the mean chromatic contrast between the central sepal area and the androecium was 0.13 HU (range: 0.06–0.24) and 0.14 TU (range: 0.09–0.27) in bee and hoverfly vision spaces, respectively; for white flowers, the corresponding values were 0.26 HU (range: 0.13–0.33) and 0.22 TU (range: 0.13–0.32). These colour loci are plotted in Figure S5c, d, g, h. In both morphs, the abaxial sides of the sepals were entirely UV-absorbing (Figure S5b, f).

Pigment methanol extracts showed an absorbance peak at 345 nm, especially pronounced in the white apices and bases, followed by the bases of the yellow morphotype and a much lower value for the yellow apices (Fig. 4a). Pigment acetone extracts from yellow sepals showed absorbance functions with a blunt peak near 420 nm and two sharper peaks at 444 and 472 nm, with values higher for yellow apices, whereas those from white sepals showed no absorbance from 400 to 700 nm (Fig. 4b).

Pollinator visits and their perception of flower colour

We recorded a total of 282 pollinator visits, 252 to yellow flowers and 30 to white flowers. When visitation rates were standardised to 100 open flower of each morph (see methods) we obtained visitation rates of 5.177 and 3.249 visits 10 $min^{-1} \cdot 100^{-1} \cdot flowers^{-1}$ respectively, which are significantly different (Wald chi-square = 51.055, 1 df, $P < 0.0001$). Visitors included Coleoptera, Hymenoptera and Diptera, the last being the most diverse (Figure S3, Table S3). *Andrena* sp. accounted for most of the recorded visits of Hymenoptera, *Episyrphus balteatus* was the most frequent visitor among Syrphidae, and *Tipula* sp. and a species of Muscidae were the most frequent in the 'Other Diptera' group (Table S3). For Hymenoptera and Syrphidae, standardised visitation rates to yellow flowers were significantly higher than those to white flowers, while the reverse pattern was found for 'Other Diptera' and no difference was found for Coleoptera (Fig. 5).

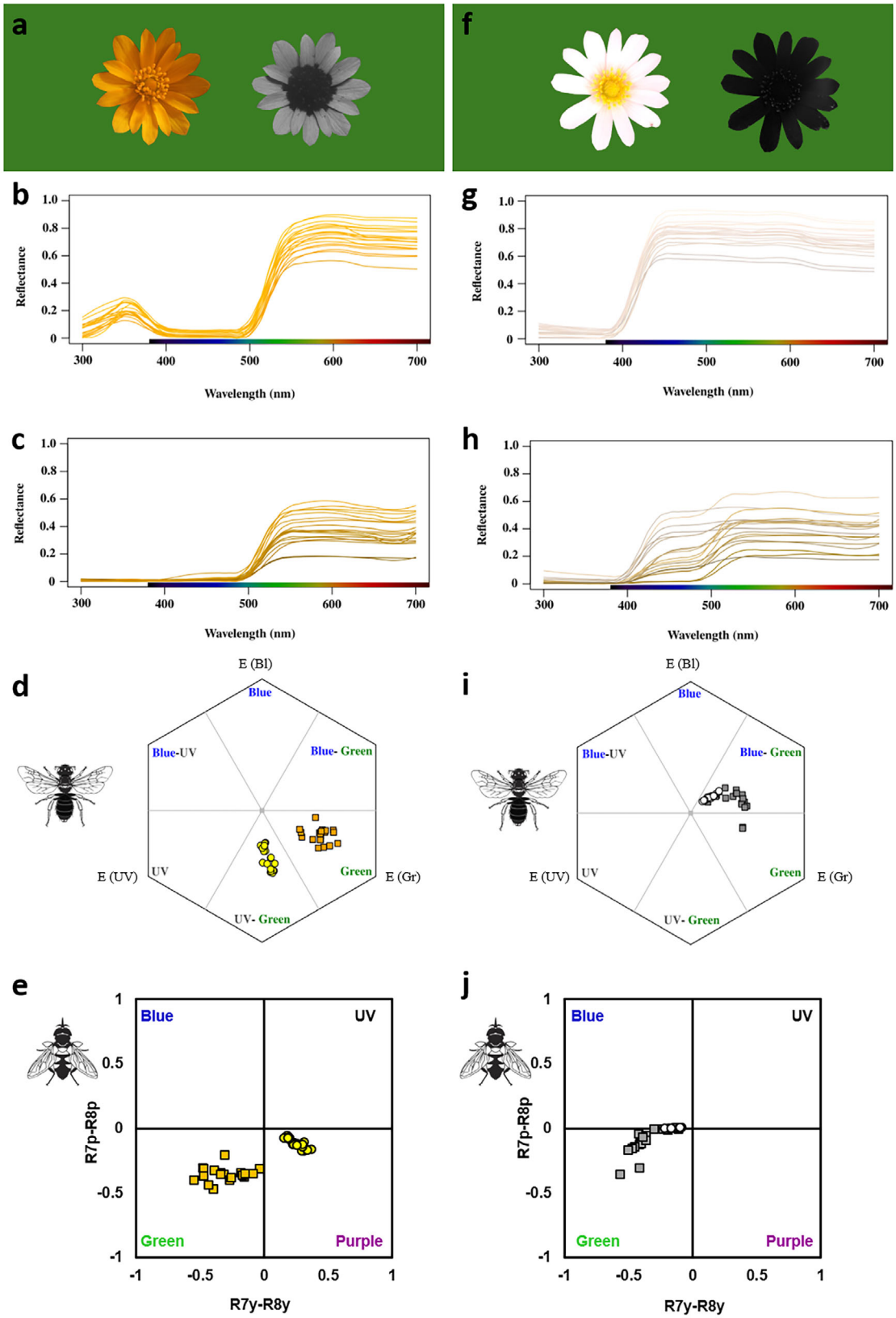

Fig. 3. Yellow (a) and white (f) morphotypes of *Anemone palmata* under visible (left) and UV (right) light. Reflectance spectra of apices (b) and bases (c) of yellow sepals of *A. palmata*, and those of apices (g) and bases (h) of white sepals. Loci of yellow (d) and white (i) sepal apices (circles) and bases (squares) in the vision space of *Apis mellifera*. Loci of yellow (e) and white (j) sepal apices (circles) and bases (squares) in the vision space of *Eristalis tenax*. Pollinator silhouettes were taken from Divulgare (www.divulgare.net) under a creative commons licence.

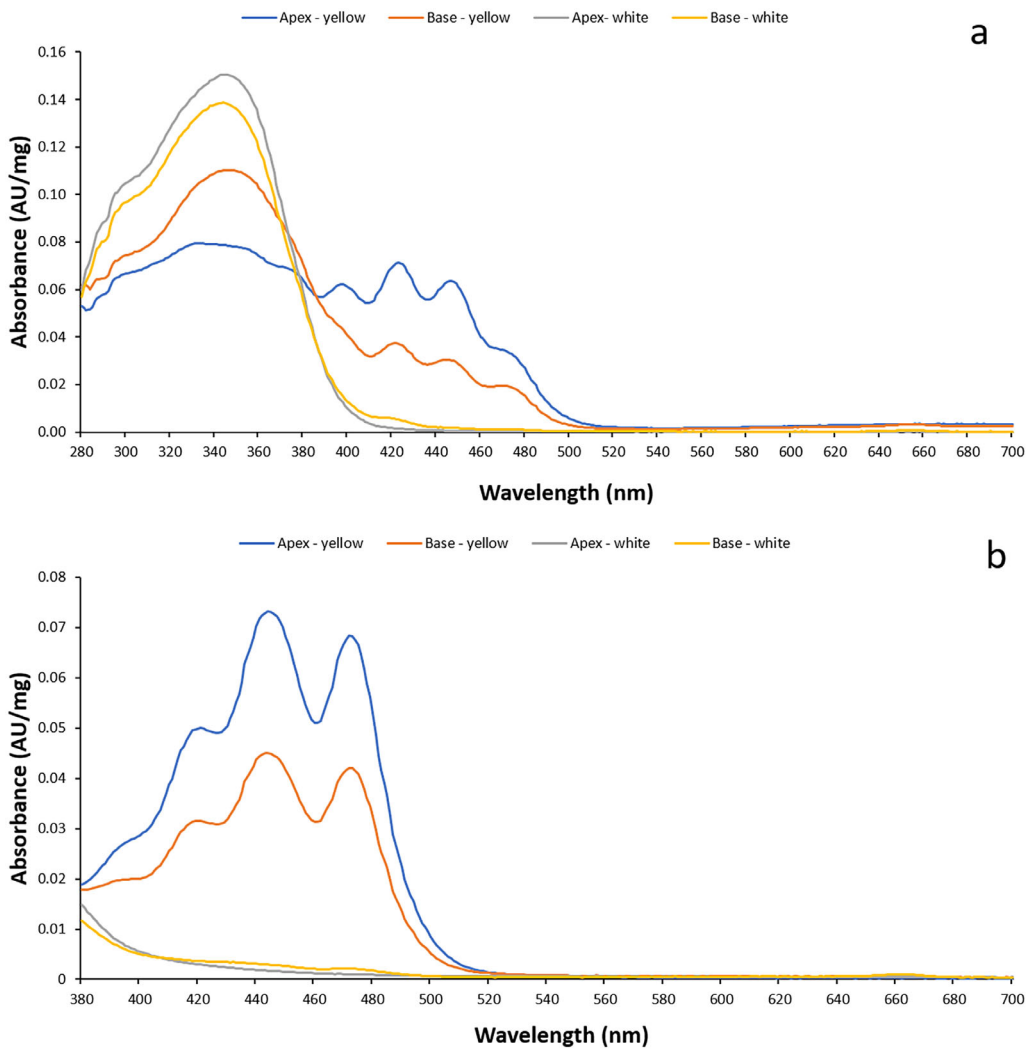

Fig. 4. Mean absorbance spectra of the floral pigments extracted with either acidified methanol (a) or pure acetone (b) from the apex and base of flower colour morphotypes of *Anemone palmata*. Absorbance values are expressed as arbitrary units per unit sample weight (AU·mg^{-1}).

We observed 73 transitions among flowers: up to 67 from yellow to yellow, three from white to white, two from yellow to white and one from white to yellow. Observed yellow–yellow transitions were higher than expected ($\chi^2 = 20.92$, $P < 0.0001$), white–white transitions were as expected ($\chi^2 = 0.36$, $P = 0.5481$) and yellow–white and white–yellow were lower than expected ($\chi^2 = 7.57$, $P < 0.01$; $\chi^2 = 9.55$, $P < 0.01$, respectively). The Bateman index for the entire set of visitors was C = 0.82.

In the bee vision model, the loci of the yellow sepal apices were located in the UV-green region and those of their bases in the green region (Fig. 3d). In contrast, loci of the white sepal apices were close to the achromatic centre in the blue-green region, and those of their bases were further away from this centre, almost all in the same region, but some in the green (Fig. 3i). In the hoverfly vision model, the loci of the yellow apices were located in the purple region and those of their bases in the green region (Fig. 3e). The loci of the white apices were located in the blue region, very close to the border with the green region (Fig. 3j), while those of their bases were located in the green region (Fig. 3j).

For both bees and hoverflies, we found significant differences between the apex and base of both morphs for the chromatic contrast to background, hue angle (Fig. 6a, b), and achromatic contrast to background (Figure S4a, b). For both insect groups, chromatic contrast to background was significantly higher for the yellow morph in either the apex or the base (Fig. 6a, b), while the reverse pattern was found for the achromatic contrast to background, the difference not being significant in the base for hoverflies (Figure S4a, b). For both insect groups, we found that the apex–base chromatic contrast was significantly higher for the yellow morph (Fig. 6a, b), and that the distances between apex pairs were much higher between morphs (means of 0.49 HU for bees and 0.40 TU for hoverflies) than within each morph (Fig. 6a, b).

Fitness measurements

We found that all three components of female fitness analysed (seeds per flower, seed set and seeds per plant) were significantly higher for yellow-flowered plants than for white-

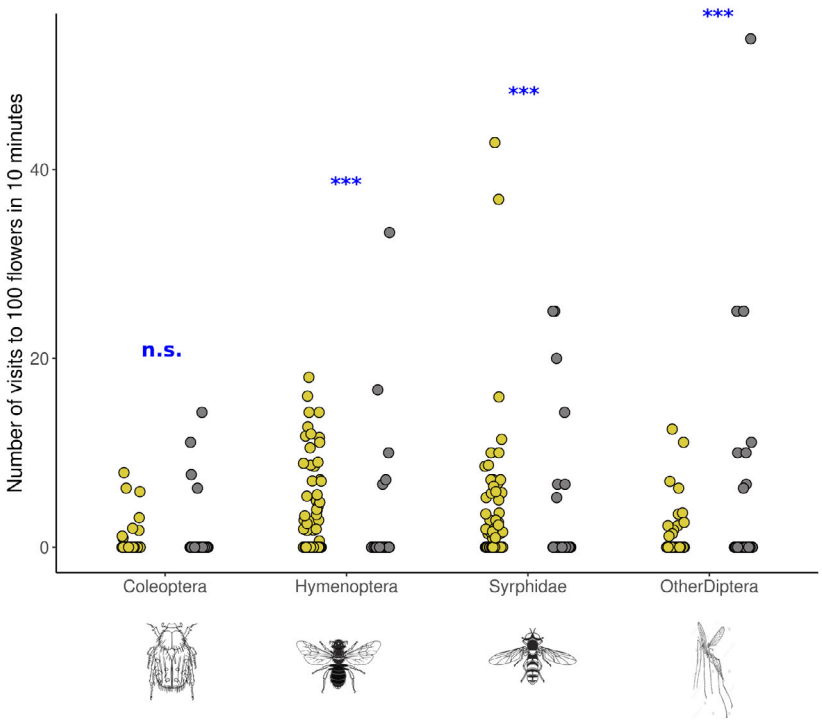

Fig. 5. Dot plots for pollinator visits to flower colour morphotypes of *Anemone palmata* recorded during censuses in spring 2019 in the Aznalcázar population. Values were standardised to 100 flowers per morph. GLM results between flower colour morphs for each pollinator group: n.s. $P > 0.05$; ***$P < 0.001$. The silhouettes of Coleoptera, Hymenoptera and Syrphidae were taken from Divulgare (www.divulgare.net) under a creative commons licence and that of "Other Diptera" taken from Shutterstock (www.shutterstock.com).

flowered plants (Table 2). The selection coefficients against the white morph were approximately 0.4 for all fitness components, with the exception of seedset, for which the values were slightly lower. In addition, although seeds per flower and per plant were slightly higher in the Punta Umbría population and seedset was slightly higher in Aznalcázar population, neither of these differences between populations nor the interactions between colour morph and population were significant (Table 2).

DISCUSSION

This study revealed that *A. palmata* exhibits a flower colour dimorphism, with yellow-flowered and white-flowered plants. Notably, the latter are considerably less prevalent and restricted to Portugal and southwestern Spain. This suggests that abiotic environmental factors may be involved in the distribution and maintenance of this polymorphism, as has been reported for other species (Arista *et al.* 2013; Vaidya *et al.* 2018; Surmacz 2023), but further work is needed to clarify this. The higher frequency of the yellow morph, which seems to be fixed in most populations of the species, appears to be supported by the high coefficients of selection we found in the studied populations, although it must be noted that this study refers to only 1 year and two populations. In the following, we will discuss in detail the differences found between morphs in terms of reproductive traits, pollinator visitation and fitness, in an attempt to unravel the underlying causes for the maintenance of the white morph, which appears to be at a selective disadvantage.

In species with floral colour polymorphism, it is common for morphs to differ in traits other than colour, and these differences may favour the performance of one morph over another (Malerba & Nattero 2012; Dafni *et al.* 2020; Rodríguez-Castañeda *et al.* 2020). *A. palmata* colour morphs, however, did not differ significantly for most of the floral traits examined. Floral anthesis occurs equally in both morphs, so that protogyny, which could be coupled with variable levels of self-incompatibility, as in other members of this genus (Müller *et al.* 2000), prevents self-fertilisation, and the long flower duration would increase the likelihood of pollinator visits and cross-fertilisation. Similarly, the main floral traits related to pollinator attraction, i.e. flower size, floral display and amount of reward (pollen volume), did not differ significantly between colour morphs; therefore, no pollinator preference for either morph is expected based on these traits. In contrast, white *A. palmata* flowers produced fewer but larger pollen grains than yellow flowers, a type of trade-off commonly reported across species (Hao *et al.* 2020). These authors argued that bees tend to avoid collecting pollen from species whose grains are large; however, the size differences found between *A. palmata* morphs are unlikely to be large enough to drive bee behaviour. Not only did white *A. palmata* flowers produce fewer pollen grains, but also fewer ovules than yellow flowers, which implies that the sexual reproductive potential of the white morph is lower. However, because of limited sample size, these differences should be interpreted with caution, as these traits show high variability. As reported for other taxa, one could expect a trade-off between clonal and sexual reproduction (Xiao

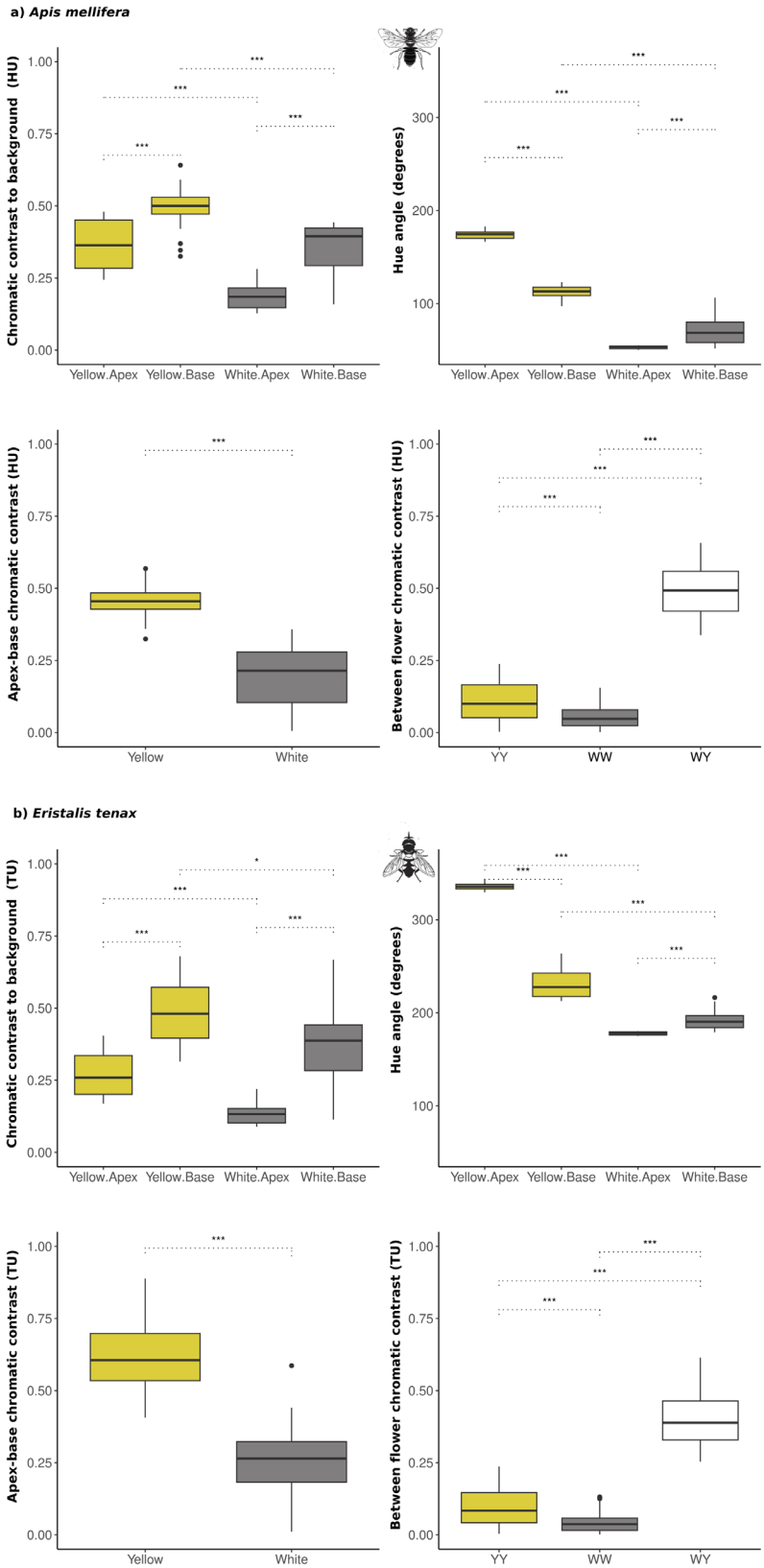

Fig. 6. Box plots for chromatic contrast to background, hue angle, intra-floral apex-base chromatic contrast, and chromatic contrast between pairs of floral apices (YY, both yellow; WW, both white; WY, one of each colour) for the colour morphs of *Anemone palmata* as perceived by *Apis mellifera* (a) and *Eristalis tenax* (b). Significance according to GLM: *$P < 0.05$; ***$P < 0.001$. Pollinator silhouettes were taken from Divulgare (www.divulgare.net) under a creative commons licence.

Table 2. Values for female fitness components, with their means ± SD and ranges, of yellow- and white-flowered plants of *Anemone palmata* in Aznalcázar and Punta Umbría populations and *P*-values from GLM for colour morph, population and their interaction on each female fitness component.

	Aznalcázar			Punta umbría			*P*-values		
fitness components	yellow (N = 42)	white (N = 18)	selection coefficient (*s*)	yellow (N = 37)	white (N = 21)	selection coefficient (*s*)	colour	population	colour × population
Seeds per flower	120.79 ± 41.68 (39–212)	70.78 ± 30.89 (9–127)	0.41	132.95 ± 45.96 (40.5–209)	85.51 ± 59.65 (3–221)	0.36	**<0.001**	0.24	0.61
Seeds per plant	236.95 ± 122.79 (53–585)	137.63 ± 87.1 (9–254)	0.42	270.65 ± 137.38 (67–835)	152.4 ± 141.39 (3–573.33)	0.44	**<0.05**	0.74	0.93
Seedset	0.77 ± 0.18 (0.29–1)	0.52 ± 0.26 (0.1–0.93)	0.32	0.59 ± 0.17 (0.25–0.93)	0.44 ± 0.25 (0.03–0.81)	0.25	**<0.001**	0.25	0.13

Because there was a significant difference between colours, selection coefficients were calculated as $1-(w_1/w_2)$, where w_1 is the disadvantaged morph and w_2 is the morph having higher fitness, following Schemske & Bierzychudek (2001). Values less than 0.5, shown in bold, are considered statistically significant.

et al. 2011; Van Drunen & Dorken 2012; Herben *et al.* 2015). However, although rhizomes of the white morph of *A. palmata* were slightly heavier, this difference was not significant. Unfortunately, measuring rhizomes is destructive, so sampling had to be very limited, but this question deserves a long-term greenhouse study to explore if such a trade-off exists in *A. palmata*. In any case, clonal propagation could be important for the persistence of the rarer white morph, thus contributing to the maintenance of polymorphism, as suggested for other polymorphic rhizomatous species (Imbert *et al.* 2014; Johnson *et al.* 2021).

The three peak absorbance functions of the acetone extracts from yellow sepals are consistent with the typical absorbance function of carotenoids, particularly β-carotene (Lee 2007; Narbona *et al.* 2021b). Therefore, carotenoids seem to be responsible for the colour of yellow flowers and are absent from white flowers. In carotenoid-based colour polymorphisms, white flowers can be caused by mechanisms that disrupt carotenoid biosynthesis and, less commonly, by subsequent degradation of carotenoids (Ohmiya *et al.* 2006; Zhu *et al.* 2010). The absorbance functions of the methanol extracts were similar for both colour morphotypes and showed a peak consistent with that of UV-absorbing flavonoids (Narbona *et al.* 2021b); this peak being similarly high in white apices and bases, less pronounced in yellow bases and reduced to a slight plateau in yellow apices. This would indicate that these flavonoids are homogeneously distributed in the white sepals, but not in the yellow ones, which present a higher concentration in their bases. Thus, the two morphotypes of *A. palmata* differ in two aspects: synthesis or not of carotenoids, and even or uneven distribution of UV-absorbing flavonoids throughout the sepal. UV reflectance is negatively correlated with the concentration of UV-absorbing flavonoids (Dudek *et al.* 2020), and thus the even distribution of highly concentrated flavonoids is responsible for the strong UV absorption throughout the entire white sepals, whereas the uneven distribution of these pigments in yellow flowers results in UV patterns hidden from the human eye (Tunes *et al.* 2021; Todesco *et al.* 2022). Yet, the presence of these flavonoids in the yellow apices, which reflect UV on their adaxial side, can account for the total UV absorption detected on their abaxial side (Figure S5b). Flower colour polymorphisms appear to be common in Ranunculaceae (Narbona *et al.* 2018), and more specifically in the genus *Anemone*, where several cases have been reported (Streinzer *et al.* 2019; Dafni *et al.* 2020), but no studies have considered possible UV patterns. Indeed, few studies have reported intraspecific variation in UV floral patterns, either continuous (Koski & Ashman 2013; Zhang *et al.* 2017) or discrete with differently sized patterns (DeMarche *et al.* 2015). Notwithstanding, the UV bull's eye observed in the yellow flowers of *A. palmata*, with a UV-reflecting periphery and a UV-absorbing centre, is common in flowers that are yellow to the human eye (Koski & Ashman 2014; Lunau *et al.* 2021; Tunes *et al.* 2021), and the uniformly UV-absorbing perianth observed in the white flowers of *A. palmata* has been reported in other species with flowers that are white to the human eye (Koski & Ashman 2014), including *Anemone pseudo-altaica* and *A. nemorosa* (Utech & Kawano 1975; Kevan *et al.* 1996).

On the other hand, the absorbances recorded in the white sepals of *A. palmata* suggest that the pigments are very similar over their entire surface, although we found significant

differences in several colour parameters between their apices and bases. These differences can be perceived by both bees (Garcia *et al.* 2017) and hoverflies (Hannah *et al.* 2019), which indicates that white flowers of *A. palmata* also have a bull's eye, which in some cases may appear yellowish to the human eye. Flower reflectance, and hence colour, results from the combination of two factors: the selective absorption of light by pigments, and the scattering and reflection of light by inner (cell layer, cell wall, vacuole, starch granules) and outer (epidermal surface) structures, both of which may be involved in the generation of colour patterns within the flower (van der Kooi *et al.* 2014, 2016; Stavenga & van der Kooi 2016). Such intrafloral structural differences have been described in Ranunculaceae (van der Kooi *et al.* 2017) and even in *Anemone* (Lunau *et al.* 2020); indeed, epidermal cells are conical in the periphery and flat in the centre of flowers of *A. palmata* (pers. obs., Figure S6). It is therefore reasonable to speculate that the observed differences between the bases and apices of white sepals of *A. palmata*, and perhaps to some extent those of yellow sepals, are due to structural rather than pigmentary features, although further observations are needed to confirm this.

Many authors have analysed the extent to which flower colour must differ, both in terms of categorical and continuous variation, for different pollinators to be able to discriminate between colours (Chittka *et al.* 1993; Troje 1993; Garcia *et al.* 2017, 2022; Hannah *et al.* 2019; Finnell & Koski 2021). Not only did the yellow and white flowers of *A. palmata* fall into different colour categories in both the bee and hoverfly vision models, but their loci were far apart (Euclidean distances >0.4) in both models. Therefore, the colour morphs of *A. palmata* were highly distinct, so that flower visitors could easily distinguish between them and use their floral colour features as cues to develop distinct visitation patterns to each colour morph.

As expected from their higher abundance, yellow flowers recorded a higher absolute number of pollinator visits. Nevertheless, even after standardising the visitation data to discount the effect of differences in abundance between morphs, yellow flowers still recorded more visits, which seems to reflect a general preference for yellow flowers. It is noteworthy that this preference is not offset by a probable higher standing crop of reward in the white morph due to fewer visits. However, censuses in balanced experimental patches would be necessary to confirm these preferences. Bees and hoverflies, the most frequent visitors to *A. palmata* flowers, were responsible for this general preference for yellow over white flowers, most likely because of their differences in chromatic contrast to background, hue and colour pattern. Yellow flowers showed a higher chromatic contrast with the green background, which has been reported to be crucial for the detectability of flowers by insects and thus for their recruitment as pollinators (Spaethe *et al.* 2001; Koski 2020). However, the chromatic contrast of white flowers with the background is more than sufficient for bees and hoverflies to detect them (Garcia *et al.* 2017; Hannah *et al.* 2019), raising the possibility that these preferences are hue-mediated; indeed, in other species with white–yellow floral dimorphism, both groups of insects have been reported to prefer yellow flowers (Stanton *et al.* 1986, 1989). In addition, only yellow flowers displayed a UV pattern, with a UV-reflecting periphery and a UV-absorbing centre, which has been proven to be highly attractive to both bees and hoverflies (Koski & Ashman 2014). Lunau *et al.* (2017, 2021) proposed that these yellow UV-absorbing centres mimic the yellow UV-absorbing stamens to simulate a higher pollen supply. Although we cannot rule out that the yellow UV-absorbing centre enhances the visual signal of the androecium (Lunau *et al.* 2024), our results do not support the existence of true mimicry in *A. palmata*, as the chromatic contrast between the perianth centre of each flower and its androecium would allow pollinators to discriminate between the two structures (Lunau *et al.* 2017). In contrast to bees and syrphids, non-syrphid dipterans preferred white flowers to yellow flowers. We can speculate that this preference is determined by the greater achromatic contrast of white flowers, although it remains unclear how dipterans use achromatic cues to detect and choose flowers (Lunau 2014). Another reason for this preference may be the striking chromatic contrast between the yellow stamens and the white sepals, which creates a particular floral pattern that has been found to be attractive to some syrphid flies (Lunau 2014; An *et al.* 2018), but we do not know whether such a pattern is attractive to other dipterans.

Overall, pollinator preferences resulted in a higher level of pollinator attention to the yellow morph and a clear partitioning of pollinators between the two colour morphs. In addition, *A. palmata* pollinators tended not to switch from one morph to another in each foraging sequence, being particularly faithful to the yellow morph. This phenomenon, termed floral constancy, seems to be widespread among pollinators, having been reported mainly in bees but also in dipterans (Goulson & Wright 1998; Grüter & Ratnieks 2011), and although it usually applies to flowers of different species (Waser 1986), it has also been observed towards the morphs of colour polymorphic species (Norton *et al.* 2015; Jiménez-López *et al.* 2020a). Pollinator partitioning between colour morphs, as well as flower colour constancy, have been proposed as mechanisms for maintaining flower colour polymorphism (Sapir *et al.* 2021). Yet, both mechanisms imply some degree of assortative mating that limits gene flow between colour morphs, which might drive speciation in the long term (Narbona *et al.* 2018; Jiménez-López *et al.* 2020a, 2023).

In the two populations studied, the white morph was selected against through female fitness; this is a result of both its lower reproductive potential (in terms of ovule number) and the lower attention it receives from pollinators. This disadvantage of the white morph could lead to its disappearance from populations unless other factors, such as the abovementioned pollinator partitioning and constancy or clonal propagation, allow it to be maintained. Temporal variation in selection regimes (Schemske & Bierzychudek 2001) could also account for the maintenance of flower colour polymorphism in the studied populations. Under this scenario, the fact that *A. palmata* is perennial could allow white-flowered individuals to compensate for fitness over the years. Still, this selection against the white morph may be the reason for its absence in most populations (Fig. 1). In any case, both morphs produced abundant seeds in the study year in both populations, indicating that sexual reproduction is important in *A. palmata*, parallel to clonal propagation, as in other species of the genus (Erbar & Leins 2013). Nevertheless, in dry years with high temperatures early in the season, sexual reproduction in *A. palmata* may be hindered (Buide, unpublished data), and under these circumstances, clonal propagation would be crucial for the maintenance of the two morphs.

In summary, *A. palmata* exhibits a remarkable dimorphism in flower colour: yellow-sepaled UV-patterned flowers versus white-sepaled UV-patternless flowers, the latter with a flower colour pattern resulting from the chromatic contrast of the yellow anthers with the white sepals. Pollinator partitioning between colour morphs and their constancy, as well as clonal propagation, seem to be important for maintaining such a dimorphism.

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AUTHOR CONTRIBUTIONS

The initial idea came from MA and PLO. Data collection was performed by NLR-C and MLB. Statistical analyses were performed by NR-C and PLO. The manuscript was prepared by MLB, PLO, NLR-C, MA and EN.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Dot plots of anthers per flower, pollen–ovule ratio and pollen grain diameter of colour morphotypes of *Anemone palmata* from the Aznalcázar population; *t* = *t*-test and W = Wilcoxon rank-sum test.

Figure S2. Floral stages of the yellow and white floral morphotypes of *Anemone palmata*. Opening: anthers closed, no stigmas receptive (a1; b1). Female phase: anthers closed, stigmas becoming receptive (a2; b2). Male phase: anthers shedding pollen, some stigmas still receptive (a3; b3).

Figure S3. Some pollinators of *Anemone palmata* from the Aznalcázar population. (a) *Andrena* sp., (b, c) *Eupeodes corollae*, (d) *Episyrphus balteatus*, (e, f) *Eristalis tenax*, (g) *Eristalinus taeniops*, (h) *Lobonyx* sp., (i) *Tipula* sp. Photographs: J. Linares (a, g), P. Fernández (b, c, e, f, i) and N.L. Rodríguez-Castañeda (d, h).

Figure S4. Box plots for achromatic contrast to background of apices and bases of flower colour morphs of *Anemone palmata* as perceived by *Apis mellifera* (a) and *Eristalis tenax* (b). Significance according to GLM: n.s. $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pollinator silhouettes were taken from Divulgare (www.divulgare.net) under a creative commons licence.

Figure S5. Reflectance spectra of the stamens of yellow (a) and white (d) flowers, and abaxial apices of yellow (b) and white (f) flowers. Distribution of stamens in the colour hexagon in comparison with the adaxial sepal bases and apices (c, yellow; g, white) and in the vision space of *Eristalis tenax* (d, yellow; h, white). Pollinator silhouettes were taken from Divulgare (www.divulgare.net) under a creative commons licence.

Figure S6. Adaxial epidermal cells of sepals of *Anemone palmata*. Top row, yellow flowers; bottom row, white flowers; left column, apical zones of sepals; right column, basal zones of sepals. Scale bars: 30 μm.

Table S1. Values for visual attraction traits, with mean ± SD and range, of yellow- and white-flowered plants of *Anemone palmata* in Aznalcázar and Punta Umbría populations.

Table S2. Summary of the floral stages of the yellow and white floral morphs of *Anemone palmata* and their approximate duration. Differences in duration between colour morphs were tested by Wilcoxon rank-sum test for each stage and by *t*-test for total flower lifespan.

Table S3. Standardised visitation rates (number of visits to 100 flowers in a 10-min census) and total number of visits in 18.66 h of census of the main pollinators of the floral colour morphotype of *Anemone palmata* in the Aznalcázar population.

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