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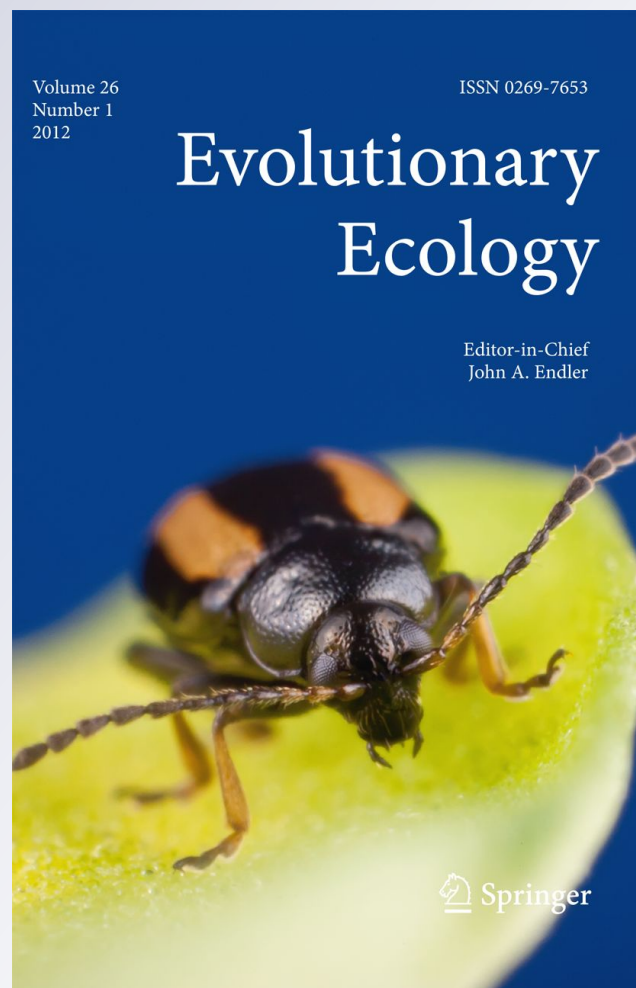
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## Beyond pollinator attraction: extra-floral displays deter herbivores in a Mediterranean annual plant

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**Abstract** The evolution of several floral traits is thought to be driven by multiple selective agents, including pollinators and herbivores. Similar combinations of selection pressures may have shaped extra-floral traits. The conspicuous purple tufts of leaves (“flags”), which often terminate vertical inflorescences in the Mediterranean annual *Salvia viridis*, were shown to attract insect pollinators to the flowering patch. Here we test whether they also function as anti-herbivore signals. We determined the aposematic potential of *S. viridis* flags on three levels: concentrations of anthocyanins, suggested to function as aposematic visual signals, in leaves and flags; spectrometry to estimate whether the color-vision system of two common Mediterranean generalist herbivores (locusts and goats) can discriminate flags from leaves; and choice experiments to determine food preferences of the same herbivores. Anthocyanin concentrations in flags were >10-fold higher than in leaves. Flags exhibited peak reflectance at 450 and 700 nm wavelengths, while leaves reflected maximally at 550 nm. According to the Vorobyev-Osorio color vision model, these differences in color reflection are likely to allow visual discrimination by herbivores. Goats preferred feeding on clipped inflorescences over control inflorescences. Locusts preferred leaves over flags. To test whether this was due to deterrence from the flags’ coloration, we also offered them choice between leaves and a rare, white morph, of the flags. The locusts chose both equally immediately after presentation, but leaves attracted more individuals after 5 min of feeding. The locusts also preferred green cabbage

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over anthocyanin-rich red cabbage. These results support the function of colorful extra-floral displays as warning signals.

**Keywords** Anthocyanin · Flag display · Goat · Locust · *Salvia*

## Introduction

The evolution of floral showy displays has traditionally been attributed to the attraction of pollinating insects (Faegri and van der Pijl 1979). Recent research has highlighted additional biotic selective agents, such as floral herbivores, that shape flower displays (reviewed by Strauss and Whittall 2006). In some cases, pollinators and herbivores exert antagonistic selective forces, i.e. traits that enhance pollination success increase the risk of herbivory and vice versa. Examples of floral traits under conflicting selective pressures include: short floral calyces in *Castilleja lineariaefolia* increase pollination success but extend pre-dispersal seed predation (Cariveau et al. 2004); longer scapes in *Primula farinosa* decrease pollen limitation but increase fruit predation (Ehrlén et al. 2002); Nectar supplementation in *Datura stramonium* increases both pollination and oviposition (and subsequently herbivory) by the moth *Manduca sexta* (Adler and Bronstein 2004); Larger inflorescences attract more pollinators than small ones, but, in *Digitalis purpurea*, also suffer higher predation by moth larvae (Sletvold and Grindeland 2008); similarly, larger plants of the Mediterranean herb *Erysimum mediohispanicum* attract pollinators as well as ungulate herbivores (Gómez 2003); Such opposing selective pressures may contribute to the large variability in some floral traits in natural populations.

Interestingly, in other cases pollinators and herbivores exert similar selective pressures, i.e., the same flower trait attracts pollinators and reduces herbivory. For example, *Hypericum* flowers have UV-reflecting markings that seem to provide a twofold adaptive benefit, since they attract pollinators while repelling caterpillars (Gronquist et al. 2001); Some floral odors both attract pollinators and deter nectar-thieving ants (Junker and Blüthgen 2008); In the genera *Dalechampia* and *Acer*, anthocyanins and resins involved in chemical defense against herbivores also provide pollinator attraction and reward, respectively (Armbruster 2002); the secondary compounds nicotine and caffeine protect vegetative tissues from herbivores, and, at low concentrations, attract pollinating honeybees (Singaravelan et al. 2005).

Colorful and highly visible plant parts occasionally surround flowers (such as in *Boguvillaea*, *Delonix* and *Limonium*), or extend as flag-like tufts of leaves at the top of inflorescences (e.g. in *Salvia viridis* and *Lavandula stoechas*). These extra-floral structures do not have a reproductive function *per-se*. The role of extra-floral displays in pollinator attraction has been demonstrated in a few species (Herrera 1997; Borges et al. 2003; Arnon et al. 2006; Keasar et al. 2009). However it is not yet known whether extra-floral displays can also provide additional selective advantages to the plants, as shown for other traits. Here we address this question by testing the potential anti-herbivore function of the extra-floral “flag” displays of *Salvia viridis* L. (Lamiaceae).

*Salvia viridis* is a Mediterranean annual plant with bright purple flag displays. Its small hermaphrodite protandric flowers are arranged along vertical inflorescences below the flags. The flowers are regularly visited by bees (mainly *Apis mellifera*), but can also spontaneously reproduce by autogamy. Previously we have demonstrated that flags in this species are important in pollinator attraction (Arnon et al. 2006; Keasar et al. 2009). Clipping of all flags from flowering patches in the field reduced the rate of approaches of potential pollinators to the patch, yielding lower seed set per inflorescence. These results

suggested that flags enhance pollination success at the patch level but not at the inflorescence level. An anecdotal result of the flag-clipping manipulation was that most (82%) of the clipped inflorescences, but only few (18%) of the non-clipped inflorescences were grazed by sheep before setting seeds. This reduced the sample sizes for seed counts in inflorescences without flags (Kearse et al. 2009). This observation fueled our hypothesis that *S. viridis* flags may also serve as an herbivore-deterrent signal.

Purple coloration in plants is often due to the presence of anthocyanins, ubiquitous flavonoid pigments in tissues such as young and senescing leaves, petals and fruit. They provide brown-reddish to violet pigmentation (to the human eye), depending on the type of molecules, their concentrations, pH and interactions with other pigments (Mateus and Freitas 2008). A wide range of physiological functions were suggested for anthocyanins. These include protection from stress associated with photo-oxidation, drought, heavy metals and low temperatures (Gould 2004), as well as anti-herbivory effects (Lev-Yadun and Gould 2008). It has been argued that anthocyanins are not harmful to herbivores by themselves, as they are probably non-toxic to mammals (Bridle and Timberlake 1997) and make up a low proportion of the total flavonoid contents of plants (Lee et al. 1987). Nevertheless, anthocyanins could provide a colorful advertisement to the presence of other flavonoids that are formed by the same metabolic pathways (Winkel-Shirley 2002). This could serve as a warning signal, since many flavonoids are toxic (e.g. quercetin, Treutter 2006). Indeed, the presence of anthocyanins is well-correlated with various anti-herbivore effects. Insect herbivores that fed on anthocyanins-rich morphs of *Raphanus sativus* showed lower feeding, growth and reproductive rates than individuals that fed on morphs without anthocyanins and probably other flavonoids (Irwin et al. 2003). Beetle larvae performed better on leaves of *Ipomopsis purpurea* mutants that lacked anthocyanin than on plants that contained high anthocyanin levels (Simms and Bucher 1996). Young red leaves suffered less herbivory by insects than green ones in several *Shorea* species (Numata et al. 2004) and in *Quercus coccifera* (Karageogou and Manetas 2006). Finally, *Betula pubescens* trees with conspicuous red autumn foliage experienced lower insect damage in the following spring compared with conspecifics with duller foliage (Hagen et al. 2003). While these findings are compatible with an aposematic function of anthocyanins, they may also be due to concealment of susceptible plant parts by these pigments. Furthermore, anthocyanin pigmentation may undermine the camouflage of small herbivores from their natural enemies (Lev-Yadun et al. 2004; Lev-Yadun and Gould 2008). In addition, the correlation between anthocyanins and reduced herbivory is not universal. For example, white-flowered *Ipomoea purpurea* plants suffer as much herbivory as plants with anthocyanin-pigmented flowers (Fineblum and Rausher 1997).

The conspicuous purple display of *S. viridis*' flags may indicate the presence of flavonoids, and may function as a visual herbivore-deterrent signal. We tested this hypothesis by (a) determining the anthocyanins content in *S. viridis* flags and leaves; (b) measuring the color reflectance spectra of flag and leaves, and modeling their discrimination by the color vision system of herbivores (Osorio and Vorobyev 2005), to evaluate whether they are perceived as differing in color; (c) comparing the herbivory preferences of a common generalist Mediterranean mammalian herbivore (the domestic goat, *Capra aegagrus hircus* L.) presented with flagged and clipped inflorescences; (d) comparing the preferences of an insect herbivore (the locust *Schistocerca gregaria* Forsskål) for feeding on leaves versus flags; (e) comparing herbivory by *S. gregaria* on leaves and flags of a rare white cultivar of *S. viridis* that contains no anthocyanin, to differentiate between "green leaf attraction" and "purple flag repulsion"; and finally (f) testing whether locusts also avoid anthocyanin-rich tissue in other plant species, by measuring their feeding choices when offered red and green cabbage (*Brassica oleracea* L.).

## Materials and methods

### Study plants

*S. viridis* seeds were collected from a field population in central Israel (Netiv Halamed He). Plants were grown from these seeds in 2009 and 2010, and were used for experiments during their peak bloom in March. In 2010, these plants were augmented with additional flowering individuals collected from wild populations in the South (Lahav forest) and the North (Mt. Gilboa) of Israel. Plants from all populations are regularly exposed to grazing by domestic cows and goats, grasshoppers and snails. Migrating locust swarms also reached the source areas of the study plants until the 1950s. While most wild individuals carry purple flags, rare varieties with white flags are cultivated for gardening purposes. White-flagged plants for feeding choice experiments were obtained from a commercial nursery (Meltzer, Kfar Yedidyah, Israel). Green and red cabbages were bought from a local grocery store.

### Quantification of anthocyanins

Anthocyanin content was determined in one flag and one leaf from each of 20 plants in 2009 and from 12 plants in 2010. Each flag/leaf was collected into a pre-weighed micro-tube and frozen ( $-20^{\circ}\text{C}$ ) until analyzed. Anthocyanin quantification followed a method adapted from Sims and Gamon (2002). Briefly, a micro-tube containing frozen plant tissue was dropped into liquid nitrogen, and ground to fine powder using a hand-held disposable grinder. Tubes were weighted and the powder resuspended in one milliliter of ice cold MeOH:HCl:H<sub>2</sub>O (90:1:1 ratio). Tubes were vortexed for 5 min, left on ice for 2 h with occasional vortex mixing, and then centrifuged (5 min, 12,000g,  $4^{\circ}\text{C}$ ). The supernatant was measured at 529 and 650 nm using a scanning spectrophotometer (UV-1650 PC, Shimadzu, Japan). The supernatant was diluted in water, when needed, to reach the spectrophotometer's linear range. Anthocyanin content was calculated as: Anthocyanin ( $\mu\text{mol/g WW}$ ) =  $[\text{Abs}_{529} - (0.228 \times \text{Abs}_{650})] \times \text{dilution/mg sample}$ . Since flags are much thinner than green leaves, we chose to report the anthocyanin content as  $\mu\text{mol/g}$  rather than as  $\mu\text{mol/m}^2$ .

### Color reflectance spectra from flags and leaves

Reflectance spectra of flags and leaves (*S. viridis*), or green and purple leaves (*B. oleracea*), were recorded and analyzed according to Chittka and Kevan (2005). A single beam UV/Vis spectrophotometer (USB-650 Red Tide, Ocean Optics, USA), equipped with 200–850 nm diffraction grating and a suitable filter (ILX511 detector and UV2/OFLV-4 filter) and 25  $\mu\text{m}$  wide slit was used. The spectrophotometer was connected by means of USB cable to a computer running SpectraSuite software (OceanOptics), and to a 600  $\mu\text{m}$  wide UV-transparent optic fiber (Ocean Optics QP-600-025-SR/BX). All measurements were conducted in a dark room, inside a matte black-painted box, to avoid unwanted light reflections. A UV lamp (Vilber Laurmat model GW, 6 W, light output at 300–400 nm, max  $\lambda$  at 365 nm) and a tungsten lamp (Philips model PF319E/44 Argaphoto 150 W) were used together as a light source. Samples were flattened on a black rubber stage covered with a black insulation tape (sticky side up) and the lamps were placed at  $0^{\circ}$  to the measured sample. The end of the optic-fiber was threaded through and covered with a

black cloth and placed at 45° to the measured surface at a distance of 5 mm. PTEE tape was used as a white reference, and dark spectra were collected by covering the end of the fiber. Integration times were adjusted (from 40 ms for tungsten white reference to 1,200 ms for UV flags/leaves) to avoid saturation, and to accommodate for the differences in brightness between the samples and the white reference. Identical integration times were used for leaf and flag and for green and red cabbage. Dark spectra were collected for all integration times. All measurements were performed in sextuplicates. For each spectrum, the corresponding dark spectrum was subtracted, and the resulting spectrum divided by the integration time. The resulting spectrum was divided by the white reference minus the dark spectrum (all according to Chittka and Kevan 2005).

### Color perception: color triangles and lines

The color vision of locusts is based on three types of photoreceptor with peak sensitivities at 360, 430 and 530 nm (Briscoe and Chittka 2001; Vishnevskaya and Shura-Bura 1990). Color vision in goats (as well as many other ungulates) is dichromatic, with receptor peak sensitivities at 443 and 553 nm (Jacobs et al. 1998; Carroll et al. 2001). The ability of animals to discriminate between color signals depends on the difference in excitation of the color receptors by each signal, and on receptor noise (Osorio and Vorobyev 2005). These data constitute the input for calculating the color distance between the two signals (Hempel De Ibarra et al. 2001). The color distances can be visualized in two-dimensional plots: color triangles for trichromats (Chittka and Kevan 2005) and color lines for dichromats. The minimal color distance that allows reliable color discrimination is determined through behavioral tests (Vorobyev et al. 2001). Color receptor excitation values, for *S. viridis* flags/leaves and red/green cabbage, were calculated for locusts using receptor sensitivity data provided by Vishnevskaya and Shura-Bura (1990). Receptor noise for locusts is difficult to determine, because it varies with the cells chosen for recording (Vishnevskaya and Shura-Bura 1990), and with temperature and light intensity (Faivre and Juusola 2008). In addition, the minimal color distance needed for discriminating two stimuli by locusts is unknown. However, these parameters have been thoroughly characterized for another insect species, the honeybee (Vorobyev et al. 2001). Locust and honeybee receptor sensitivity are similar (comparing data in Vishnevskaya and Shura-Bura 1990 and in Chittka and Kevan 2005). We therefore approximated the color distance between flags/leaves and red/green cabbage, as perceived by locusts, by plotting them on the honeybee color triangle (Chittka and Kevan 2005). We also used receptor error rates for honeybees to estimate the color difference in receptor noise-limited (RNL) units for locusts (Hempel De Ibarra et al. 2001). Color lines for goats (dichromatic) were calculated using receptor sensitivity data from Jacobs et al. (1998), using a similar approach but for only two receptors.

### Feeding choice experiments

#### *Trials with goats*

Each of 63 domestic goats (*C. aegagrus hircus*) from two herds was presented with two bouquets of five *S. viridis* inflorescences. The bouquets were presented to the goats prior to their morning meadow outing, to minimize variability in hunger level. We removed all flags from one of the bouquets, and 1–2 arbitrarily chosen leaves per inflorescence from the other bouquet, to control for possible odor effects due to tissue injury. The left–right positions of the bouquets were varied randomly between tests. We recorded whether the

goats directed their first bite at the flagged bouquet or at the clipped one. When the flagged inflorescences were chosen, we noted whether the goats first fed on flags or on the leaves. Each goat participated in one choice trial.

### *Trials with locusts*

Around 200 insectary-reared fourth- and fifth- instar nymphs of *S. gregaria* were housed in a  $50 \times 50 \times 50$  cm cage, kept at  $33 \pm 2^\circ\text{C}$ , and a 16:8 L:D schedule. The locusts were maintained on grass *ad lib* between experiments, and were starved for 24 h before each feeding trial. Trials were conducted by presenting two 9-cm open petri dishes, each containing a different type of potential food. The locusts were offered three kinds of choices: (1) Purple *S. viridis* flags versus green *S. viridis* leaves—10 replicates, (2) white *S. viridis* flags versus green leaves of the same morph—five replicates, (3) red cabbage leaves versus green cabbage leaves—five replicates. The leaves of each food type were spread out in a single layer on the bottom of the petri dish, and the right-left location of the dishes within the cage was determined by a coin toss. We recorded the number of locusts feeding on each of the dishes at 1-min intervals, for 10 min. We also noted the time of food depletion in the dishes, if it occurred within the 10-min observation period. We weighed the food before and after each feeding trial (except for the first replicate of each choice test), and calculated the proportion consumed during the trial. Therefore, sample sizes for the proportions of biomass consumed are nine for purple flags versus leaves, four for white flags versus leaves, and four for red versus green cabbage.

### Statistical analysis

Both flags and leaves were sampled for anthocyanin content from each *S. viridis* plant. We therefore used a paired *t* test to compare anthocyanin levels between tissues. We calculated Pearson's coefficients to determine correlations in anthocyanin level between flags and leaves within a plant. This was done separately for each year, since concentrations differed significantly between the two study seasons (see results). Anthocyanin levels in red and green cabbage were compared using Wilcoxon tests due to small sample sizes ( $n = 5$ ). In the feeding trials with goats, we treated each feeding choice as a Bernoulli experiment, and defined the outcome "choice of clipped bouquets" as success. We employed a binomial test to calculate the probability for the observed number of successful experiments, if the goats choose randomly between bouquets (i.e., under the null hypothesis that  $p(\text{choice of clipped}) = 0.5$ ). In the feeding trials with locusts we used Wilcoxon matched-rank paired tests to determine whether leaves were preferred over flags (in *S. viridis*), and whether green leaves were preferred over purple ones (in cabbage). Both hypotheses are one-tailed, because they predict reduced predation on purple flags and leaves. We therefore tested them using one-tailed tests.

## Results

### Anthocyanins content

The mean levels of anthocyanins in both flags and leaves varied significantly between the two study years ( $t_{22} = 1.72$ ,  $P = 0.05$  for leaves,  $t_{23} = 3.78$ ,  $P < 0.001$  for flags). Nevertheless in both years concentrations in flags were significantly higher than in leaves



(2009:  $t_{13} = 4.58$ ,  $P = 0.001$ , 2010:  $t_{15} = 3.44$ ,  $P = 0.004$ , Fig. 1). Anthocyanin levels in flags and leaves were significantly correlated within individual plants in 2010 ( $n = 12$ ,  $r = 0.666$ ,  $P = 0.018$ ), but not in 2009 ( $n = 20$ ,  $r = -0.158$ ,  $P = 0.517$ ). Green and red cabbage leaves, which were used as additional test plants in the feeding trials, also differed significantly in anthocyanin contents ( $n = 5$  for both green and red cabbage, Wilcoxon:  $W = 15$ ,  $P = 0.003$ ) with much higher levels in the red cabbage. Anthocyanin levels in both flags and leaves of *S. viridis* were higher than in green cabbage, and lower, about a third, than in red cabbage (Fig. 1).

### Reflectance spectra

Relative reflectance data for *S. viridis* flags and leaves, and for green and red *B. oleracea* leaves, are presented in Fig. 2. A color triangle, providing a visualization of the color signals for locusts and honeybees, is presented in Fig. 3a. The estimated color distances between leaves and flags are 6.36 RNL units (locust) and 8.06 (honeybees), and the distance between green and red cabbage is 7.71 units (locust). Color lines for goats are presented in Fig. 3b.

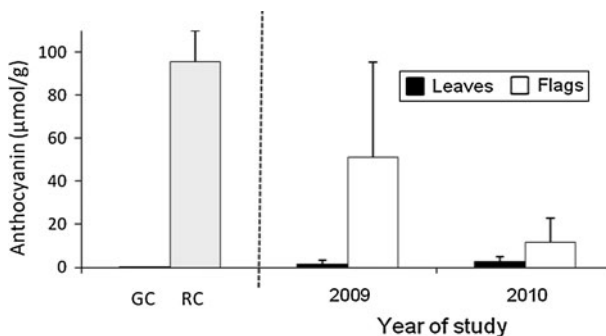
### Feeding choice experiments

#### *Trials with goats*

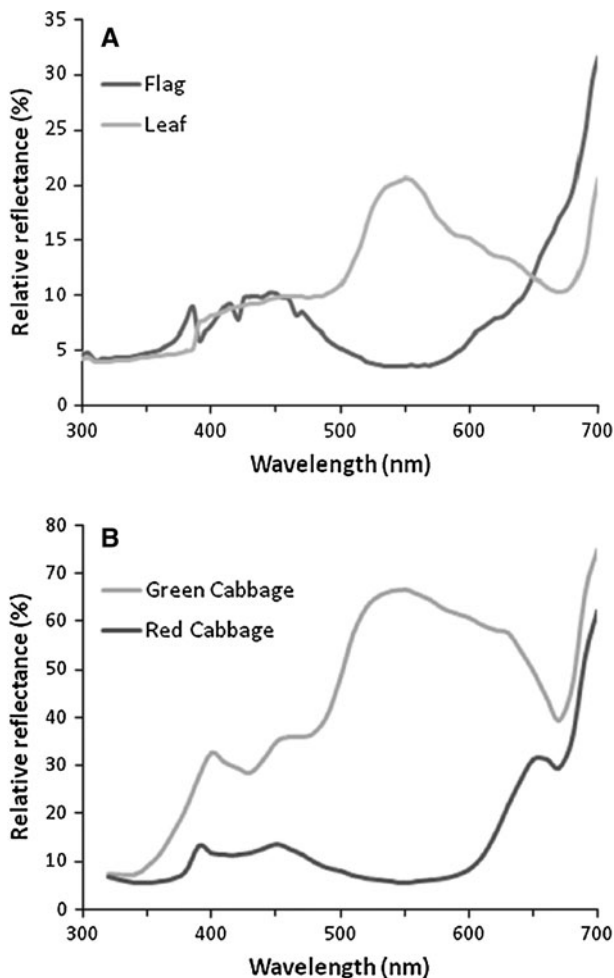
Sixty-three goats were tested. Out of these 30 chose the clipped inflorescences and 12 chose the flagged ones. Nine of the latter goats consumed the bouquet's flags, and the remaining three fed on the leaves at the bottom of the bouquet (Fig. 4). 21 goats chose not to feed on either bouquet and were excluded from the data analysis due to ambiguity in interpretation. Thus, 30 of 42 choosing individuals selected the clipped inflorescences, indicating a statistically significant preference (one-tailed binomial test,  $P = 0.004$ ).

#### *Trials with locusts*

**Purple flags versus leaves** One minute following the presentation of both potential food types, a significantly larger number of locusts fed on leaves than on flags (Fig. 5a, one-tailed Wilcoxon matched-rank test for paired samples:  $n = 10$ ,  $Z = 1.93$ ,  $P = 0.03$ ). After



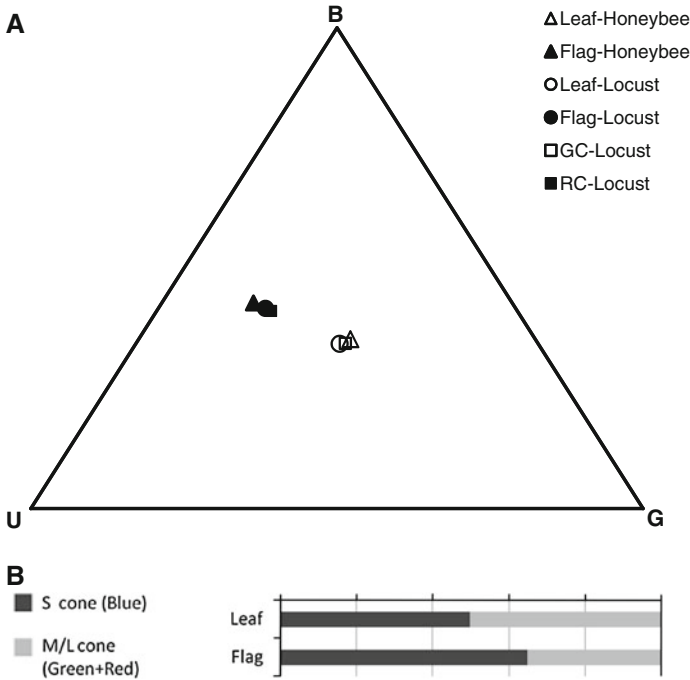
**Fig. 1** Mean (SD) anthocyanin contents in *S. viridis* leaves and flags as measured in spring 2009 ( $n = 20$ ) and 2010 ( $n = 13$ ), and in green (GC) and red cabbage (RC) leaves ( $n = 5$  for each)



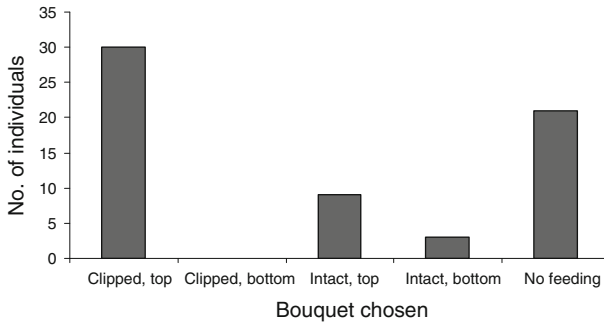
**Fig. 2** Normalized reflected intensity spectra: **a** *S. viridis* flag versus leaf, **b** red versus green cabbage leaf. Lines were smoothed using a 5-point moving average. Each line represents an average of six measurements. Average coefficients of variation of the original values were 18% for flags, 17% for leaves, 8.7% for green cabbage and 39% for red cabbage. Error bars are not plotted for graphical clarity

6 min of feeding, the locusts completely consumed one of the food types in half of the 10 trials. We therefore compared the number of locusts on both food types at the end of the 5th min as the latest time that two feeding choices were available, in all 10 trials. At this time point, the preference for leaves over flags was more pronounced ( $n = 10$ ,  $Z = 2.41$ ,  $P = 0.008$ ).  $55.9 \pm 14.1\%$  and  $40.4 \pm 13.3\%$  (means  $\pm$  SEs) of the initial mass of leaves and flags, respectively, were consumed during the 10-min trials. These percentages did not differ significantly ( $n = 9$ ,  $Z = 1.24$ ,  $P = 0.11$ ).

To maintain consistency in data analysis among all three sets of locust choice tests, we compared the number of individuals feeding on both potential food types, one and five minutes after food exposure in all tests.



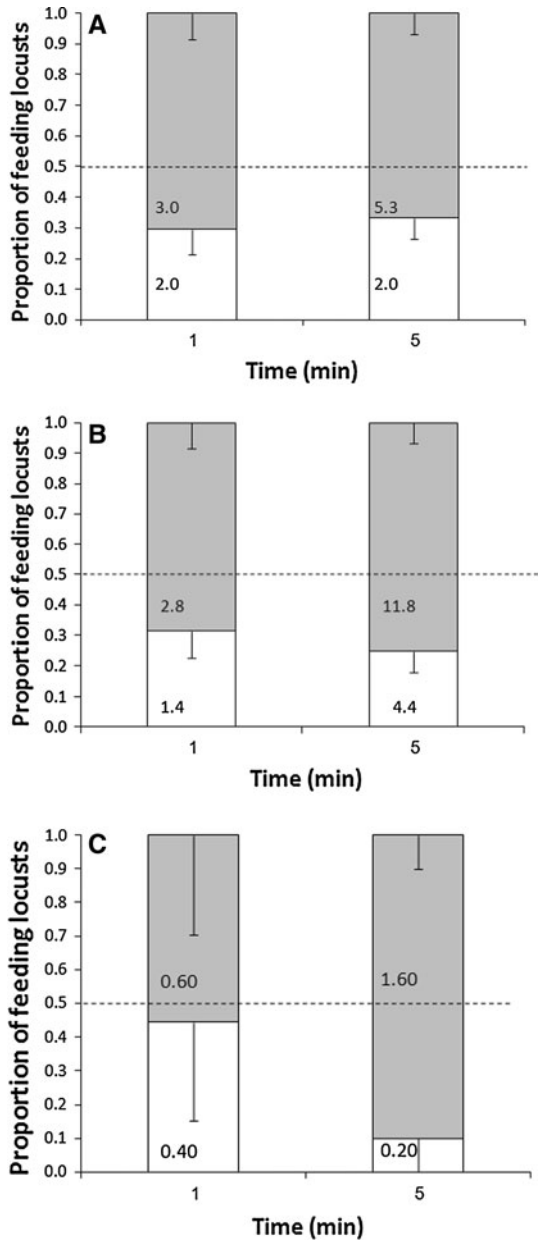
**Fig. 3** An approximate visualization of the relative excitation of the (a) UV, green and blue photoreceptors of locusts and honeybees by *S. viridis* flags/leaves and by red/green cabbage; b the short (S-cone)- and medium (M/L cone)-wavelength receptors of goats by *S. viridis* flags versus leaves



**Fig. 4** The distribution of first feeding choices of goats that were presented with clipped and flagged *S. viridis* inflorescences

*Red versus green cabbage* A significantly larger number of locusts fed on green cabbage than on red cabbage, both 1 min ( $n = 5$ ,  $Z = 1.84$ ,  $P = 0.03$ ) and 5 min ( $n = 5$ ,  $Z = 2.02$ ,  $P = 0.02$ ) after food presentation (Fig. 5b). Much more of the initial biomass of green cabbage ( $47.3 \pm 3.9\%$ ) was consumed within 10 min, compared to red cabbage ( $17.3 \pm 10.1\%$ ). The difference indicates higher consumption of green leaves relative to purple ones ( $n = 4$ ,  $Z = 1.83$ ,  $P = 0.04$ ). Neither food type was completely devoured during the 10-min observation period in any of the five replicates.

**Fig. 5** Mean (SE) proportions of locusts feeding on **a** *S. viridis* flags (empty bars) and leaves (shaded bars),  $n = 10$  feeding trials; **b** red (empty) versus green (shaded) cabbage leaves,  $n = 5$  feeding trials; **c** white morph *S. viridis* flags (empty) versus leaves (shaded),  $n = 5$ . Dotted lines mark the expected choices of both feeding options, if they are equally favored. The numbers within the bars denote the absolute mean number of individuals recorded on each feeding option



*White flags versus leaves* To test whether locusts avoided flags *per-se* or due to their purple color, we determined their response toward flags versus leaves of the rare white cultivar of *S. viridis*. The number of individuals on both food types was similar after 1 min ( $n = 5$ ,  $Z = 0.58$ ,  $P = 0.28$ ) of exposure. After 5 min, more *S. gregaria* nymphs fed on leaves than on white flags (Fig. 5c,  $n = 5$ ,  $Z = 1.84$ ,  $P = 0.03$ ). The total proportion of biomass consumed did not differ between leaves ( $50.1 \pm 28.8\%$ ) and white flags

( $59.4 \pm 18.0\%$ ,  $n = 4$ ,  $Z = 0$ ,  $P = 1$ ). The green leaves were completely consumed after 8 min in two of the replicates.

## Discussion

Our results provide tentative evidence for the dual selective benefit of *S. viridis*' flag displays. Previously we demonstrated the importance of the flag in attracting pollinators (Arnon et al. 2006; Keasar et al. 2009). Here we show first evidence for its importance in reducing foraging by insect and mammalian herbivores. The combined data thus suggest that two selective forces, herbivory and pollination, have influenced the evolution of this extra-floral visual display. A growing body of research focuses on the evolution of flower traits in response to the combined (and often opposing) selective pressures of pollinators and herbivores (Strauss and Whittall 2006). The present study extends this view to a plant structure that has no direct reproductive function.

The biochemical analysis, in agreement with our working hypothesis, confirmed the presence of high anthocyanin concentrations in *S. viridis* flags. The food choice trials with goats and locusts provide circumstantial evidence for the hypothesis that these anthocyanins form a visual signal that deters herbivores. This is supported by the locusts avoiding an additional anthocyanin-rich food source (red cabbage) in favor of a green cultivar. Moreover, locusts did not initially prefer *S. viridis* leaves to white flags lacking the anthocyanin signal, although this preference formed later along the assay. This suggests that foragers are deterred by the purple cue when present, rather than being attracted to the green one. Non-palatable secondary metabolites that may be present in the white flags (and which were not analyzed here) could have caused increased feeding on leaves 5 min later. Alternatively, flags may be less nutritive than leaves, and anthocyanins in the purple varieties could serve as a visual cue indicating less nutritive tissue, rather than as a deterrent in itself. Flags could also signal defended flowers, and be avoided as an indication of other defenses rather than a defense in themselves. In both cases, the white flags may provide weaker warning signals than the purple ones. Locusts were chosen as a representative insect herbivore for the feeding trials because of their generalist diet, crowded (gregarious-like) lifestyle and local distribution. The locusts preferred feeding on leaves over purple flags but much less over white, anthocyanins-less flags, suggesting avoidance of flags.

Anthocyanin levels in flags and leaves were positively correlated in one of the study years, but not in the other. This inconclusive finding justifies further study in additional seasons, populations and plant parts. A generally positive relationship between flag and leaf anthocyanins could be exploited by herbivores to identify and avoid anthocyanin-rich individuals by their bright flags. Flags would thus function as reliable signals that advertize the plants' chemical defense state. However, if this correlation is usually weak, then flag color is not expected to provide information to herbivores as to the plant's palatability. Previous studies have shown a positive correlation between secondary metabolite concentrations in different tissues in other plant species, for example in *Gelsemium semper-virens* (Irwin and Adler 2006). Similarities in the secondary metabolites between nectar and leaves were also found in *Nicotiana tabacum* (Adler et al. 2006) and in several species of *Asclepias* (Manson 2010). These examples suggest that the correlation between anthocyanin level in flags and leaves that we observed in 2010 may not be coincidental.

In spite of preference to *S. viridis* leaves over flags, locusts consumed similar proportions of both food types by the end of the 10-min assay. This probably reflects the fact that

under the experimental conditions the amount of leaves was limited, driving individuals to eat the less-preferred food source that was left available.

To affect feeding choices, anthocyanin-generated aposematic signals must be perceptible by the visual system of herbivores. Although there are no direct data on locust and goat color discrimination, analyzing reflectance data according to organisms with similar color vision (i.e. bees and horses) would suggest that indeed flags are perceived as different from leaves by both locusts and goats. In support of this assumption, the predicted flag-leaf color distance for locust is 6.36 RNL units, much higher than the (bee) minimum which is 2.3 (Vorobyev et al. 2001). The calculated color distance for honeybees (8.06) fits well with previous experimental data where flags were demonstrated to enhance attraction of pollinating bees (Keasar et al. 2009). Although we do not know the minimal color distance for goats, such that will allow for reliable color discrimination, these and other ungulates are known to have two types of color sensing cells, with max responses at  $\sim 450$  and  $\sim 550$  nm (Jacobs et al. 1998; Carroll et al. 2001). Interestingly, those two maxima fit well with the peak wavelengths of reflectance for leaves and flags (Fig. 2a).

The importance of herbivore deterrence in promoting the evolution of flags is also indirectly supported by two characteristics of these displays. First, the development of flags and flowers on an inflorescence is incompletely synchronized, since flags often develop before the first flowers open, and wilt a few days after the end of flowering (Arnon et al. 2006). This asynchrony is likely to be non-adaptive from a pollination point of view (as it provides pollinators with false signals as to the state of blooming), but may be beneficial to the plant by reducing herbivory. Second, clipped inflorescences that grow within a patch of flag-carrying individuals produce as many seeds as their flagged neighbors (Keasar et al. 2009). Thus, the selective benefit of flag production for any individual in the patch is low from a pollination point of view. This would be expected to select for “cheater” genotypes that do not invest resources in flag production. Such selection may be counteracted, however, by the fitness advantage of flags as anti-herbivore signals.

In summary, our previous work (Keasar et al. 2009) and the current results circumstantially support a dual role of the *Salvia viridis* flag display, as both anti-herbivory, aposematic signal and a pollinator attractant. The anti-herbivore role of the flag displays can be tested further by measuring predation on flag-clipped plants in the field, and quantifying the resulting reduction in fitness (see anecdotal results in the introduction). Our results exemplify the complexity of multiple selective forces in shaping extra-floral structures in plants.

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