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Flower color–flower scent associations in polymorphic *Hesperis matronalis* (Brassicaceae)

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Abstract

Floral scent emission rate and composition of purple and white flower color morphs of *Hesperis matronalis* (Brassicaceae) were determined for two populations and, for each, at two times of day using dynamic headspace collection and GC–MS. The floral volatile compounds identified for this species fell into two main categories, terpenoids and aromatics. Principal component analysis of 30 compounds demonstrated that both color morphs emitted more scent at dusk than at dawn. Color morphs varied in chemical composition of scent, but this differed between populations. The white morphs exhibited significant differences between populations, while the purple morphs did not. In the white morphs, one population contains color–scent associations that match expectations from classical pollination syndrome theory, where the flowers have aromatic scents, which are expected to maximize night-flying moth pollinator attraction; in the second population, white morphs were strongly associated with terpenoid compounds. The potential impact that pollinators, conserved biosynthetic pathways, and the genetics of small colonizing populations may have in determining population-specific associations between floral color and floral scent are discussed.
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Keywords: *Hesperis matronalis*; Brassicaceae; GC–MS; Terpenoids; Aromatics; Flower color polymorphism

1. Introduction

Floral traits have long been recognized as important targets of pollinator-mediated selection. Pollinators respond to visual cues such as floral shape, size, and color (e.g., Waser and Price, 1981; Stanton, 1987; Campbell, 1991; Rausher and Fry, 1993; Conner et al., 1996; Caruso, 2000; Jones and Reithel, 2001), as well as olfactory cues (reviewed in Raguso, 2001), but the latter have received much less attention. In the past, researchers have focused on pollinator attraction through combinations of specific floral traits, such as scent and color, in the form of pollination syndromes (Baker, 1961; Knudsen and Tollsten, 1993; Raguso et al., 2003). For example, studies of pollination syndromes give rise to the hypothesis that white (null pigment) flower morphs should emit more of the aromatic compounds (especially alcohols and esters) that characteristically attract nocturnal moths as pollinators (Baker, 1961; Haynes et al., 1991; Plepys et al., 2002; Raguso et al., 2003). While such color–scent relationships are generally more complex than predicted by simple ecological observation, recent studies have shown that visual and olfactory cues often function synergistically to attract pollinators. For instance, in several well-studied day-flying insect pollinators, visual cues elicit long-range attraction while scent provides a landing cue; in contrast, night-flying pollinators are initially attracted by scent and land or probe in response to visual cues (Ômura et al., 1999; Raguso and Willis, 2002, 2005; Andersson and Dobson,

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2003). Moreover, the combination of scent and visual cues increases the number of visits and degree of foraging activity for many pollinators (Honda et al., 1998; Kunze and Gumbert, 2001; Raguso and Willis, 2002, 2005; Andersson and Dobson, 2003), as well as floral constancy (Gegear, 2005). Thus, evidence is accumulating that specific associations between floral scent and visual stimuli such as flower color can form a complex selection target, maximizing attractiveness to pollinators and potentially enhancing plant reproductive success.

Biochemical processes might also account for associations between floral scent and color. Recent work suggests a link between constitutive chemical herbivore defense systems and plant pigmentation via shared substrates and conserved metabolic pathways (Coley and Kursar, 1996; Armbruster et al., 1997; Fineblum and Rausher, 1997; Agrawal and Karban, 2000; Clegg and Durbin, 2000). Many of the precursors, products, and/or by-products of these biosynthetic systems may be volatilized into recognized olfactory stimuli (Linhart and Thompson, 1995; Raguso and Pichersky, 1999). In this way, scent and color combinations may be passive consequences of conserved biochemical pathways (Armbruster, 2002) or their regulatory elements, and may not be easily dissociated by natural selection. This outcome may be particularly clear in a species that is polymorphic for flower color, where null mutants, often represented by individuals lacking pigment, may display radical changes in the amount or type of volatile compounds emitted as compared to brightly colored morphs, due to changes in metabolic flux (e.g., Zuker et al., 2002).

We sought to determine whether there are associations between floral color and scent in hermaphroditic *Hesperis matronalis* L. (Brassicaceae). This plant is a biennial, introduced from Eurasia, and commonly found in disturbed areas throughout the northeastern United States (Mitchell and Ankeny, 2001). In all populations surveyed in the study area of western Pennsylvania, *H. matronalis* displays a striking flower color polymorphism consisting of purple and white petaled morphs (C. Majetic, unpub. data), although other studies have documented a pink intermediate (Dvorak, 1982; Mitchell and Ankeny, 2001; Rothfels et al., 2002). Initial crossing experiments suggest that color in this plant species is determined by a simple one or two locus Mendelian system, with white dominant to purple (C. Majetic, unpub. data). A previous study of greenhouse-reared plants in the European range documented diurnal variation in floral scent emission, but no evidence of a difference between color morphs (Nielsen et al., 1995).

We addressed three questions: (1) Does overall floral volatile emission rate or chemical composition differ between purple and white flowered *H. matronalis*? (2) Do *H. matronalis* color morphs differ in diurnal pattern of floral volatile emission or composition? (3) Are there population-level differences in floral scent emission or composition between *H. matronalis* color morphs?

2. Results and discussion

2.1. Characterization of H. matronalis scent

We identified 33 volatile compounds from *H. matronalis* flowers (Table 1) and these generally fell into two categories – those consisting of ester or alcohol-modified compounds with benzene rings (hereafter "aromatics"; Nielsen et al., 1995; Honda et al., 1998; Raguso et al., 2003), and those composed of isoprene units (hereafter "terpenoids"; Linhart and Thompson, 1995; Nielsen et al., 1995; Honda et al., 1998; Raguso and Pichersky, 1999; Raguso et al., 2003). All 17 compounds previously identified by Nielsen et al. (1995) from the floral scent of Danish populations of *H. matronalis* were also detected in our samples (Table 1). We identified an additional seven aromatic compounds, including those derived from benzoic acid (benzaldehyde, benzyl proprionate, and benzyl acetate) and from phenylalanine (phenyl acetonitrile and eugenol), as well as one compound produced as an intermediate of tryptophan biosynthesis (methyl anthranilate). We also identified terpenoid compounds with irregular carbon skeletons, such as *E*-4,8-dimethyl-1,5,7-nonatriene, and oxidized derivatives of the two most abundant volatiles, pyranoid linalool oxide ketone (from linalool) and *E*-β-ocimene epoxide.

Principal components analysis (PCA) was performed on the 30 most abundant compounds. PCA is a multivariate method for rotating axes in the original multidimensional data space to find the orthogonal (i.e., statistically independent) axes of variation among a set of partially co-varying traits. PCA, with its eigenvalues and eigenvectors, can be of value in two ways. First, scent compounds that partially share biochemical production pathways can be highly correlated, obscuring the true patterns in the data (Gotelli and Ellison, 2004) by making statistical analysis difficult or misleading. PCA provides a reduced number of independent axes of variation, principal components (PCs). A PC's eigenvalue is the variance explained by that PC and this can be tested for significance. Second, the eigenvectors consist of coefficients that indicate how much each scent compound influences the PC. Interpretation of the patterns of variation among the scent compounds' coefficients can give some biological insight into the observed variation in scent composition.

PCA yields a single set of PCs for an entire data set, and therefore provides no parametric test of significance. Therefore, the significance of the PC eigenvalues was tested using a randomization test (Tonsor unpub. program a) in SAS (2001) macro language. Our significance test randomly permutes each column of scent values, thus breaking up any real associations between compounds in the permuted data set. The randomly associated trait values are then subjected to PCA. This is done 1000 times, each time using a newly permuted data set. This provides a distribution of possible values for the PCs given the null hypothesis. The actual PC values are then compared to

Table 1
Summary of *H. matronalis* scent chemical composition

Compound	PC 1	PC 2	Retention times	RM mean (SE) emission rate		RD mean (SE) emission rate	
				Purple (N = 7)	*White (N = 8)*	*Purple (N = 8)*	*White (N = 8)*
Aromatics							
Benzaldehyde	0.17	0.07	12.69	0.976 (0.286)	1.324 (0.387)	0.823 (0.248)	1.335 (0.449)
Phenylacetaldehyde	0.24	0.01	14.23	0.163 (0.077)	0.220 (0.091)	0.186 (0.072)	0.555 (0.278)
Benzyl acetate	0.18	0.30	15.24	3.756 (1.534)	6.828 (3.698)	4.858 (1.946)	4.361 (2.442)
Benzyl proprionate	0.04	0.19	16.02	0.010 (0.004)	0.073 (0.034)	0.006 (0.005)	0.025 (0.024)
Phenylethyl actetate	0.21	0.21	16.28	0.048 (0.018)	0.128 (0.061)	0.090 (0.035)	0.106 (0.054)
Benzyl alcohol	0.17	0.29	16.86	0.427 (0.084)	0.947 (0.320)	0.504 (0.230)	0.582 (0.341)
2-Phenylethanol	0.25	0.09	17.27	0.065 (0.019)	0.118 (0.060)	0.119 (0.043)	0.197 (0.095)
Phenylacetonitrile	0.19	0.04	17.52	0.031(0.011)	0.019 (0.007)	0.011 (0.004)	0.026 (0.012)
Unidentified aromatic ($m/z = 43$, 57, 77, 92, 105, 115)	0.11	0.34	19.75	0.017 (0.011)	0.196 (0.144)	0.024 (0.015)	0.045 (0.018)
Eugenol	0.13	0.37	19.92	0.264 (0.148)	0.891 (0.515)	0.127 (0.046)	0.274 (0.202)
Methyl anthranilate	0.23	−0.09	21.07	0.028 (0.012)	0.054 (0.014)	0.067 (0.014)	0.120 (0.030)
Benzyl benzoate	0.23	0.16	24.11	0.529 (0.217)	0.839 (0.351)	0.481 (0.161)	1.146 (0.564)
Methyl salicylate*	N/A	N/A	15.9	0.008 (0.004)	0.033 (0.020)	0.009 (0.007)	0.032 (0.017)
Terpenoids							
α-Pinene	0.07	−0.19	4.84	0.469 (0.000)	0.336 (0.021)	0.352 (0.031)	0.533 (0.046)
β-Pinene	0.20	−0.05	6.27	0.193 (0.000)	0.169 (0.027)	0.189 (0.036)	0.308 (0.047)
Sabinene	0.17	−0.27	6.54	0.163 (0.032)	0.091 (0.015)	0.309 (0.036)	0.543 (0.087)
β-Myrcene	0.23	−0.09	7.33	0.137 (0.042)	0.111 (0.024)	0.298 (0.080)	0.399 (0.131)
Limonene	0.20	−0.15	7.87	0.089 (0.030)	0.087 (0.026)	0.137 (0.027)	0.295 (0.108)
1,8 Cineole	0.15	−0.28	8	0.798 (0.211)	0.545 (0.080)	2.104 (0.249)	3.728 (0.682)
***Z*-β-Ocimene**	0.20	−0.16	8.5	0.336 (0.075)	0.315 (0.092)	0.819 (0.162)	0.805 (0.195)
***E*-β-ocimene**	0.16	0.08	8.75	8.488 (1.837)	8.628 (1.755)	11.346 (2.305)	8.400 (1.564)
E-4 dimethyl 1,3,7 nonatriene	0.21	−0.05	9.64	0.025 (0.005)	0.023 (0.005)	0.043 (0.007)	0.058 (0.020)
6-Methyl 5-hepten-2-one	0.22	−0.17	10.07	0.124 (0.031)	0.129 (0.025)	0.243 (0.046)	0.388 (0.099)
***Z*-furanoid linalool oxide**	0.16	−0.07	11.56	0.059 (0.011)	0.054 (0.018)	0.130 (0.041)	0.129 (0.038)
***E*-furanoid linalool oxide**	0.12	−0.10	11.96	0.146 (0.037)	0.116 (0.035)	0.179 (0.044)	0.263 (0.075)
Pyranoid linalool oxide ketone	0.15	0.06	12	0.144 (0.026)	0.126 (0.028)	0.080 (0.018)	0.115 (0.052)
Linalool	0.16	−0.02	12.93	5.804 (1.066)	4.330 (0.591)	3.543 (0.663)	12.276 (3.658)
E-β-ocimene epoxide	0.18	0.21	12.2	0.393 (0.083)	0.405 (0.085)	0.157 (0.039)	0.329 (0.116)
α-Terpineol	0.24	−0.14	14.84	0.161 (0.061)	0.124 (0.051)	0.283 (0.057)	0.782 (0.218)
E,*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene	0.21	−0.22	16.22	0.062 (0.008)	0.070 (0.012)	0.151 (0.044)	0.311 (0.086)
Unidentified terpenoid ($m/z = 41$, 43, 55, 67, 69, 83, 95, 119, 123, 137)	−0.03	0.12	10.95	0.300 (0.065)	0.316 (0.068)	0.370 (0.110)	0.142 (0.070)
Z-pyranoid linalool oxide*	N/A	N/A	15.35	0.051 (0.033)	0.016 (0.009)	0.014 (0.007)	0.018 (0.012)
E-pyranoid linalool oxide*	N/A	N/A	15.59	0.014 (0.009)	0.001 (0.001)	0.021(0.012)	0.043 (0.019)

Principal component scores for each scent compound emitted from flowers are given in the first two columns; compounds followed by an asterisk were omitted from principal components analysis. Retention times and mean emission of volatile compounds (reported in nanograms of scent per gram fresh mass per hour) found in color morphs (purple and white) across two populations (RM and RD) are reported in the remaining columns; values in parentheses are standard errors. Compounds in bold were also found by Nielsen et al. (1995).

this null hypothesis distribution. If the observed PC lies beyond the central 95% of this distribution, it is considered significant (i.e., $P < 0.05$). Of the six factors identified with eigenvalues greater than one, only the two largest PCs were significant based on randomization tests (observed PC 1 eigenvalue = 14.27, null hypothesis confidence intervals: upper 95% CI = 4.06, lower 95% CI = 3.02; observed PC 2 eigenvalue = 4.70, null hypothesis confidence intervals: upper 95% CI = 3.39, lower 95% CI = 2.71), and together explained 86% of the variance in floral scent.

The eigenvector coefficient scores of the first two PCs were next examined for their biological meaning. While the *set* of eigenvector coefficients is significant for PC 1 and PC 2 (where the eigenvalue presents the variance explained by the eigenvector), interpreting which of the coefficients within that vector have meaning is difficult. Bootstrap confidence intervals appear to be the best means of interpretation (Peres-Neto et al., 2003). They are nevertheless of low power, especially in an experiment such as this one where sample size is small. In addition, for PCs of relatively low magnitude, axis reflection and axis reordering can inflate the estimated bootstrap confidence interval (Jackson, 1995). We tested the eigenvector coefficients of each scent compound for both significant PCs using bootstrap 95% confidence intervals (Tonsor, unpub. program b). For the first PC, where the variance explained is greatest (60%), these confidence intervals are useful. For the second smaller PC (only 26% of variance explained), they are not. The only practical option for PC 2 is to use an arbitrary cut-off value. We used a cut-off of ±0.15 because this gave us the approximate upper 50th percentile. Less stringent cut-offs have unacceptable type I error rates. Any more stringent cut-off provides the same clear biochemical interpretation as ±0.15 (this can seen by inspection of the coefficient scores in Table 1).

All compounds loaded positively onto PC 1. In studies of floral and vegetative traits, PCs where all factors load positively are often interpreted to represent plant size (e.g., Gotelli and Ellison, 2004). Similarly, positive loading onto PC 1 (outside the 95% confidence intervals expected by chance alone) by all but one of our chemical compounds (an unidentified terpenoid) indicates that this factor reflects overall volatile emission rate (Table 1).

The compounds with the greatest effect on PC 2 fell into two chemically distinct categories; terpenoid compounds generally associated negatively and aromatics generally associated positively with this factor (Table 1). Seven compounds displayed negative coefficients beyond a threshold value of ±0.15: α-pinene, sabinene, limonene, 1,8 cineole, *Z*-β-ocimene, 6-methyl 5-hepten-2-one, and *E*,*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene. These compounds all fall into the terpenoid compound category. In contrast, eight compounds had positive coefficients above 0.15: benzyl acetate, benzyl proprionate, phenylethyl actetate, benzyl alcohol, unidentified aromatic ($m/z = 43$, 57, 77, 92, 105, 115), eugenol, benzyl benzoate, and *E*-β-ocimene epoxide. All but the last of these compounds fall into the aromatic category. When the threshold value is made more stringent (e.g., ±0.20), the general result does not change. Thus, PC 2 can be interpreted as a crude descriptor of the chemical composition of volatile emission: plants that have a more negative principal component score are associated with greater amounts of terpenoid-derived compounds in scent emission, while plants with a more positive score are associated with greater amounts of aromatic-derived compounds. Such an interpretation does not suggest that plants with a negative score do not emit aromatic-based compounds, but that the floral scent of these individuals has a stronger association with terpenoid compounds relative to other plants in our study.

Because the PCs are uncorrelated, they can be used in independent statistical tests (Gotelli and Ellison, 2004). The potential sources of variation in PC scores were then analyzed with individual fixed effects ANOVAs on the two significant PCs (PROC GLM, SAS, 2001), and the effects of color morph (purple, white), time of day (dawn, dusk), source population (RM, RD), and their two-way interactions tested. The three-way interaction was never significant and was eliminated from analyses. Post hoc tests for significant differences were conducted using Tukey's tests on least squares means (LSMEANS statement, SAS, 2001).

2.2. Does overall floral volatile emission rate or chemical composition differ between *H. matronalis* color morphs?

Analysis of variance on PC values shows that color morphs on average did not differ significantly in their overall scent emission rates (PC 1; Table 2) or in scent composition (PC 2; Table 2). This result is consistent with the results of Nielsen et al. (1995), who found no differences between the color morphs when using compound-by-compound comparisons.

Table 2
F-statistics and *P*-values from ANOVAs on the two principal components of floral scent in *H. matronalis* flowers

Source	PC 1 (60%)[a]		PC 2 (26%)	
	$F_{(Ndf,Ddf)}$	*P*	$F_{(Ndf,Ddf)}$	*P*
Model	$3.66_{(7,23)}$	0.008	$5.18_{(7,23)}$	0.001
Color	$2.44_{(1,23)}$	0.13	$0.06_{(1,23)}$	0.81
Time of day	$13.32_{(1,23)}$	0.001	$4.49_{(1,23)}$	0.05
Source population	$4.45_{(1,23)}$	0.05	$21.19_{(1,23)}$	0.0001
Color × time	$1.09_{(1,23)}$	0.31	$0.85_{(1,23)}$	0.37
Color × source	$1.52_{(1,23)}$	0.23	$7.00_{(1,23)}$	0.01
Time × source	$1.76_{(1,23)}$	0.20	$1.18_{(1,23)}$	0.29

The complete model includes three main effects and their interactions, the main effect variables being color (purple vs. white); time of day (dawn vs. dusk); and source population (RM vs. RD).

[a] Values in parentheses represent percent of total variance in floral scent described by each principal component.

2.3. Do H. matronalis color morphs differ in diurnal pattern of floral volatile emission or composition?

Total volatile emission rate and composition of scent varied with time of day (PC 1; Table 2): *H. matronalis* flowers emitted more scent at dusk than at dawn, as evidenced by higher values for PC 1 in both study populations (Fig. 1), and scent emitted at dusk was composed of a significantly higher proportion of aromatic compounds (positive scores for PC 2) and a lower proportion of terpenoid compounds than scent emitted at dawn (PC 2; Table 2; Fig. 1). However, across time periods the color morphs, on average, did not differ significantly in their bulk emission (PC 1; Table 2) or in scent composition (PC 2; Table 2).

The marked increase in the emission of aromatic compounds at night by both color morphs of *H. matronalis* corroborates the findings of Nielsen et al. (1995), who charted scent emission on plants in Denmark across the course of a 24-h period. We chose dawn and dusk as time points that were biologically relevant (to pollinator visitation) and thus could give higher priority to the number of plants sampled. The dusk-emitted compounds identified here are similar to those emitted on nocturnal rhythms in night-pollinated *Nicotiana* spp. (Kolosova et al., 2001; Raguso et al., 2003). The terpenoid compounds that dominate dawn-emitted scent are similar to those in day-pollinated species including *Brassica rapa*, *Ligustrum japonicum*, and *Fragaria virginiana* (Honda et al., 1998; Ômura et al., 1999; Ashman et al., 2005). This suggests that timing of emission of different floral volatile compounds in *H. matronalis* may reflect patterns of diurnal vs. nocturnal pollinator fauna attraction in either the introduced or native range of this species.

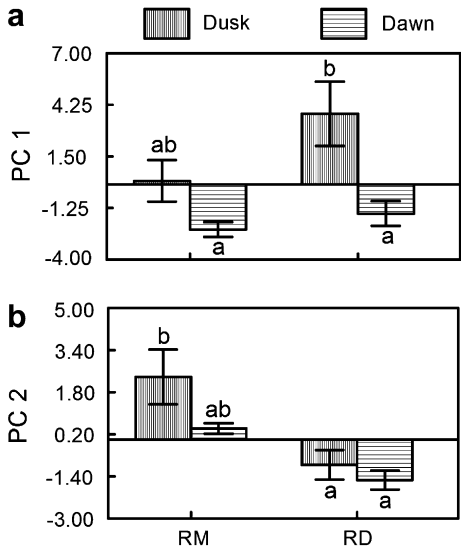

Fig. 1. Mean (±SE) PC 1 (A) and PC 2 (B) of floral scent for both time periods (dawn, dusk) and populations (RM, RD) of *H. matronalis*. PC 1 describes overall amount of scent emitted. PC 2 represents scent composition; a bar presenting a negative mean suggests a more aromatic-based scent, while a positive mean suggests a more terpenoid-rich scent (Table 1). Means not sharing letters are significantly different ($P < 0.05$) as determined by Tukey's tests.

Indeed, the *H. matronalis* populations described here are visited by both diurnal and nocturnal pollinator species (C. Majetic, *personal obs.*; H. Sahli, *personal obs.*), making the pattern of emission found in this study relevant to potential pollinator attraction in the species' introduced range. However, it is interesting to note that 1,8-cineole and several other monoterpenoid compounds found to be emitted at daytime in both Nielsen et al. (1995) and this study are entrained to a striking nocturnal rhythm in all species of Brazilian *Nicotiana* (Raguso et al., 2003, 2006). Such differences in emission patterns across species suggest that both ecological and phylogenetic contexts are important in understanding the function and origin of floral scent in *H. matronalis*.

2.4. Are there population-level differences in floral scent emission or composition between H. matronalis color morphs?

Populations differed in their bulk emission rates and scent composition (PC 1 and PC 2; Table 2; Fig. 2): plants from population RM emitted significantly less scent that was more strongly dominated by aromatics, while plants from population RD emitted large amounts of terpenoid-dominated scent (Table 2; Fig. 2). In addition, there was a significant interaction between population and flower color in floral scent composition (PC 2; Table 2, $P = 0.01$). Plants in population RD, the smaller of the two populations, tended to have a scent composition rich in terpenoid compounds (negative PC 2 values), but white morphs had a much stronger terpenoid component than purple-flowered individuals (Fig. 3). In contrast, population RM contained plants with more aromatic-dominated floral scent; here again, the white morphs had a stronger association with the dominant compound type (aromatics) as compared to their purple counterparts (Fig. 3). Consequently, white morphs differed significantly between populations in terms

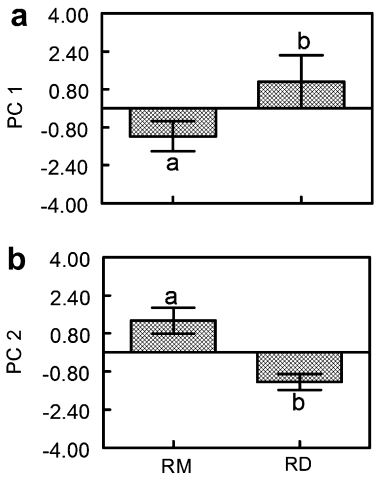

Fig. 2. Mean (±SE) PC 1 (A) and PC 2 (B) of floral scent for populations of *H. matronalis*. Means not sharing letters are significantly different ($P < 0.05$) as determined by individual ANOVAs.

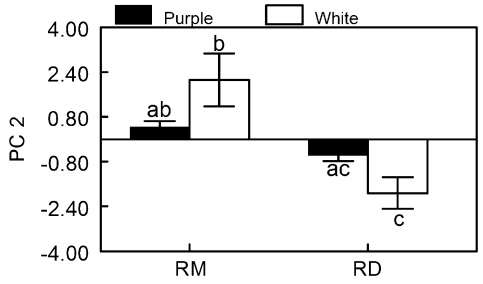

Fig. 3. Mean (±SE) PC 2 of floral scent for color morphs and populations for *H. matronalis*. PC 2 describes predominantly positive loading of terpenoids and predominantly negative loading of aromatics (Table 1). Bars not sharing letters are statistically significant at $P < 0.05$.

of scent composition while purple morphs scent composition did not. These differences may be explained by population dynamics. As with many invasive species, *H. matronalis* populations are often small and transient (C. Majetic, pers. obs.; Meekins and McCarthy, 2002). These small populations may be composed of few founders and thus be subject to genetic drift (Conner and Hartl, 2004). Differences in fragrance of purple and white morphs in a population thus may be a result of genetic drift from founders with specific color–scent combinations. However, for some invasive species, disturbance and recolonization may also actually serve as a source of genetic variation (Dietz et al., 1999). Given that in Denmark Nielsen et al. (1995) uncovered unusually high variance in scent between individuals of *H. matronalis*, the between-population differences in floral scent and color combinations recorded here may represent differences in founding and/or recolonization events. At the same time, the two morphs showed similarity in their floral scent within populations, which may reflect some form of stabilizing selection on floral scent across the color morphs in each locale. However, this explanation does not account for the fact that white morphs differ strongly in scent composition between populations, unless the optimal phenotype differs between populations or white morphs reflect nulls from different colored backgrounds.

2.5. The potential impact of pollinators and the role of biosynthetic pathways

The results presented here deviate from predictions derived from pollination syndromes, where white morphs are expected to have scents of greater aromatic composition in order to maximize attraction of night-flying moth pollinators (e.g., Loughrin et al., 1990; Raguso et al., 2003; Huber et al., 2005). This is true only in one population (RM), where both morphs have an aromatic-biased scent but white morphs tend to have a stronger association with these compounds. In this population, the presence of aromatic-associated scent across all color morphs may illustrate a method of pollinator assurance by providing the purple morphs with some ability to compensate for reduced visual contrast at night (compared with white flowers) and thus achieve some level of crepuscular pollination. A similar situation may be taking place in the terpenoid-associated scent population (RD) – here purple morphs have a weaker association to terpenoid compounds as compared to their white counterparts. Such a weak association may make any aromatic compounds they produce more noticeable to pollinators, ensuring visitation to these dark-colored morphs. One caveat to this prediction is that several hawkmoth species possess true color vision even under very dark conditions, and appear to innately prefer blue-colored flowers in many cases (White et al., 1994; Cutler et al., 1995; Kelber et al., 2002, 2003). Additionally, although noctuid and sphingid moths are highly attracted to oxygenated aromatic compounds, they can also learn to associate nectar rewards with terpenoids such as α-pinene and linalool (Daly et al., 2001; Cunningham et al., 2004, in press), compounds that are present day and night in both color morphs of *H. matronalis*. Populations of *H. matronalis* are visited by both day and night-flying pollinators, including bumblebees, small bee species, butterflies, syrphid flies, and sphingid moths (C. Majetic, *personal obs.*). Such a variety of pollinators may be supported, in part, by morph-specific combinations of floral traits that cater to pollinators with different preferences. Future experimentation is needed to assess how different suites of pollinators impact fitness of floral color–scent variants.

While the potential for biotic agents such as pollinators to select for particular floral scent–color combinations is great, there remains the possibility that scent–color correlations are influenced predominantly by innate biochemical processes. We suggested earlier that, in some cases, white flower morphs might release more aromatic scent compounds because they represent null mutants with blocked biosynthetic pigment pathways. Scent production often involves multiple pathways (Dudareva et al., 2004). In mutants, the compounds that are normally processed to generate pigment may be diverted to other pathways within the network. Increased use of these alternative pathways could then lead to changes in the type or amount of volatile compounds produced. Such a pattern has been found in carnations: anti-sense suppression of flavanone-3-hydroxylase, a gene encoding a critical enzyme in anthocyanin biosynthesis, resulted in increased emissions of methyl benzoate and methyl salicylate, whereas emissions of β-caryophyllene, a terpenoid compound unrelated to anthocyanin metabolism, remained unchanged (Zuker et al., 2002). However, this combination of floral cues was observed in the white morphs of only one of our populations of *H. matronalis*. Initial crossing experiments suggest a simple Mendelian inheritance for flower color in *H. matronalis*, with white dominant to purple, although the specific genetic model (single-locus vs. two locus) for flower color in this species has not yet been determined (C. Majetic, unpub. data). In some Brassicaceae, namely wild radish (*Raphanus sativus*), pink pigmentation is dominant to white phenotype (Stanton, 1987). However, other members of this family are thought to show dominance of white

petal color (Anstey, 1955; Stanton et al., 1986; Séguin-Swartz et al., 1997; Gomez, 2000). In either case, blockage of biochemical synthesis can often be caused by any of a number of mutations throughout the pathway, as studies of induced mutations, gene insertions, and spontaneous mutants in pigmentation synthesis have shown (e.g., Nakatsuka et al., 2005; Nishihara et al., 2005). It is possible then that the white morphs observed in our two populations are the result of different mutations. While molecular mechanisms regulating production of volatile compounds tend to be similar within and across species (Kolosova et al., 2001), different mutations in a biochemical pathway might have a variety of effects on volatile production, leading to striking differences in scent due to increased production of certain by-product compounds (e.g., Zuker et al., 2002; Verdonk et al., 2003). Selection by pollinators may not effectively decouple these two traits, leading to the maintenance of floral scent and color polymorphism within populations. The identity of the persisting scent–color combinations within a particular population may be determined by the local pollinator fauna or gene flow from other populations.

3. Concluding remarks

This study demonstrates naturally occurring variation in flower color and scent in two non-native populations of *H. matronalis*, with populations differing in both emission and chemical composition of the floral scent. Although plants in both populations emit a greater total amount of scent at dusk compared to dawn, the dusk emissions are more heavily dominated by aromatics. Interestingly, the color morphs do not differ overall in their bulk emission of volatile compounds, but do exhibit contrasting patterns in the two populations, where the white morphs show much differentiation between populations, but the purple morphs do not. Indeed, the white morphs have more extreme associations with certain classes of scent compounds than do purple morphs. The causes of these color–scent associations may include natural selection on one or both traits by pollinators, as well as neutral mutations in metabolic pathways and/or genetic drift. Future studies of pollinator response to, and fitness consequences of, all possible floral scent and color combinations, as well as floral scent analyses of individuals with known mutations in the anthocyanin pathway, are needed to understand the degree of association between these two traits in *H. matronalis*.

4. Experimental

4.1. Floral scent collection

Plant material for this experiment was obtained from two source populations in northwestern PA (Crawford County): RM consisted of ~300 individuals and was located on the slope of a highly disturbed road shoulder (N 41°36.156′; W 080°25.788′); RD (~50 individuals) was located on the edge of a disturbed drainage ditch (N 41°37.152′; W 080°27.155′). Both populations experienced moderate levels of shade throughout the day and minor flooding events following heavy rain (C. Majetic, pers. obs.)

On June 1–3, 2004, dynamic headspace scent collection (Raguso and Pellmyr, 1998) was performed indoors at the Pymatuning Laboratory of Ecology (PLE – Crawford County, Pennsylvania) on harvested inflorescences of each color morph across two time periods, dawn (6 a.m.–9 a.m.) and dusk (6 p.m.–9 p.m.). Harvesting inflorescences does not cause any significant changes in the composition of floral volatiles emitted by *H. matronalis* (Majetic et al., unpub. data; Nielsen et al., 1995); thus, inflorescences from four purple and four white-flowered plants were harvested 10 min prior to sampling. Sixteen plants (four per morph per time period) were sampled per population. The number of open flowers on each inflorescence was recorded (a range of 12–24 flowers per inflorescence) and the fresh mass of flowers per inflorescence was determined to the nearest 0.10 g using a Sartorius balance (Sartorius Research, Goettingen, Germany).

To collect fragrances, the inflorescences were placed into vials of water and covered with a 0.5L Reynolds Oven Bag (Reynolds Inc., Richmond, VA, USA) following Raguso and Pellmyr (1998). Each bag was secured with a plastic tie around the stem, thus slowing the flow of air into and out of the bag. Bagging the inflorescences in this way limits potential external contaminants and ensures that the volatile headspace of the plant is appropriately sampled. A scent trap, consisting of a Pasteur pipette containing 10 mg Porapak Super Q adsorbent (Alltech Associates, Inc, Deerfield, IL, USA) and a plug of silanized quartz wool, was attached to each bag. Each scent trap was then connected to a vacuum pump (model number 2522B-01, Welch Vacuum/Thomas Industries, Skokie, IL, USA) using Tygon tubing. Inflorescences were sampled for 1 h, with a flow rate from the bag through the scent trap of 250 mL air/min. After this sampling period, volatiles were eluted from the scent traps using 300 μL of pure hexane. Two control air samples (empty bags) at each time period were also collected. All samples were stored in a −20 °C freezer in glass vials with Teflon caps until GS–MS analysis.

4.2. Quantitative analysis of scent samples

Thirty-one floral scent samples (one sample dried out) were analyzed at the University of South Carolina. To determine chemical composition and total emission, we rapidly (~20 s) concentrated our samples from 300 μL to a volume of 75 μL using N_2 gas and added 5 μL of 0.03% toluene (16 ng) as a standard as in Raguso et al. (2003), resulting in quantification of all compounds as toluene equivalents. This can cause complications when composition is determined, as many biochemical products volatilize differently, leading to partial or total loss of certain

compounds. It is unlikely that we have completely lost compounds; however, the possibility that certain compounds may have been volatilized at different rates in different samples suggests that analysis based on this technique must be interpreted with caution. An aliquot (1 μL) of each sample was injected into a Shimadzu GC-17A with a QP5000 quadrupole, electron impact MS detector for analysis (Shimadzu Corporation, Kyoto, Japan). The oven was then heated to 240 °C to vaporize each sample for separation of components on an EC wax GC column (Alltech Associates, Inc., Deerfield, IL, USA).

Thirty-three scent compounds were identified using computerized mass spectroscopic libraries and retention times; all identified compounds are known terpenoid or aromatic floral volatiles. The MS compound peaks for each sample were then integrated using Shimadzu GC–MS Solutions Software (version 1.02A, Shimadzu Corporation, Kyoto, Japan), and the amount of each compound in a sample was quantified through comparison to the 16 ng internal toluene standard as in Ashman et al. (2005).

For each plant, the emission rate of each compound was determined as the amount (in μg) per gram fresh mass or number of flowers per hour (Table 1). These values were normally distributed and largely homoscedastic, and thus did not require any transformations for statistical analysis. Analyses on emission rates standardized by fresh mass and number of flowers were similar; thus for simplicity, only the results controlling for fresh mass are reported.

4.3. Statistical analysis

To determine the separate effects of floral scent emission and composition, a correlation-based principal components analysis (PCA) in SAS (PROC PRINCOMP; SAS, 2001) was performed on emission rates per sample for the 30 most abundant volatile compounds identified (Table 1). The three rarest compounds (contributing less than <0.22% total to overall scent emission: *Z*-pyranoid linalool oxide, *E*-pyranoid linalool oxide, and methyl salicylate) were removed from the data set for PCA analysis. Because they were entirely absent in some samples, their inclusion could have biased the analysis, giving these compounds more prominence than is biologically relevant (Pielou, 1984).

The significance of PC eigenvalues and coefficient scores were determined using randomization (Tonsor unpub. program a) and bootstrap confidence interval tests (Tonsor unpub. program b), respectively (see Section 2.1 for details). The two PCs found to be significant were then examined further using individual fixed effects ANOVA (PROC GLM, SAS, 2001).

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References

Agrawal, A.A., Karban, R., 2000. Specificity of constitutive and induced resistance: pigment glands influence mites and caterpillars on cotton plants. Entomologia Experimentalis et Applicata 96, 39–49.

Andersson, S., Dobson, H.E.M., 2003. Behavioral foraging responses by the butterfly *Heliconus melpomene* to *Lantana camara*. Journal of Chemical Ecology 29, 2303–2318.

Anstey, T.H., 1955. Inheritance of white petal in green sprouting broccoli. Canadian Journal of Agricultural Science 35, 573–578.

Armbruster, W.S., 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. Journal of Evolutionary Biology 15, 468–486.

Armbruster, W.S., Howard, J.J., Clausen, T.P., Debevec, E.M., Loquvam, J.C., Matsuki, M., Cerendolo, B., Andel, F., 1997. Do biochemical exaptations link evolution of plant defense and pollination systems? Historical hypotheses and experimental tests with *Dalechampia* vines. American Naturalist 149, 461–484.

Ashman, T.-L., Bradburn, M., Cole, D.H., Blaney, B.H., Raguso, R.A., 2005. The scent of a male: the role of floral volatiles in pollination of a gender dimorphic plant. Ecology 86, 2099–2105.

Baker, H.G., 1961. The adaptation of flowering plants to nocturnal and crepuscular pollinators. The Quarterly Review of Biology 36, 64–73.

Campbell, D.R., 1991. Effects of floral traits on sequential components of fitness in *Ipomopsis aggregata*. American Naturalist 137, 713–737.

Caruso, C.M., 2000. Competition for pollination influences selection on floral traits of *Ipomopsis aggregata*. Evolution 54, 1546–1557.

Clegg, M.T., Durbin, M.L., 2000. Flower color variation: a model for the experimental study of evolution. Proceedings of the National Academy of Science 97, 7016–7023.

Coley, P.D., Kursar, T.A., 1996. Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological trade-offs. In: Mulkey, S.S., Chazdon, R.L., Smith, A.P. (Eds.), Tropical Forest Plant Eco-physiology. Chapman and Hall, New York, pp. 305–335 (Chapter 11).

Conner, J.K., Hartl, D.L., 2004. Population genetics II: changes in allele frequency. A Primer of Ecological Genetics. Sinauer Associates, Inc., Sunderland, Massachusetts, pp. 47–95 (Chapter 3).

Conner, J.K., Rush, S., Jennetten, P., 1996. Measurement of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). I. Selection through lifetime female fitness. Evolution 50, 1127–1136.

Cunningham, J.P., Moore, C.J., Zalucki, M.P., West, S.A., 2004. Learning, odour preference, and flower foraging in moths. The Journal of Experimental Biology 207, 87–94.

Cunningham, J.P., Moore, C.J., Cribb, B.W., in press. Context and complexity in odour learning: moths learn single volatiles during flower visiting. The Journal of Experimental Biology.

Cutler, D.E., Bennett, R.R., Stevenson, R.D., White, R.H., 1995. Feeding behavior in the nocturnal moth *Manduca sexta* is regulated by blue receptors, but where are they in the retina? Journal of Experimental Biology 198, 1909–1917.

Daly, K.C., Durtschi, M.L., Smith, B.H., 2001. Olfactory based discrimination learning in the moth *Manduca sexta*. Journal of Insect Physiology 47, 375–384.

Dietz, H., Fischer, M., Schmid, B., 1999. Demographic and genetic invasion history of a 9-year-old roadside populations of *Bunias orientalis* L. (Brassicaceae). Oecologia 120, 225–234.

Dudareva, N., Pichersky, E., Gershenzon, J., 2004. Biochemistry of plant volatiles. Plant Physiology 135, 1893–1902.

Dvorak, F., 1982. Some results of the study of *Hesperis matronalis* L. from the Belanske Tatry Mountains. Biologia Bratislava 37, 441–447.

Fineblum, W.L., Rausher, M.D., 1997. Do floral pigmentation genes also influence resistance to enemies? The *W* locus in *Ipomoea purpurea*. Ecology 78, 1648–1654.

Gegear, R.J., 2005. Multicomponent floral signals elicit selective foraging in bumblebees. Naturwissenschaften: Published online, doi:10.1007/s00114-005-0621-5.

Gomez, J.M., 2000. Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. Journal of Evolutionary Biology 13, 689–699.

Gotelli, N.J., Ellison, A.M., 2004. The analysis of multivariate data. In: Gotelli, N.J., Ellison, A.M. (Eds.), A Primer of Ecological Statistics. Sinauer Associates, Inc., Sunderland, Massachusetts, pp. 406–415 (Chapter 12).

Haynes, K.F., Zhao, J.Z., Latif, A., 1991. Identification of floral compounds from *Abelia grandiflora* that stimulate upwind flight in cabbage looper moths. Journal of Chemical Ecology 17, 637–646.

Honda, K., Ômura, H., Hayashi, N., 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower-visiting by cabbage butterfly, *Pieris rapae*. Journal of Chemical Ecology 24, 2167–2180.

Huber, F.K., Kaiser, R., Sauter, W., Schiestl, F.P., 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). Oecologia 142, 564–575.

Jackson, D.A., 1995. Bootstrapped principal component analysis—reply to Mehlman et al. Ecology 76, 644–645.

Jones, K.N., Reithel, J.S., 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). American Journal of Botany 88, 447–454.

Kelber, A., Balkenius, A., Warrant, E.J., 2002. Scotopic colour vision in nocturnal hawkmoths. Nature 419, 922–925.

Kelber, A., Balkenius, A., Warrant, E.J., 2003. Colour vision in diurnal and nocturnal hawkmoths. Integrative and Comparative Biology 43, 571–579.

Knudsen, J., Tollsten, L., 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. Botanical Journal of the Linnean Society 113, 263–284.

Kolosova, N., Gorenstein, N., Kish, C.M., Dudareva, N., 2001. Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. The Plant Cell 13, 2333–2347.

Kunze, J., Gumbert, A., 2001. The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. Behavioral Ecology 12, 447–456.

Linhart, Y.B., Thompson, J.D., 1995. Terpene-based selective herbivory by *Helix aspersa* (Mollusca) on *Thymus vulgaris* (Labiatae). Oecologia 102, 126–132.

Loughrin, J.H., Hamilton-Kemp, T.R., Andersen, R.A., Hildebrand, D.F., 1990. Volatiles from flowers of *Nicotiana sylvestris*, *N. otophora*, and *Malus* × *domestica*: headspace components and day/night changes in their relative concentrations. Phytochemistry 29, 2473–2477.

Meekins, J.F., McCarthy, B.C., 2002. Effect of population density on the demography of an invasive plant (*Allaria petolata*, Brassicaceae) population in a southeastern Ohio forest. American Midland Naturalist 147, 256–278.

Mitchell, R.J., Ankeny, D.P., 2001. Effects of local conspecific density on reproductive success in *Penstemon digitalis* and *Hesperis matronalis*. Ohio Journal of Science 101, 22–27.

Nakatsuka, T., Nishihara, M., Mishiba, K., Yamamura, S., 2005. Two different mutations are involved in the formation of white-flowered gentian plants. Plant Sciences 169, 949–958.

Nielsen, J.K., Jakobsen, H.B., Friis, P., Hansen, K., Moller, J., Olsen, C.E., 1995. Asynchronous rhythms in the emission of volatiles from *Hesperis matronalis* flowers. Phytochemistry 38, 847–851.

Nishihara, M., Nakatsuka, T., Yamamura, S., 2005. Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. FEBS Letters 579, 6074–6078.

Ômura, H., Honda, K., Hayashi, N., 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. Journal of Chemical Ecology 25, 1895–1906.

Peres-Neto, P.R., Jackson, D.A., Somers, K.M., 2003. Giving meaningful interpretation to ordination axes: assessing loading significance in principal component analysis. Ecology 84, 2347–2363.

Pielou, E.C., 1984. Ordination. In: Pielou, E.C. (Ed.), The Interpretation of Ecological Data: A Primer on Classification and Ordination. Wiley, New York, pp. 133–165 (Chapter 4).

Plepys, D., Ibarra, F., Löfstedt, C., 2002. Volatiles from flowers of *Platanthera bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae). Oikos 99, 69–74.

Raguso, R.A., 2001. Floral scent, olfaction, and scent-driven foraging behavior. In: Chittka, L., Thompson, J.D. (Eds.), Cognitive Ecology of Pollination. Cambridge University Press, Cambridge, pp. 83–105.

Raguso, R.A., Pellmyr, O., 1998. Dynamic headspace analysis of floral volatiles: a comparison of methods. Oikos 81, 238–254.

Raguso, R.A., Pichersky, E., 1999. A day in the life of a linalool molecule: chemical communication in a plant-pollinator system. Part 1: Linalool biosynthesis in flowering plants. Plant Species Biology 14, 95–120.

Raguso, R.A., Willis, M.A., 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Maduca sexta*. Animal Behaviour 64, 685–695.

Raguso, R.A., Willis, M.A., 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. Animal Behaviour 69, 407–418.

Raguso, R.A., Levin, R.A., Foose, S.E., Holmberg, M.W., McDade, L.A., 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. Phytochemistry 63, 265–284.

Raguso, R.A., Schlumpberger, B.O., Kaczorowski, R.L., Holtsford, T.P., 2006. Phylogenetic fragrance patterns in *Nicotiana* sections *Alatae* and *Suaveolentes*. Phytochemistry 67, 1931–1942.

Rausher, M.D., Fry, J.D., 1993. Effects of a locus affecting floral pigmentation in *Ipomoea purpurea* on female fitness components. Genetics 134, 1237–1247.

Rothfels, C.J., Beaton, L.J., Dudley, S.A., 2002. The effects of salt, manganese, and density on life history traits in *Hesperis matronalis* L. from oldfield and roadside populations. Canadian Journal of Botany 80, 131–139.

SAS, 2001. SAS Version 8.2 for Windows. SAS Institute, Inc., Cary, NC.

Séguin-Swartz, G., Warwick, S.I., Scarth, R., 1997. Cruciferae: Compendium of Trait Genetics. Agriculture and Agri-Food Canada. <http://www.brassica.info/resources/crucifer_genetics/compend.pdf>.

Stanton, M.L., 1987. Reproductive biology of petal color variants in wild populations of *Raphanus sativus*: I. Pollinator response to color morphs. American Journal of Botany 74, 178–187.

Stanton, M.L., Snow, A.A., Handel, S.N., 1986. Floral evolution: attractiveness to pollinators increases male fitness. Science 232, 1625–1627.

Tonsor, S., Unpublished program a. PCA Randomization Confidence Interval Generation for SAS Version 8.2 for Windows <http://www.pitt.edu/~biology/tonsor>.

Tonsor, S., Unpublished program b. PCA Bootstrap Confidence Interval Generation for SAS Version 8.2 for Windows <http://www.pitt.edu/~biology/tonsor>.

Verdonk, J.C., Ric De Cos, C.H., Verhoeven, H.A., Haring, M.A., Van Tunen, A.J., Schuurink, R.C., 2003. Regulation of floral scent production in petunia revealed by targeted metabolomics. Phytochemistry 62, 997–1008.

Waser, N.M., Price, M.V., 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. Evolution 35, 376–390.

White, R.H., Stevenson, R.D., Bennett, R.R., Cutler, D.E., Haber, W.A., 1994. Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. Biotropica 26, 427–435.

Zuker, A., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkmann, G., Martens, S., Neta-Sharir, J., Weiss, D., Vainstein, A., 2002. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Molecular Breeding 9, 33–41.