Annals of Botany **102**: 911–922, 2008
doi:10.1093/aob/mcn181, available online at www.aob.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

The Impact of Biochemistry vs. Population Membership on Floral Scent Profiles in Colour Polymorphic *Hesperis matronalis*

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Received: 20 May 2008 Returned for revision: 14 July 2008 Accepted: 5 August 2008 Published electronically: 26 September 2008

• *Background and Aims* Studies of floral scent evolution often attribute variation in floral scent to differences in pollinator behaviour, ignoring the potential for shared biochemistry between floral scent and floral colour to dictate patterns of phenotypic variation in scent production. To determine the relative effects of shared biochemistry and/or localized population-level phenomena on floral scent phenotype, floral scent composition and emission rate were examined in five wild populations of colour polymorphic *Hesperis matronalis* (Brassicaceae).
• *Methods* Floral scent was collected by *in situ* dynamic headspace extraction on purple and white colour morphs in each of five wild populations. Gas chromatography–mass spectroscopy of extracts allowed determination of floral scent composition and emission rate for all individuals, which were examined by non-metric multidimensional scaling and analysis of variance (ANOVA), respectively, to determine the contributions of floral colour and population membership to scent profile variation.
• *Key Results* Despite the fact that colour morph means were very similar in some populations and quite different in other populations, colour morphs within populations did not differ from each other in terms of scent composition or emission rate. Populations differed significantly from one another in terms of both floral scent composition and emission rate.
• *Conclusions* Shared biochemistry alone cannot explain the variation in floral scent phenotype found for *H. matronalis*. Such a result may suggest that the biochemical association between floral scent and floral colour is complex or dependent on genetic background. Floral scent does vary significantly with population membership; several factors, including environmental conditions, founder effects and genetics, may account for this differentiation and should be considered in future studies.

Key words: *Hesperis matronalis*, floral scent, floral colour, plant volatiles, population differentiation, scent composition, scent emission rate, terpenoids, aromatics.

INTRODUCTION

For many angiosperms, flowers function as 'sensory billboards', attracting pollinators via combinations of several different types of cues (visual, tactile, olfactory; Raguso, 2004). Two characters often considered from this perspective are floral scent and floral colour. Pollinators show preferences for certain variants of these traits (e.g. colour in Stanton, 1987; colour and scent in Galen and Kevan, 1980), and this could lead to the evolution of specific colour–scent combinations via the process of pollinator-mediated natural selection (Waser and Price, 1981; Miyake and Yafuso, 2003; Salzmann *et al.*, 2007). However, few studies consider the hypothesis that physiology or biochemical pathways limit the possible combinations of floral colour and floral scent such that colour–scent patterns in a given species reflect these constraints.

In many plant tissues, purple pigment results from the synthesis of anthocyanin through the shikimate pathway (Taiz and Zeiger, 1998; Clegg and Durbin, 2000); plants that fail to make this pigment, due to any of a number of mutations, appear white (e.g. Levin and Brack, 1995; Nakatsuka *et al.*, 2005). These mutations can involve non-functional structural genes (those encoding biosynthetic enzymes), as well as alterations in transcription factors and *cis*-regulatory regions (Rausher, 2008). Floral scent is complex and can be composed of volatiles produced by several different pathways; the most common volatiles include terpenoids and aromatics (Knudsen *et al.*, 2006). Aromatics, like anthocyanin pigments, are produced by branches of the shikimate pathway (Dudareva *et al.*, 2004; Schuurink *et al.*, 2006), suggesting the potential for correlated response by colour and scent to a single change in biochemistry. Such a biochemical connection, though not well studied, is supported by some preliminary evidence. In a study manipulating pigmentation in *Dianthus caryophyllus* petals, Zuker *et al.* (2002) found that removal of petal pigment by antisense suppression of the flavanone 3-hydroxylase gene in the anthocyanin pathway led to changes in floral scent emission of an aromatic volatile (methyl benzoate). Other studies also suggest a connection between pigmentation and aromatic volatile production: introduction of an anthocyanin pigment transcription factor into *Petunia hybrida* led to both enhanced colour and a 10-fold increase in the production of some aromatic compounds (Zvi *et al.*, 2008*a*, *b*). Previous research on the floral scent of *Hesperis matronalis* (Brassicaceae), a plant with a purple–white floral colour polymorphism, further supports a biochemical association between floral scent and floral colour. A study of two wild

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populations of this species found that the floral scent composition of purple morphs was quite similar between populations, whereas scent composition in white morphs was disparate and population specific (Majetic *et al.*, 2007). Taken together, these results suggest that if shared biochemistry indeed defines both floral scent and floral colour for a species, then plants producing purple pigmentation will have similar floral scent profiles, particularly in the identity and abundance of aromatic volatiles (Fig. 1A). In contrast, white morphs, due to changes in metabolic flux through the shikimate pathway, may differ from purple morphs in scent composition. This outcome is unlikely for other floral volatiles, such as terpenoids, as their biosynthetic pathways are not directly related to anthocyanin production.

Moreover, the above predictions should hold regardless of the scale of study: if shared biochemistry strongly defines floral scent, then purple morphs from a single population would probably have similar scent profiles (Fig. 1A), as would purple morphs sampled from across several populations (Fig. 1B). White morphs, if derived from null (i.e. loss-of-function) mutations, will continue to differ from purple morphs, but may also diverge from each other if mutations differ among populations (i.e. a non-functional enzyme in one population vs. a non-functional transcription factor in another population; Fig. 1B). Previous research on *H. matronalis* suggests that this is possible, but the small number of populations in that study limited the extent to which this hypothesis could be evaluated. In contrast, if shared biochemistry only weakly constrains floral scent–floral colour associations, then evolutionary phenomena such as founder effects, natural selection or genetic drift (Conner and Hartl, 2004) may shape scent profiles at the population level. This leads to the alternative prediction that floral scent will be determined by population differentiation regardless of pigmentation (Fig. 1C). Few studies have attempted to explore variation in floral scent among populations, but those that have found mixed results in terms of significant scent variation among populations (Knudsen *et al.*, 2006, and references therein).

The rarity of population-level studies of floral scent is due in large part to the logistical and technological costs of the required chemical analysis. Despite recent technological advances (reviewed by Tholl and Röse 2006), techniques such as gas chromatography–mass spectrometry (GC-MS) are expensive, suffer from low sample throughput (30–120 min per sample after extraction and concentration steps; Dobson *et al.*, 2005) and can be time-consuming in terms of post-chromatographic data analysis and interpretation (van Dam and Poppy, 2008). Thus, researchers are faced with trade-offs in sampling intensity within vs. among populations. For instance, of the ten population-level studies cited in Knudsen *et al.* (2006) that explicitly report sample size, only two sampled >5 populations, and these sampled only 2–3 individuals per population (Azuma *et al.*, 2001; Dötterl *et al.*, 2005). Four other studies sampled 3–5 populations, with an average of seven individuals studied per population (Knudsen, 1994, 2002; Tollsten and Øvstedal, 1994; Pettersson and Knudsen, 2001). The most extensive sampling is found in two more recent studies: Svensson *et al.* (2005), who examined an average of nine individuals across ten populations of *Yucca filamentosa*, but found no significant differentiation among populations in floral scent composition, and Mant *et al.* (2005), who sampled eight populations with an average of 29 *Ophrys* species individuals per population and found significant population differentiation both within and among species. In the face of the significant individual and environmental variation in floral scent for many species, the sample sizes of most of the studies cited here would be too low to address both the biochemical and population-level hypotheses outlined above, yet were acquired only through considerable effort and expense by the authors of these studies.

Therefore, to understand the relative contributions of biochemistry and population-level phenomena in shaping floral scent profiles, floral scent–colour associations were examined in five populations across part of the North American range of the introduced species *H. matronalis* (Brassicaceae), using an intensive within-population sample size ($n = 20$ plants per population). *Hesperis matronalis* provides an excellent system in which to examine the shared biochemistry hypothesis because it is polymorphic for anthocyanin-based flower colour (i.e. purple or white flowers, Majetic, 2008). Additionally, populations of this species are extremely widespread within the introduced range; multiple locations can therefore be examined and it

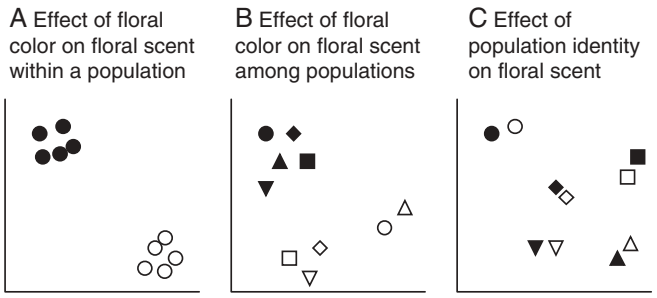

FIG. 1. Graphical hypotheses explaining variation in floral scent (A) within a population due to floral colour and shared biochemistry; (B) among populations due to floral colour and shared biochemistry; and (C) among populations due to population membership, i.e., localized environmental or evolutionary events (i.e., drift, selection, founder events). Circles in (A) represent scent for individual plants within a population; varying symbol shapes in (B) and (C) represent separate population means. Filled symbols represent purple individuals or population means, and open symbols represent white individuals or population means. Axes are hypothetical, representing measures of distance (e.g., similarity/dissimilarity) between individuals or means.

is possible to determine whether the biochemical predictions are upheld across a broad geographic scale or whether population membership plays a more important role in defining floral scent variation. Specifically, floral scent was examined for both colour morphs in all populations, and the following questions were asked. (1) Is variation in floral scent composition within populations of *H. matronalis* explained by floral colour polymorphism? If so, are colour-associated patterns found only for aromatic volatiles, as predicted by the shared biochemistry hypothesis, or do other volatile compounds (i.e. terpenoids) show colour-associated patterns? (2) Is variation in floral scent composition among populations of *H. matronalis* explained by floral colour and/or population membership? Are colour-associated patterns (if present) found only for aromatic volatiles? (3) Is variation in overall floral scent emission rate, aromatic emission rate or terpenoid emission rate of *H. matronalis* explained by floral colour and/or population membership?

MATERIALS AND METHODS

Study species

Hesperis matronalis (Brassicaceae) is a herbaceous biennial that has been introduced to the USA from Eurasia and is found in disturbed areas throughout all but the southernmost parts of the country (US Department of Agriculture, 2007). It has been designated as an invasive plant in some areas due to increased spread in recent years (US Department of Agriculture, 2007; PA Department of Conservation and Natural Resources, 2007). Plants overwinter as vegetative rosettes before bolting in mid to late spring in Pennsylvania (hereafter PA). Inflorescences reach a maximum height of 20–100 cm when flowering (C. Majetic, University of Pittsburgh, pers. obs.), with floral displays reaching 20 flowers per inflorescence open at a time (Mitchell and Ankeny, 2001). Flowers of *H. matronalis* are hermaphroditic and at least partially self-compatible in western PA (Majetic, 2008). However, studies in other locations (Mitchell and Ankeny, 2001; Weeks and Frey, 2007) find *H. matronalis* to be self-incompatible. Daytime pollinators in the introduced range include bees (including *Bombus* and *Apis* species), lepidopterans and syrphid flies, with occasional evening moth visitation documented in some locales (Mitchell and Ankeny, 2001; Majetic, 2008); insects do not display any colour preferences (Majetic, 2008).

In all populations surveyed in western PA, as well as many populations throughout its northern range, *H. matronalis* displays a flower colour polymorphism consisting of purple or white petaled morphs (Majetic, 2008), although other studies have documented a pink intermediate (Dvořák, 1982; Mitchell and Ankeny, 2001; Rothfels *et al.*, 2002). Purple flowers contain high levels of anthocyanin pigments while white flowers contain little or no anthocyanins (Majetic, 2008). No associations have been found between flower colour and flower size or shape (Majetic, 2008). Previous studies of this species suggest that floral volatile emission in *H. matronalis* peaks at dusk (Nielsen *et al.*, 1995; Majetic *et al.*, 2007), and floral colour morphs differ in aspects of their scent composition (Majetic *et al.*, 2007).

Plant material

In May and June of 2006, a survey of five wild populations of *H. matronalis* was conducted across part of its introduced geographical range in North America: two populations in southern Ontario (‘ONT1’, 44º01′N, 79º31′W; and ‘ONT2’, 43º32′N, 79º31′W), two populations in northwestern PA (‘PA1’, 41º36′N, 80º25′W; and ‘PA2’, 41º36′N, 80º27′W) and a population in northern Virginia (‘VA’, 39º05′N, 78º04′W). All of these populations were polymorphic for floral colour (50–80 % purple morphs; Majetic, 2008) and chosen to represent much of the latitudinal distribution of *H. matronalis*. During peak flowering in each population, ten purple morphs and ten white morphs were selected at random and marked for study.

Floral scent collection

Floral scent was collected using dynamic headspace extraction for 1 h at dusk (between 1800 and 2100 h) following the protocol outlined by Majetic *et al.* (2007); this protocol was modified slightly for outdoor use, using vacuum pumps powered by portable batteries rather than direct electrical outlet power, and bagging rooted inflorescences rather than harvested flowers. Ambient air controls were collected concurrently with floral scent samples at all locations; controls were collected daily at dusk at each population for a total of two environmental controls per population. For the purposes of this study, the decision was made to focus on scent emitted at dusk, given that it is the time of peak production. Floral scent collections took place over a 2–3 d period in each wild population, coinciding with peak flowering time at a particular site. Temperatures at the time of collection were similar for most populations (~16–23 ºC), with the exception of PA2, which was somewhat cooler (~13 ºC on the days of sampling). To calculate emission rates based on the amount of floral tissue sampled, the number of flowers on each inflorescence was counted. Rates based on flower number are comparable with those based on fresh biomass (Majetic *et al.*, 2007).

GC-MS characterization of floral volatiles

To determine the chemical composition of the sampled volatiles, GC-MS was performed at the University of South Carolina using a Shimadzu GC17A gas chromatograph with a QP5000 quadrupole mass spectrometer as a detector, as described in Majetic *et al.* (2007) and Schlumpberger and Raguso (2008). Aliquots of scent samples (1 μL) were injected (splitless) at 240 °C on to an EC-wax fused capillary GC column, held and exposed to a 10 °C temperature increase per minute from 40 to 260 °C, holding for 3 min at the front end and 5 min at the back end of the temperature ramp. Thirty-nine scent compounds (see Appendix) were identified from their

mass spectra and retention times using NIST and Wiley computerized mass spectral libraries; most identified compounds are known floral volatiles identified from laboratory standards, of which 32 were found in a previous study of *H. matronalis* (Majetic *et al.*, 2007). The compounds fall into two categories (terpenoids and aromatics) based on their biochemical pathway of origin (as in Knudsen and Tollsten, 1993; Knudsen *et al.*, 2006). Resulting MS compound peaks for each sample were then integrated using Shimadzu GC-MS Solutions Software (version 1·02A, Shimadzu Corporation, Kyoto, Japan). The hypotheses were then tested using the two major components of the floral volatile profile (composition and emission rate) separately. The data manipulations and analyses are each considered in turn below.

Patterns of floral scent composition

To assess whether floral scent composition was associated with floral colour on two scales (i.e. within and among populations), non-metric multidimensional scaling analyses (NMDS) were performed (Borg and Lingoes, 1987). For this technique, chromatograms were assessed and all 39 floral volatiles were coded as either present ('1') or absent ('0'). Aromatic and terpenoid data sets were analysed separately via NMDS analysis (PC-ORD; Borg and Lingoes, 1987; McCune and Mefford, 2006). For each compound category, a compositional similarity matrix was first calculated between all plants using Sørensen's dissimilarity index, defined as $(100 \times 2a)/(2a + b + c)$, where a is the number of compounds in both plants, b is the number present in one plant but absent in the second, and c is the number absent in the first but present in the second (Clarke and Gorley, 2006; NMDS using Euclidean distance instead of Sørensen's index produced similar results). The resulting matrix from all possible comparisons was then used in an iterative process to generate a new set of axes (here, 50 iterations of the axis-fitting process), placing the individuals sampled on these axes in a way that represents their compositional similarity spatially (as in Jürgens *et al.*, 2002). Analysis can result in any number of axes, so the most appropriate number of axes for display was determined by examining the stress coefficient. This coefficient is a measure of how much the resulting ordination relationship departs from relationships found in the original data set; generally, the lower the coefficient, the better the fit of the ordination, although interpretation is somewhat subjective (Borg and Lingoes, 1987). Stress coefficients in this analysis resulted in a model with two axes to represent aromatic composition (stress = 16·96) and a model with three axes for terpenoid composition (stress = 14·13; see McCune and Mefford, 2006). From these models, a score was obtained for each plant and each was plotted in ordination space. While descriptive in nature, NMDS is a commonly used technique that provides unbiased insight into patterns of association in the 'phenotype space' of floral scent or other multivariate characters (e.g. Clarke, 1993; Jürgens *et al.*, 2002; Jürgens, 2004; Dötterl *et al.*, 2005; Castilho *et al.*, 2007; Davies *et al.*, 2007; Laughlin and Abella, 2007; Roberts *et al.*, 2007).

To determine whether compositional differences are driven by floral colour within a population, individual plant ordination scores were plotted for each population separately and the graph was visually inspected for clustering by colour morph. Plants that cluster together are interpreted as more similar in their scent composition than those that are very distant (Borg and Lingoes, 1987); thus, clustering of purple or white morphs in space suggests scent composition similarity due to pigmentation within a population (Fig. 1A). To assess the relative role of floral colour vs. population membership in determining floral scent composition among multiple populations, plant scores were averaged by flower colour within each source population and these were visualized graphically. Clustering of means for purple or white colour morphs, regardless of population membership, suggests support for the shared biochemistry hypothesis for floral scent composition (Fig. 1B). In contrast, clustering of purple and white means from the same population suggests an important role for ecological processes driving population differentiation (Fig. 1C).

Following graphical representation of NMDS, it was necessary to determine whether potential clustering patterns identified visually were statistically different. Thus, analysis of similarity (ANOSIM) was performed on the presence/absence data used in NMDS, using the ANOSIM procedure in PRIMER v6 (Clarke and Gorley, 2006). ANOSIM is a non-parametric permutation analysis used to assess the similarity between two or more groups in terms of a set of independent variables, such as the abundance or presence/absence of a given taxa or species (Clarke, 1993; Clarke and Gorley, 2006). This technique is increasingly used in scent studies to identify statistically meaningful patterns, substituting the presence/absence or abundance of a specific scent compound for a taxa or species (e.g. Jürgens *et al.*, 2006). Individual plants are assigned to a group corresponding to colour morph and/or population. Distances are calculated between and within groups; the distances are ranked, and these ranks are used to calculate a value 'R', between -1 and 1, for each group comparison. An R-value of zero suggests no difference between the two groups, while a large positive R suggests dissimilarity. The significance of R-values is tested by a permutation test of group membership (Hammer *et al*, 2001). The identity of the major compounds contributing to differences between groups was also determined using the Similarity Percentage (SIMPER) procedure in PRIMER v6, a sorting technique based on the distances between groups (Hammer *et al*, 2001). This procedure provides a list of the compounds used in the analysis, their individual contributions (overall and percentage) to the dissimilarity between two groups and their mean abundances in each group.

Techniques such as NMDS and ANOSIM allow comparison of not only quantitative data, but also data sets that contain compounds that are non-normally distributed and/or categorical (i.e. zeros are prevalent). While principal components analysis (PCA) might allow quantitative

analysis of data, the presence of such conditions in this data set represents two major violations of the assumptions of PCA (Borg and Lingoes, 1987; Gotelli and Ellison, 2004), making NMDS/ANOSIM a particularly appropriate approach for the data here.

Patterns of scent emission rate

To quantify the emission rate of each floral volatile accurately, serial dilutions [240–320 μg (0·03 %); 24–32 μg (0·003 %); 2·4–3·2 μg (0·0003 %); 0·24–0·32 μg (0·00003 %)] of seven external standards (1,8 cineole, *E*-β-ocimene, benzaldehyde, linalool, benzyl acetate, 2-phenylethanol and eugenol) were created; these dilutions were injected into the GC and run on the same program as the *H. matronalis* samples. The GC-MS peaks were then integrated and standardized for subtle differences in sample volume by dividing by a sample-specific internal standard peak area. The resulting area values were used in conjunction with the known compound concentrations to make dose–response curves. Each floral volatile was then assigned an external standard (Appendix) based on knowledge of vapour pressures and structural similarity (Debbrecht, 1977; Jennings *et al.*, 1997), and peak area was transformed to micrograms of volatile emitted per flower per hour using the dose–response best-fit line equations. When an appropriate external standard was not available, the data were converted to toluene equivalents using the internal standard, as described in Majetic *et al.* (2007). Correlation of emission rates calculated by external standard dose–response curve and rates calculated based on toluene standards suggested that the two approaches yield highly similar results (all $R > 0{\cdot}7$, $P < 0{\cdot}0001$). In some cases (~20 % of data points), use of external standards led to underestimations when peaks on chromatograms were present but too small to quantify accurately with the present equipment; such a phenomenon is likely to be due to a compound concentration lower than those used in the dose–response curve. When transformation of a peak's area to amount resulted in a zero or negative value, the value was replaced by the arbitrary but reasonable value 0·0001 μg per flower h^{-1}. By doing so, the presence of all compounds could be accounted for, including those too small to quantify accurately, as contributors to total scent emission rate. Compound amounts were summed to obtain emission rates (μg per flower h^{-1}) for aromatics, terpenoids and total scent. Prior to statistical analysis, emission rate data were natural-log-transformed to improve normality and conform to the assumptions of ANOVA.

To determine the effects of floral colour and population identity on floral scent emission rates, individual fixed factor ANOVAs were conducted for aromatics, terpenoids and total scent (PROC GLM, SAS, 2007). Because a non-random sample of populations along a latitudinal gradient was selected in this study, population was treated as a fixed effect (Gotelli and Ellison, 2004). An overall significant effect of floral colour suggests a pigment-derived influence on floral scent emission rate across all populations, whereas a significant effect of source population suggests that populations are differentiated regardless of colour. A significant interaction suggests population-specific differences between colour morphs. When any significant population or interaction effects were found, *post hoc* Tukey's tests were performed.

RESULTS

Question 1: floral scent composition – floral colour within populations

NMDS analysis of floral scent composition within populations was similar for all five populations examined in the survey; therefore, the graphical results for population PA2 are presented as a representative example (Fig. 2; for figures displaying the results from the remaining populations, see Supplementary Information Figs S1–S4, available online). Examination of the NMDS results suggests no distinct clustering patterns of individual plants associated with floral colour. Purple and white morphs are generally intermixed in terms of both aromatic (Fig. 2A) and terpenoid (Fig. 2B) floral scent composition, suggesting significant within-population variation in composition independent of floral colour identity. This outcome is generally supported by individual ANOSIM analyses for each population. In three populations (ONT2, PA2 and VA), there are no significant differences between colour morph aromatic and terpenoid scent composition (Table 1). In ONT1 and PA1, marginally significant differences are seen between colour morphs in aromatics (for ONT1) and terpenoids (for PA1; Table 1).

Question 2: floral scent composition – floral colour across populations

Mean aromatic compound NMDS values cluster based on population, regardless of floral colour (Fig. 3A); indeed, ANOSIM analysis finds no significant differences between colour morphs across all populations ($R = -0{\cdot}003$, $P = 0{\cdot}49$). However, the proximity of purple and white mean values differs among populations. In particular, mean values for purple and white morphs in populations VA and ONT2 are quite close to one another; in contrast, purple and white means are quite distant in populations ONT1 and PA1 (Fig. 3A). Together, this spatial arrangement suggests distinct compositional differences due to population membership. ANOSIM and SIMPER analyses generally support this conclusion as the populations all differ significantly from one another (global test for population differences, $P = 0{\cdot}001$, $R = 0{\cdot}187$; pairwise tests, $P < 0{\cdot}05$, $R > 0{\cdot}1$ for all comparisons); these differences are driven in part by the more likely presence of methyl anthranilate in ONT1 and ONT2 relative to PA2 and VA, as well as a higher likelihood of phenylacetonitrile in VA; these compounds are nitrogen-bearing aromatics derived from the amino acids tryptophan and phenylalanine, respectively. Likewise, PA1 is significantly different from PA2 due to the greater presence of eugenol (i.e. eugenol is found in the scent of more PA1 than PA2 plants).

A similar pattern emerges when terpenoids are considered. Again, ANOSIM tests for global differences between colour morphs are non-significant ($R = 0{\cdot}011$, $P = 0{\cdot}30$). Mean NMDS values for purple and white

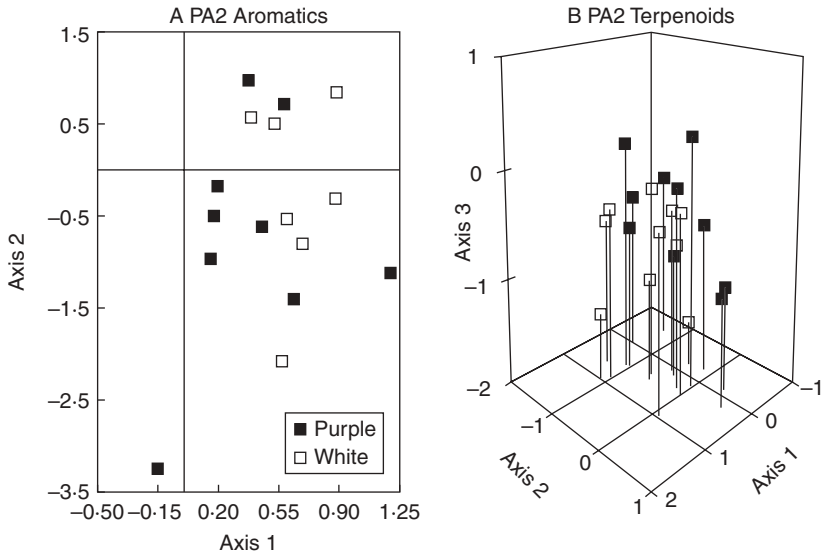

FIG. 2. Representative within-population NMDS plots of *Hesperis matronalis* scent composition (population PA2) in terms of (A) aromatics and (B) terpenoids. Filled symbols represent purple plants and open symbols represent white plants.

TABLE 1 *Individual ANOSIMs assessing the similarity of floral scent composition between colours (purple vs. white) within* H. matronalis *populations* (n = *20 plants per population for all but VA, where* n = *19)*

	Aromatic composition		Terpenoid composition	
Population	*R*	*P*	*R*	*P*
ONT1	0·092	0·05	0·035	0·23
ONT2	−0·054	0·77	−0·017	0·60
PA1	0·007	0·37	0·072	0·05
PA2	−0·028	0·63	−0·005	0·48
VA	−0·033	0·67	−0·036	0·79

Estimated *R*-values and calculated *P*-values are presented for both aromatic and terpenoid composition.

morphs cluster by population, and the degree of divergence between colour morph means varies among populations (Fig. 3B). In particular, purple and white means from PA1 are very close to one another and means from PA2 are quite far apart. The ANOSIM results suggest that all populations are significantly different from one another (global test for population differences, $P = 0{\cdot}001$, $R = 0{\cdot}218$; pairwise tests, $P < 0{\cdot}003$, $R > 0{\cdot}113$ for all comparisons). These differences are driven by differences in the presence of *E*, *E*-α-farnesene and modified linalool products (namely furanoid linalool oxides and linalool epoxide) among populations.

Question 3: floral scent emission rate – floral colour vs. population identity

There were no significant differences in aromatic, terpenoid or total scent emission rates between colour morphs, but highly significant differences among populations for all three rate variables (Table 2, Fig. 4). In particular, PA1 and VA consistently produced the largest amount of floral scent per flower, regardless of chemical type. PA2 emitted a low level of terpenoid compounds and consequently had a low overall emission rate. Populations ONT1 and ONT2, in contrast, had low emission rates for aromatic compounds and intermediate rates for terpenoids; however, as aromatics contribute less to overall scent emission rate than terpenoids, these two populations had resulting total emission rates that were also intermediate in value. The interaction between floral colour and source population also was not significant for total scent and terpenoids, and only marginally significant for aromatics (Table 2).

DISCUSSION

The results of the present study generally do not support a strictly biochemical hypothesis for the pattern of floral scent variation in *H. matronalis*. However, support was found for the population differentiation hypothesis. Such an outcome may be attributed to a number of causal factors, including variation in the genetic underpinnings of floral colour and floral scent, environmental variation, and aspects of population establishment and evolutionary divergence. While the current experiment cannot determine the relative effects of each of these factors, their possible roles are considered in detail below.

Floral colour, floral scent and shared biochemistry

In general, the data do not support the hypothesis that shared biochemistry between floral colour and floral scent is the primary determinant of floral scent variation in *H. matronalis* (Fig. 1A, B). Considerable variation is seen in scent composition among individuals regardless of floral colour within populations (Table 1, Fig. 2) and only limited spatial patterning based on colour across

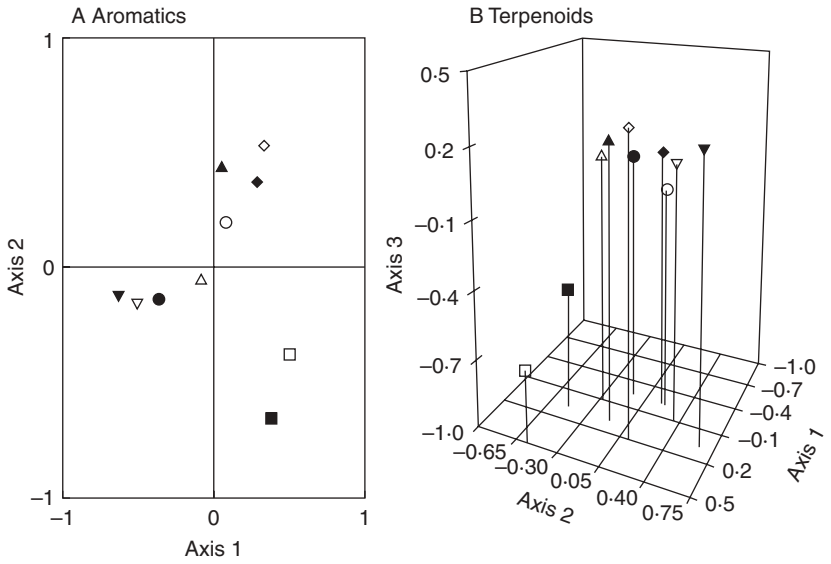

FIG. 3. NMDS plots of mean scent composition for populations of *Hesperis matronalis* in terms of (A) aromatics and (B) terpenoids. Filled symbols represent means for purple plants and open symbols represent means for white plants. Populations are represented as follows: triangles, PA1; circles, ONT1; diamonds, VA; squares, PA2; and inverted triangles, ONT2.

TABLE 2 *Individual ANOVAs assessing the effects of floral colour (purple vs. white) and population identity (*n = *5 populations) on three categories of floral scent emission rates in* H. matronalis *(*n = *99 plants)*

		Aromatics		Terpenoids		Total scent	
Variable	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Model	9	8·83	0·0001	4·32	0·0001	5·84	0·0001
Colour	1	0·42	0·52	1·90	0·17	1·32	0·25
Population	4	17·52	0·0001	8·12	0·0001	10·95	0·0001
Colour × population	4	2·10	0·09	0·99	0·42	1·69	0·16

Aromatic and terpenoid scent emission rates represent the sum of all floral volatile emission rates within each category; total scent emission rates represent the grand sum of all individual floral volatile emission rates.

populations (Fig. 3). Specifically, composition means for purple and white morph cluster based on population membership, but there is variation in how similar those means are within populations (Fig. 3). No significant differences between colour morphs are found in terms of emission rates (Table 2); among populations, no overall effect of floral colour on emission rate was find (Table 2, Fig. 4).

The general lack of support for the shared biochemistry hypothesis may indicate that the predictions regarding the specificity of biochemical connection between floral colour and floral scent are oversimplified. For instance, while white flowers in many plant species are associated with a lack of pigment production, they could arise through any of a number of enzyme or transcription factor mutations in the anthocyanin pigmentation pathway, as found for other plant species (e.g. van Houwelingen *et al.*, 1998; Clegg and Durbin, 2000; Nakatsuka *et al.*, 2005; Rausher, 2008). It is possible that the colour morphs found in different *H. matronalis* populations result from different mutations in the anthocyanin pathway, which may vary in their potential for pleiotropic impact on aromatic scent emissions. For example, if a mutation occurs upstream of the production of scent compounds and anthocyanin pigment, white flowers with no scent might be seen due to a lack of flux through the pathway. However, a mutation downstream of the production of scent compounds that blocks pigmentation may change floral scent due to an added flux of precursors. There is some evidence for the latter scenario in at least one case: in the *D. caryophyllus* study by Zuker *et al.* (2002), suppression of the enzyme flavanone-3-hydroxlyase (an enzyme whose activity occurs relatively late in the anthocyanin biosynthetic pathway) resulted in a white morph that produces increased amounts of the aromatic volatile methyl benzoate. Multiple mutations in the anthocyanin pathway also explain disparate results found for *Petunia* scent: two studies finding no differences in scent focus on mutations of two transcription factors affecting pigmentation production (AN2 and ODO1; Verdonk *et al.*, 2005; Hoballah *et al.*, 2007), while those that find changes in scent emission focus on a third transcription factor (Pap1) derived from *Arabidopsis thaliana* and introduced into plant tissue (Zvi *et al.*, 2008*a*, *b*). The presence of more than one mutation in a population or different

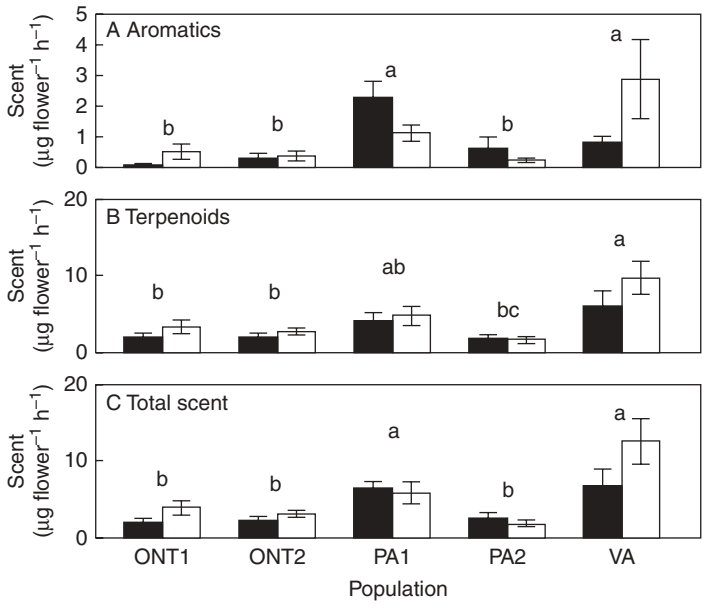

FIG. 4. Scent emission rates for populations of *Hesperis matronalis* in terms of (A) aromatics, (B) terpenoid and (C) total scent. Data have been untransformed for presentation. Purple bars represent means for purple plants and white bars represent means for white plants; error bars represent the standard error. Letters over bars represent Tukey's test differences between population means at $P \leq 0{\cdot}05$.

mutations in different populations would lead to associations between floral scent and floral colour that are much less transparent than those hypothesized in Fig. 1.

Genetic variation in other biochemical aspects of floral scent production may also explain the degree of variation between colour morphs within populations. While characterization of genetic background or identification of specific mutants have not been conducted in any wild populations, several aspects of the genetic control of floral scent production are becoming well known in a handful of model systems under greenhouse conditions; these have documented a number of enzymes that contribute to volatile production and a plant's overall floral scent phenotype (e.g. Lewinsohn *et al.*, 2000; Barkman, 2003; Dudareva *et al.*, 2003; Negre *et al.*, 2003; Dudareva *et al.*, 2005). The results of these studies suggest that biosynthetic pathways are extremely complex, such that altering any one step leads to overall changes in scent phenotype (e.g. van Schie *et al.*, 2006); such epistatic effects are likely to cause variation. There is also significant potential for pleiotropy: certain genes code for single enzymes that can be capable of converting several substrates into their associated volatile products (as reviewed in Pichersky *et al.*, 2006); modification of these enzymes could lead to radical changes in a number of compounds that define overall floral scent. Such biochemical connectedness would make it quite difficult to ascertain whether pigmentation or other genetic elements most define floral scent. No research has been conducted to determine the extent of genetic variation for floral scent in wild populations; scientists would gain much from understanding this important source of phenotypic variation in natural settings.

Previous work examining floral colour and floral scent in *H. matronalis* would appear to contradict the findings here in regards to the shared biochemistry hypothesis. Specifically, the earlier study suggests a floral colour–floral scent association, with purple morphs harvested from two populations having similar scent composition and white morphs having population-specific scent (Majetic *et al.*, 2007). However, one distinct difference between these studies is the manner in which scent was characterized and analysed. In the present study, each plant's floral scent profile was broken into two parts prior to qualitative (for composition) and quantitative (for emission rate) analyses. In Majetic *et al.* (2007), PCA was used to create orthogonal composition and emission rate variables which were then examined using ANOVA – a completely quantitative set of analyses. It is therefore possible that the differences seen in the results are driven not by the data alone, but by the way in which the data were analysed. One population, PA1, was examined in both studies and provides a test of this idea. The colour morphs in this population show a significant quantitative divergence in mean scent composition in Majetic *et al.* (2007) and a large but non-significant qualitative composition divergence in the present study (Fig. 3, triangles). The maintenance of this pattern between studies suggests that while there may be some variation in results due to quantitative vs. qualitative data analysis, other factors must come into play as well. One such factor is environmental variation. Volatile emissions are known to be sensitive to abiotic factors such as air temperature and humidity (i.e. Jakobsen and Olsen, 1994; Nielsen *et al.*, 1995). While weather conditions were more or less similar in all sites, populations probably differed in several other environmental features (e.g. soil nutrient levels or moisture), which might impact floral scent production and/or emission. If environmental contributions to variation in floral scent are high, then they may have obscured patterns of floral colour–floral scent association. The previous study

involved collecting floral scent from cut inflorescences in a controlled lab environment. While there may have been lingering effects of the growing environment on scent emission, the environmental conditions during scent sampling were less variable and may have allowed better visualization of the scent–colour associations present in this species. This fact is most likely to be the predominant reason for researchers interested in floral scent biochemistry to perform their experiments under controlled conditions in greenhouses and growth chambers (e.g. Lewinsohn *et al.*, 2000); associations between floral scent and other biochemical characteristics are more easily elucidated without environmental noise. However, these artificial conditions do not provide an ecologically accurate representation of floral scent phenotype. To understand truly how the scent phenotype is determined, more studies asking both biochemical and ecological questions must be conducted in natural settings.

Floral scent and population differentiation

The results of this study provide some support for an effect of population membership on floral scent: populations do differ significantly in both floral scent composition and emission rates (Figs 3 and 4, Table 2), although there tends to be some variance in how similar colour morph means are within populations. Several factors are known to contribute to phenotypic differentiation among populations, including differences in environment and genetics (Conner and Hartl, 2004). Most studies examining floral scent variation among populations focus on potential differences in pollinator fauna as the selective agent causing genetic differentiation (e.g. Svensson *et al.*, 2005; Schlumpberger *et al.*, 2008). However, herbivores, pathogens and abiotic factors are also known to cause phenotypic differences within and among populations for a number of floral traits (Schemske and Bierzychudek, 2001; Frey, 2004; Strauss and Whittall, 2006). Some casual preliminary observations were conducted at each site during scent collection, but these did not suggest any large-scale differences in terms of site quality and pollinator fauna (Majetic, 2008). In general, little is known about the degree of genetic vs. environmental control of scent in natural settings. To understand fully which environmental characteristics might cause population differentiation, future studies must examine both biotic and abiotic factors that could act as selective agents on floral scent. Moreover, incorporating genetics into such field experiments would allow researchers to partition phenotypic variance further into genetic and environmental components.

Population differentiation can also be caused by genetic bottlenecks and founder effects, particularly when populations of introduced plant species are established by only one or two individuals (Durka *et al.*, 2005, and references therein). It is then possible that these initial genetic differences, coupled with increased self-fertilization and genetic drift over time, cause populations to diverge (Walker *et al.*, 2003). If most populations of *H. matronalis* have been founded by single individuals, then it is quite possible that population differentiation is a result of founder effects. Alternatively, some populations of invasive species are known to be established through multiple introductions to the same area, leading to high within-population variation in a number of traits (Walker *et al.*, 2003, and references therein). A given population of *H. matronalis* may be composed of individuals from several sources of origin, particularly given that this species often occurs in road-side ditches and disturbed areas (Rothfels *et al.*, 2002). Such a scenario could also explain high levels of phenotypic variation within populations (e.g. Fig. 2), because founder individuals from different origins may have different scent or pigmentation mutations. Finally, multiple introductions might explain population variation in the observed 'distance' between colour morphs in mean scent composition (Fig. 3). Populations in which mean scent composition of purple and white morphs are more disparate (i.e. ONT1, Fig. 3A) may result from introduction events in which purple and white morphs came from different origins, whereas when colour morphs are very similar in scent composition (i.e. ONT2, Fig. 3A), they may reflect a single founding family. None of these tantalizing possibilities has been explored in terms of floral scent.

CONCLUSIONS

In *H. matronalis*, much of the variation seen in floral scent profiles is due to population membership rather than an association between floral scent and floral colour. This population differentiation may be due to any of a number of environmental and genetic factors; if the relationship between floral colour and floral scent is more complex than initially hypothesized here, then there may be no single unifying pattern of colour–scent association in the wild. The present study is a good first step in determining whether shared biochemistry or population-level phenomena are more influential in shaping floral scent phenotypes in a biologically relevant setting. It is suggested that progress will be made by conducting experiments with known biochemistry mutants in ecologically relevant environments. Only by using such an approach will the causes of floral scent phenotypic variation in the wild be able to be fully assessed.

SUPPLEMENTARY INFORMATION

Supplementary information can be found online at http://aob.oxfordjournals.org/ and consists of four figures as follows. Fig. S1: within-population NMDS plots of *Hesperis matronalis* scent composition for population ONT1. Fig. S2: within-population NMDS plots of scent composition for population ONT2. Fig. S3: within-population NMDS plots of *Hesperis matronalis* scent composition for population PA1. Fig. S4: within-population NMDS plots of *Hesperis matronalis* scent composition for population VA. For all populations, composition is presented in terms of (A) aromatics and (B) terpenoids.

ACKNOWLEDGEMENTS

We thank the Ashman, Tonsor, Traw and Kalisz labs (University of Pittsburgh) for their comments and discussion on this research, the staff of PLE for logistical support, and D. Carr, C. Caruso and A. Zimmerman for help in identifying focal plant populations. Many thanks to the staff at PRIMER-E Ltd for helping us to obtain their statistics package. Input from two anonymous reviewers greatly improved this manuscript. This is contribution number 226 to the Pymatuning Laboratory of Ecology. Funding is gratefully acknowledged from the National Science Foundation (DEB-0449488 and DEB-0108099 to T.L.A., DEB-0317217 and IBN-0444163 to R.A.R.); University of Pittsburgh's McKinley-Darbaker Grants (to C.J.M); Sigma Delta Epsilon/Graduate Women in Science Eloise Gerry Fellowship (to C.J.M.).

APPENDIX

Volatile compounds identified in *H. matronalis* floral scent by GC-MS and standards used for quantification. Compounds in bold were found in previous studies of *H. matronalis* (Nielsen *et al.*, 1995; Majetic *et al.*, 2007). Retention times indicate time of GC-MS elution for a specific compound

Compound	Retention time	External/ internal standard?	Name of standard
Aromatics			
Benzenoid compounds			
Benzaldehyde	12·8	External	Benzaldehyde
Phenylacetaldehyde	14·38	External	2-Phenylethanol
Benzyl acetate	15·4	External	Benzyl Acetate
Methyl salicylate	16	External	Benzyl Acetate
Benzyl propionate	16·17	External	Benzyl Acetate
Phenylethyl actetate	16·41	External	Benzyl Acetate
Benzyl alcohol	17	External	2-Phenylethanol
2-Phenylethanol	17·43	External	2-Phenylethanol
Benzyl benzoate	24·46	External	Eugenol
Phenyl propanoid compounds			
Methyl eugenol	18·48	External	Eugenol
Eugenol	20·06	External	Eugenol
Nitrogen-containing benzenoid compounds			
Phenylacetonitrile	17·67	External	2-Phenylethanol
Benzothiazole	18	Internal	Toluene
Methyl anthranilate	21·1	External	Eugenol
Terpenoids			
Monoterpenoids			
α-Pinene	4·84	External	*E*-β-Ocimene
β-Pinene	6·3	External	*E*-β-Ocimene
Sabinene	6·58	External	*E*-β-Ocimene
β-Myrcene	7·36	External	*E*-β-Ocimene
Limonene	7·91	External	*E*-β-Ocimene
***Z*-β-Ocimene**	8·55	External	*E*-β-Ocimene
***E*-β-Ocimene**	8·82	External	*E*-β-Ocimene
Oxygenated monoterpenoids			
1,8 Cineole	8·03	External	1,8 Cineole
***Z*-Furanoid linalool oxide**	11·66	External	Linalool
***E*-Furanoid linalool oxide**	12·05	External	Linalool
***E*-β-Ocimene epoxide**	12·2	External	1,8 Cineole
Linalool	13	External	Linalool
Linalool epoxide	13·54	External	Linalool
α-Terpineol	14·93	External	1,8 Cineole
***Z*-Pyranoid linalool oxide**	15·35	External	Linalool
***E*-Pyranoid linalool oxide**	15·59	External	Linalool
Irregular and sesquiterpenoids			
***E*-4 Dimethyl 1,3,7 nonatriene**	9·7	External	*E*-β-Ocimene
6-Methyl 5-hepten-2-one	10·16	External	*E*-β-Ocimene
E, *E*-α-Farnesene	15·6	Internal	Toluene
***E,E*-4,8,12-Trimethyl-1,3,7,11-tridecatetrane**	16·81	Internal	Toluene
Unknown compounds			
Unidentified terpenoid 1 (*m/z* = 41, 43, 55, 67, 69, 83, 95, 119, 123, 137)	11·02	Internal	Toluene
Unidentified terpenoid 2 (*m/z* = 43, 57, 69, 85, 109, 151)	19·18	External	Linalool
Unidentified terpenoid 3 (*m/z* = 43, 57, 69, 85, 109, 151)	19·23	External	Linalool
Unidentified terpenoid 4 (*m/z* = 43, 57, 69, 85, 109, 151)	21·64	External	Linalool
Unidentified aromatic (*m/z* = 43, 77, 115, 133)	19·75	Internal	Toluene

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