

**THE EVOLUTIONARY ECOLOGY OF FLORAL SCENT IN *HESPERIS*
MATRONALIS: ASSESSING THE POTENTIAL FOR POLLINATOR-MEDIATED
NATURAL SELECTION**

by

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Heritable trait variation and differential fitness among trait variants are conditions required for pollinator-mediated natural selection on attractive traits like floral scent. However, previous studies of floral scent have focused on assessing evolution through stereotypical pollination syndromes and often fail to evaluate the conditions of natural selection. I assess the potential for pollinator-mediated natural selection on the floral scent of color polymorphic *Hesperis matronalis* (Brassicaceae). A study that assessed the importance of shared biochemistry between floral scent and color found significant diurnal variation in scent emission and a population-specific effect of floral color on floral scent composition. Specifically, purple morphs tended to be similar, while white morphs tended to differ significantly. A survey of five wild populations across part of *H. matronalis*'s introduced range supported this trend, particularly for aromatic composition; both scent composition and overall emission rates varied among populations. An experiment comparing scent profiles of plants grown in a common garden environment suggested both environmental and genetic causes of among-population variation. A three-part study assessed the relationship between scent and fitness. Experimental augmentation of floral targets with color-specific floral scent revealed increased syrphid fly visitation in response to increased scent emission rate, predicting a positive linear relationship between plant fitness and emission rate. An experiment limiting pollinator access to plants showed this expected relationship for plants exposed to diurnal pollinators, but no relationship for plants exposed to

night pollinators. In contrast, I found a negative quadratic relationship between daytime emission rate and fitness across plants in four large wild populations, suggesting possible costs of scent production under wild conditions, i.e., attraction of herbivores or energetic expenditures.

Overall, this dissertation suggests strong potential for pollinator-mediated natural selection on *H. matronalis* floral scent. Additionally, the results illustrate the importance of assessing all conditions necessary for natural selection of floral scent rather than relying on the observational pollination syndrome framework to describe the evolutionary trajectory of a species.

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PREFACE

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1.0 INTRODUCTION

Three conditions are necessary for any trait to evolve through the process of natural selection: trait variation, fitness differences between the trait variants (as mediated by some selective agent), and the ability for the trait to be inherited (Endler, 1986). For floral characteristics, it is thought that pollinators play the role of selective agent; one of the main functions of floral traits such as shape, size, and color is to attract vectors that can carry pollen to suitable mates, potentially maximizing the fitness of an individual and their offspring (Waser and Price, 1981; Stanton, 1987; Nagy, 1997; Schemske and Bradshaw, 1999; Gómez, 2000; Ashman, 2003; Irwin and Strauss, 2005). This is epitomized in pollination ecology by the classic concept of “pollination syndromes”: plants are thought to evolve a suite of traits that is maximally attractive to a specific effective pollinator (Baker, 1961; Knudsen and Tollsten, 1993; Raguso et al., 2003; Fenster et al., 2004). Thus, it is assumed that by simply defining the floral characteristics of a species, you can determine the most closely associated pollinators.

Floral scent is thought to play a particularly important role in defining pollination syndromes, and thus has been a focus for many studies (i.e., Knudsen and Tollsten, 1993; Knudsen et al., 2006 and references therein). However, research suggests that for many species, defining a specific pollination syndrome does not completely capture the complex relationships between floral traits and pollinator behavior. Instead, it may be more appropriate to describe clusters of interacting traits as “sensory billboards” (Raguso, 2004); in some cases, floral scent

acts as one of several redundant signals to draw in pollinators, whereas in others floral scent serves as a long distance attraction cue while visual characteristics elicit feeding and landing behaviors (Lunau, 1992; Ômura et al., 1999; Kunze and Gumbert, 2001; Raguso and Willis, 2002; Raguso, 2004 and references therein). Such a perspective allows researchers to both consider the interaction of attractive characteristics and to focus on features that are not traditionally incorporated into the scope of pollination syndromes.

The concept of pollination syndromes is further complicated by the potential for floral trait correlations; if two traits are associated due to genetic and/or physiologic constraints (e.g., Armbruster, 2002), pollinators may be unable to effectively select for traits independently of one another. This may be particularly true for characteristics such as floral scent and floral color, as some floral scent volatiles are produced by branches of biochemical pathways that produce certain types of floral pigments; changes in pigmentation can lead to concurrent changes in floral scent (e.g., Zuker et al., 2002). In cases such as this, a grouping of trait characteristics may not be representative of simultaneous natural selection by a specific pollinator, but rather correlated selection due to preferences for one specific characteristic (reviewed in Ashman and Majetic, 2006). A pollination syndrome viewpoint limits one's ability to explore this possibility.

Few studies have attempted to examine floral scent evolution beyond descriptive studies based on pollination syndromes (e.g., Knudsen and Tollsten, 1993; Andersson et al., 2002) or pollinator physiology and behavior (Knudsen et al., 1999; Kunze and Gumbert, 2001; Raguso and Willis, 2002; Schiestl, 2004; Ashman et al., 2005). Rather than relying on a limited pollination syndrome based framework, scientists can more effectively study floral scent evolution by examining plant-pollinator systems for the objective conditions required for natural selection outlined by Endler (1986), while actively considering the possibility for trait

associations or interactions (see Galen and Newport, 1988; Valdivia and Niemeyer, 2006; Salzmann et al., 2007b). In this way, we can explicitly determine whether floral scent is a target of natural selection, as is commonly assumed by pollination syndrome theory. In this dissertation, I assess the potential for pollinator-mediated natural selection on the floral scent of the introduced species *Hesperis matronalis* within the context of a possible trait association between floral scent and floral pigmentation. *H. matronalis* is an extremely fragrant species that displays a floral color polymorphism (purple vs. white flowers) throughout its introduced range, making this an excellent focal species for such a study.

In Chapter 2, I report the results of a study that examines the nature of a possible association between floral color and floral scent profile (including composition of volatiles and amount of scent emitted) in *H. matronalis*. This research was conducted in collaboration with Dr. Tia-Lynn Ashman and Dr. Stephen Tonsor at the University of Pittsburgh and Dr. Robert Raguso at Cornell University; it was published in *Phytochemistry* (Majetic et al., 2007).

In Chapter 3, I present the results of a survey study assessing the contribution of within- and among-population variation to overall phenotypic variation in floral scent profiles of *H. matronalis* across a geographic gradient. This study further examines whether among-population variation is driven by environment, genetics, or a combination of both factors. This research, conducted with assistance in the field from Sarah Papperman, represents collaboration with Dr. Tia-Lynn Ashman and Dr. Robert Raguso and is currently in preparation for the *American Journal of Botany*.

In Chapter 4, I report the results of a manipulative experiment that determines the relationship between variation in *H. matronalis* floral scent profiles and pollinator behavior. Furthermore, I examine the relationship between floral scent variation and female fitness in an

array experiment limiting pollinator access and in wild populations of *H. matronalis* to determine whether these relationships coincide with the outcomes predicted by floral scent-pollinator relationships. This research was conducted in collaboration with Dr. Tia-Lynn Ashman and Dr. Robert Raguso, with field assistance from Sarah Papperman and Rachel Pileggi. It is in preparation for *Ecology*.

In Chapter 5, I review my findings, discussing the significance of this work to the study of plant evolutionary ecology. I also describe several potential future directions for my research.

2.0 FLOWER COLOR-FLOWER SCENT ASSOCIATIONS IN POLYMORPHIC *HESPERIS MATRONALIS* (BRASSICACEAE)

2.1 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.

2.2 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, 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).

I 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 (Appendix A), 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 (Appendix C). 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).

I 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?

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization of *H. matronalis* scent

I identified 33 volatile compounds from *H. matronalis* flowers (Table 2.1) 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 my samples (Table 2.1). I identified an additional 7 aromatic compounds, including those derived from benzoic acid (benzaldehyde, benzyl propionate, and benzyl acetate) and from phenylalanine (phenyl acetonitrile and eugenol), as well as one compound produced as an intermediate of tryptophan biosynthesis (methyl anthranilate). I 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*-beta-ocimene epoxide.

Principal components analysis (PCA) was performed on the thirty 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. This 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 1,000 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 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 6 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). I 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. I used a cut-off of ± 0.15 because this gave us the approximate upper 50th percentile. Less stringent cutoffs have unacceptable type I error rates. Any more stringent cutoff provides the same clear biochemical interpretation as ± 0.15 (this can be 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 my chemical compounds (an unidentified terpenoid) indicates that this factor reflects overall volatile emission rate (Table 2.1).

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 2.1). Seven compounds displayed negative coefficients beyond a threshold value of ± 0.15 : alpha-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 propionate, phenylethyl acetate, 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 my 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).

2.3.2 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 2.2) or in scent composition (PC 2; Table 2.2, but see below). 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.

2.3.3 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 2.2): *H. matronalis* flowers emitted more scent at dusk than at dawn, as evidenced by higher values for PC 1 in both study populations (Fig. 2.1), 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 2.2; Fig. 2.1). However, across time periods the color morphs, on average, did not differ significantly in their bulk emission (PC 1; Table 2.2) or in scent composition (PC 2; Table 2.2).

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-hour period. I 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. (Kolossova 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. Indeed, the *H. matronalis* populations described here are visited by both diurnal and nocturnal pollinator species (Appendix B; Appendix D; C. Majetic, University of Pittsburgh, *pers. obs.*; H. Sahli, Michigan State University, *pers. 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; Raguso et al., 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*.

2.3.4 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 2.2; Fig. 2.2): 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 2.2; Fig. 2.2). In addition, there was a significant interaction between population and flower color in floral scent composition (PC 2; Table 2.2, $p=0.01$). 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. 2.3). 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. 2.3). Consequently, white morphs differed significantly between populations in terms 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, University of Pittsburgh, *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 (see below).

2.3.5 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; 2006). 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., 1993; 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 alpha-pinene and linalool (Daly et al., 2001; Cunningham et al., 2004; Cunningham et al., 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 (Appendix D; C. Majetic, University of Pittsburgh, *pers. 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. I 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 beta-caryophyllene, a terpenoid compound unrelated to anthocyanin metabolism, remained unchanged (Zucker et al., 2002). However, this combination of floral cues was observed in the white morphs of only one of my 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, University of Pittsburgh, *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., 1999; Gómez, 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 my two populations are the result of different mutations. While molecular mechanisms regulating production of volatile compounds tend to be similar within and across species (Kolossova 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.

2.4 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*.

2.5 EXPERIMENTAL

2.5.1 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, University of Pittsburgh, *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 (6am-9am) and dusk (6pm-9pm). 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 ten minutes 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 0.10g 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, Virginia, 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 10mg Porapak Super Q adsorbent (Alltech Associates, Inc, Deerfield, Illinois, 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, Illinois, USA) using Tygon tubing. Inflorescences were sampled for one hour, with a flow rate from the bag through the scent trap of 250mL air/minute. After this sampling period, volatiles were eluted from the scent traps using 300 μ L 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.

2.5.2 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, I rapidly (~20 seconds) concentrated our samples from 300 μ L to a volume of 75 μ L using N₂ gas and added 5 μ L of 0.03% toluene (16ng) 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 I 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 μ L) 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, Illinois, 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

GCMS Solutions Software (version 1.02A, Shimadzu Corporation, Kyoto, Japan), and the amount of each compound in a sample was quantified through comparison to the 16ng 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 2.1). 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.

2.5.3 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 2.1). 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. The two PCs found to be significant were then examined further using individual fixed effects ANOVA (PROC GLM, SAS, 2001).

Table 2.1 Summary of *H. matronalis* scent chemical composition. 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.

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)
<i>Aromatics</i>							
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 propionate	0.04	0.19	16.02	0.010 (0.004)	0.073 (0.034)	0.006 (0.005)	0.025 (0.024)
Phenylethyl acetate	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)
<i>Terpenoids</i>							
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)

Table 2.1 continued

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)
<i>Terpenoids</i>							
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)
<i>E</i> -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)
<i>E</i> - 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)
<i>E,E</i> -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)
<i>Z</i> -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)
<i>E</i> -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)

Table 2.2 *F*-statistics and *P*-values from ANOVAs on the two principal components of floral scent in *H. matronalis* flowers. 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).

Source	PC 1 (60%) ¹		PC 2 (26%)	
	<i>F</i> _(Ndf,Ddf)	<i>P</i>	<i>F</i> _(Ndf,Ddf)	<i>P</i>
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 x Time	1.09 _(1,23)	0.31	0.85 _(1,23)	0.37
Color x Source	1.52 _(1,23)	0.23	7.00 _(1,23)	0.01
Time x Source	1.76 _(1,23)	0.20	1.18 _(1,23)	0.29

¹Values in parentheses represent percent of total variance in floral scent described by each principal component.

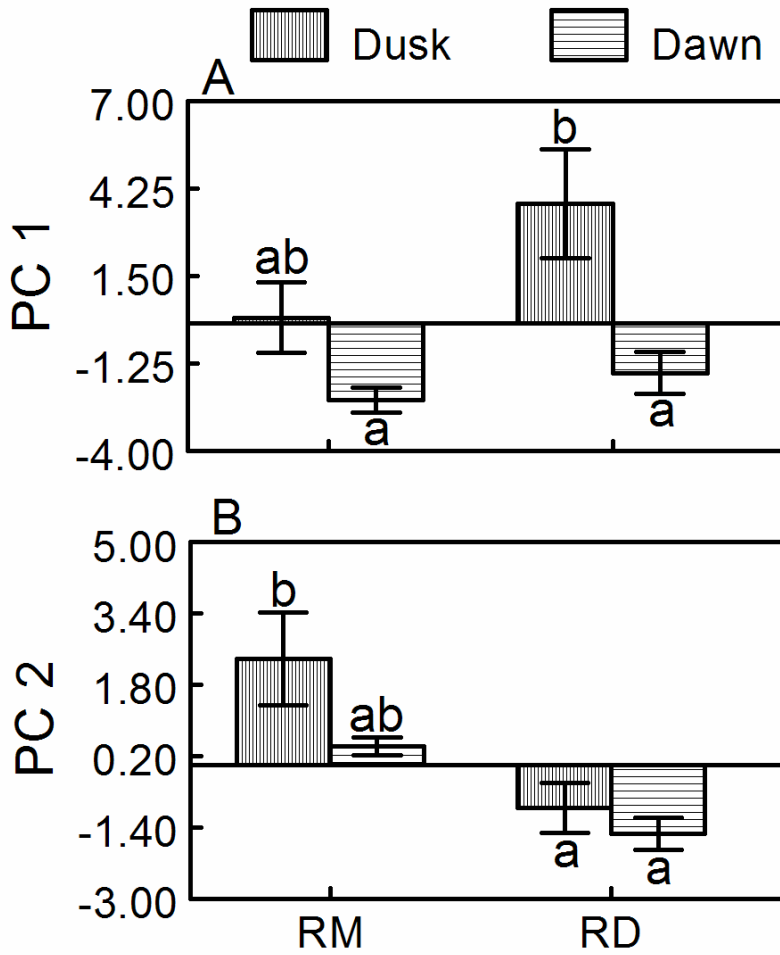


Figure 2.1 Mean (\pm SE) PC 1 (A) and PC 2 (B) of floral scent for both time periods (dawn, dusk) and populations (RM, RD) of *H. matronalis*. PC 1 describes overall amount of scent emitted. PC 2 represents scent composition; a bar presenting a negative mean suggests a more terpenoid-based scent, while a positive mean suggests a more aromatic-rich scent (Table 1). Means not sharing letters are significantly different ($P < 0.05$) as determined by Tukey's tests.

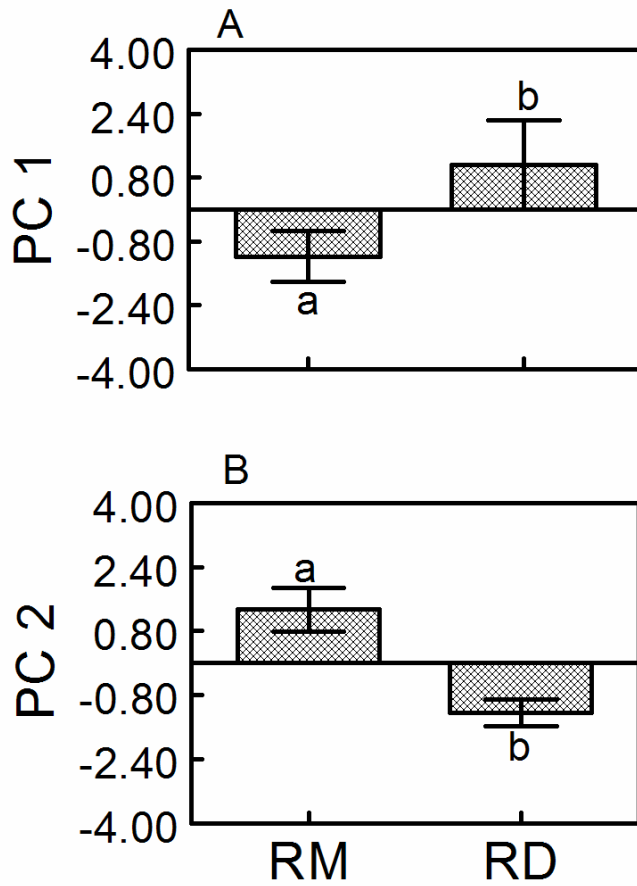


Figure 2.2 Mean (\pm SE) PC 1 (A) and PC 2 (B) of floral scent for populations of *H. matronalis*. Means not sharing letters are significantly different ($P < 0.05$) as determined by individual ANOVAs.

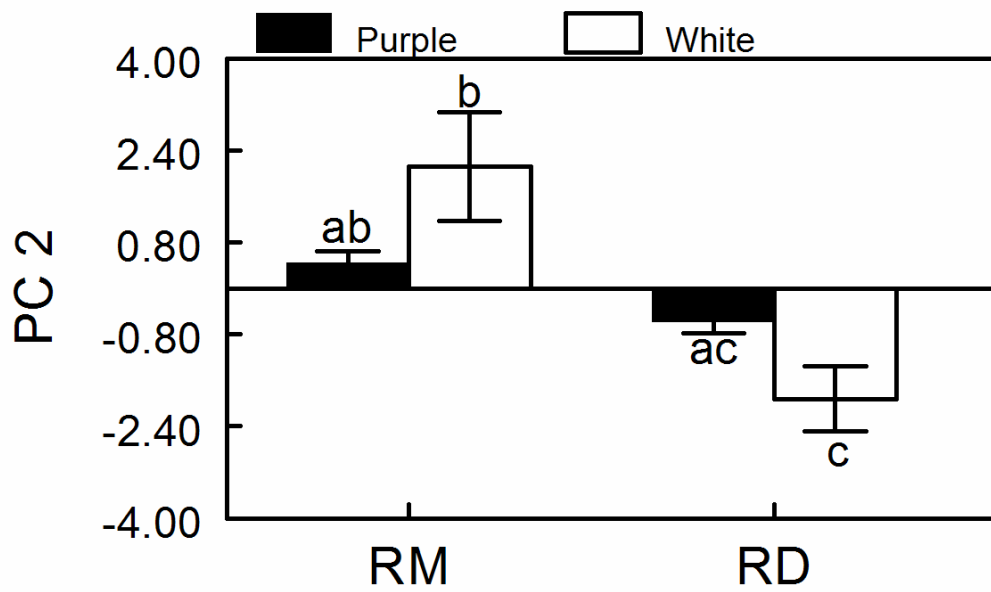


Figure 2.3 Mean (\pm SE) PC 2 of floral scent for color morphs and populations for *H. matronalis*. PC 2 describes predominantly negative loading of terpenoids and predominantly positive loading of aromatics (Table 1). Bars not sharing letters are statistically significant at $P < 0.05$.

**3.0 FLORAL SCENT VARIATION IN *HESPERIS MATRONALIS*
(BRASSICACEAE): ASSESSING POTENTIAL CAUSES OF WITHIN- AND AMONG-
POPULATION VARIATION**

3.1 ABSTRACT

Phenotypic variation in floral scent is well-documented for a large number of species and largely attributed to pollinator-mediated selection. However, very few studies attempt to partition this variation into within-population and among-population components. In this study, I examine the contributions of these components to floral scent profiles of color polymorphic *Hesperis matronalis*. Measurement of *in situ* floral scent from five populations suggests that patterns of correlation with floral pigmentation contribute to within-population variation in scent composition, but not scent emission rates. I also find significant among-population variation in both composition and emission rate in these wild populations, but this variation is not associated with population geography. I further explore scent variation by comparing volatile profiles of purple morphs from two populations, grown in common garden conditions. This comparison suggests that environment contributes to between-population variation, i.e., floral scent may be plastic. In addition, I find evidence for a possible genes-by-environment interaction in floral scent composition. These results suggest that future experiments on floral scent should assess

the possibility of both within- and among-population effects before solely focusing on pollinator-driven hypotheses of trait evolution.

3.2 INTRODUCTION

For evolution by natural selection to occur, there must be heritable phenotypic variation for the particular trait under consideration (Endler, 1986). Because this condition forms such a basic requirement for evolutionary research, a vast number of studies have been published documenting phenotypic variation in a variety of floral traits. Floral scent is no exception; emission rate and/or composition vary widely, and researchers often draw on known pollinator relationships or pollination syndromes to explain such variation (e.g., Raguso et al., 2003). These studies occur at many levels of biological organization, including among species, among populations of a species, and among individuals in a population (e.g., Knudsen and Tollsten, 1993; Grison-Pigé et al., 2001a; Knudsen, 2002) with studies documenting variation among species being particularly common. However, studies at the population level, where natural selection occurs, tend to be limited in scope and sample size (Knudsen et al., 2006 and references therein). Perhaps more importantly, these studies rarely attempt to assess potential sources or causes of phenotypic variation in floral scent within populations.

Phenotypic variation among populations may reflect phenotypic plasticity (an effect of environment, e.g., Jakobsen and Olsen, 1994; Nielsen et al., 1995; Agrawal and Karban, 2000; Schemske and Bierzychudek, 2001; Coberly and Rausher, 2003), genetic differentiation (a genetic effect, e.g., Galen and Kevan, 1980; Ackerman et al., 1997), genetic variation in

phenotypic plasticity (a genes by environment interaction, e.g., Pigliucci, 2001), or all three. In some cases, geography may provide insight into trait variation patterns among populations. Factors related to location, such as gene flow or natural selection by similar forces, may lead proximal plant populations to have similar scent composition or emission rates (Knudsen, 2002; Svensson et al., 2005). Genetic differentiation among populations, in contrast, can be the result of differential selection, genetic drift, or founder effects (Conner and Hartl, 2004). The casual observer thus cannot discern whether variation is caused by environmental effects, genetic differences among populations, or an interaction between the two.

Genetic differences among individuals in a population (and therefore within-population variation) can occur in quantitatively varying traits (e.g., flower size or number: Ashman, 2003) and polymorphic qualitative traits (e.g., flower color: Stanton, 1987; Rausher and Fry, 1993). Floral color is a particularly tractable trait polymorphism to study, as the genetics of pigmentation are often known. More importantly, variation in floral color may contribute, albeit indirectly, to floral scent variation. For instance, purple pigmentation in most plants is derived from the synthesis of a subset of anthocyanin pigments by a branch of the shikimate pathway (Taiz and Zeiger, 1998; Clegg and Durbin, 2000). The shikimate pathway is also involved in the production of a large class of volatile compounds, the aromatics (Schuurink et al., 2006). One may expect purple-flowered plants in a polymorphic population to produce anthocyanins in combination with certain aromatic floral volatiles due to their shared biosynthetic pathway. In contrast, if white-flowered plants owe their phenotype to null mutations in pigment biosynthesis (e.g., Levin and Brack, 1995; Nakatsuka et al., 2005), pleiotropic effects may result in the release of different kinds or quantities of volatile compounds. In this way, an association between floral color and some aspects of floral scent may contribute significantly to variation in floral scent

within populations. However, this relationship may not hold for all floral scent compounds. Another major class of volatiles, the terpenoids, is produced by both the mevalonate and methylerythritol phosphate pathways (Dudareva et al., 2004). Neither of these pathways is connected explicitly to anthocyanin pigment production; therefore, one might expect terpenoid volatile production to be more independent of floral pigmentation.

Given that variation in floral scent could be accounted for by genetic and/or environmental factors, research must not only document variation in floral scent amount and composition, but attempt to assess the relative contribution of among- and within-population effects to trait variation. This is especially true in a species where composition in floral scent may be influenced by floral color. Thus, color polymorphic plant species provide an attractive system for addressing these questions. Examining the floral scent profiles of color morphs across multiple populations may show a pattern in which individuals from the same population cluster regardless of floral color (Table 2.1a). Such a result suggests that population differentiation contributes strongly to floral scent variation. However, patterns of similarity between individuals of the same color morph regardless of population (Table 2.1b) suggest that floral color, a genetic polymorphism within populations, explains at least some of the variation in floral scent.

In the case described above, population differentiation occurs when there is significant variation or differences among populations. Variation among populations can be driven by environmental or genetic differences. A classic technique for assessing the contribution of these to variation in any given trait is to grow plants from multiple populations in a common garden (e.g., Núñez-Farfán and Schlichting, 2001 and references therein; Stinchcombe et al., 2004; Thorpe et al., 2005). For example, if floral scent varies based on environmental factors alone,

plants reared in the same environment will have similar volatile profiles regardless of their origin (Table 2.2a), indicating plasticity. If floral scent variation is caused by genetic differences between populations, plants reared in a common garden environment will have a volatile profile similar to that of plants reared in their home environment (Table 2.2b), assuming a random and representative sample of plants assessed in both sites. Finally, if floral scent variation is due to both genetic and environmental effects, response of volatile profile to rearing environment will vary among populations (Table 2.2c). This last outcome is consistent with genetic variation in phenotypic plasticity.

An excellent plant system in which to explore both within and among population effects is *Hesperis matronalis* (Brassicaceae). This non-native biennial is polymorphic for flower color, with anthocyanin-producing (purple) morphs and non-anthocyanin-producing (white) morphs (Mitchell and Ankeny, 2001; Majetic et al., 2007). Previous studies, although limited in scope, suggest there is variation in floral scent within and between populations (Nielsen et al., 1995; Majetic et al., 2007), and phenotypic association between floral scent and floral color (Majetic et al., 2007). In addition, *H. matronalis* is widespread in its introduced range in North America. I used *H. matronalis* as a model system to conduct a two-part study consisting of an *in situ* scent survey of both color morphs in wild populations across a latitudinal gradient and a comparative study of wild and garden reared purple-flowered plants from two source populations. In my previous work, I found that *H. matronalis* color morphs differed in their floral scent composition; the scent of purple morphs tended to be similar across two populations, while the scent of white morphs differed between the populations. By utilizing a more comprehensive sampling protocol across a larger geographic range, coupled with a manipulative experiment and more sophisticated analysis techniques that are strongly suited to floral scent data, I aimed to extend

our knowledge about the causes of floral scent variation in this species. Specifically by examining patterns in both terpenoid and aromatic scent compounds, I sought to answer the following questions: 1) In wild populations of *H. matronalis*, is variation in *in situ* volatile profile attributable to flower color and/or population membership? If population identity is a source of variation, then are populations that are geographically distant less similar than those that are in close geographic proximity? 2) Are differences between populations that are observed *in situ* maintained when plants are grown in a common environment? That is, is volatile profile of *H. matronalis* affected by rearing environment, and if so, do populations respond in the same manner?

3.3 MATERIALS AND METHODS

3.3.1 Study Species

Hesperis matronalis (Brassicaceae) is an herbaceous biennial that has been introduced to the United States and is found in disturbed areas throughout all but the southernmost parts of the country (United States Department of Agriculture, 2007). It has been designated as an invasive plant in some areas due to increased spread in recent years (United States Department of Agriculture, 2007; Pennsylvania Department of Conservation and Natural Resources, 2007). Plants over-winter as vegetative rosettes before bolting in mid to late spring in Pennsylvania. 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 Pennsylvania (individual flowers that are hand-pollinated with self pollen or allowed to autonomously self produce seeds, although the seed set is reduced; Appendix B). 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; Appendix B, Appendix D).

In all populations surveyed in western Pennsylvania, as well as many populations throughout its northern range, *H. matronalis* displays a flower color polymorphism consisting of purple or white petaled morphs (Appendix A), 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 (Appendix A). No associations have been found between flower color and flower size or shape (Appendix A). Previous studies of this species suggest that floral volatile emission (and therefore production) in *H. matronalis* peaks at dusk (Nielsen et al., 1995; Majetic et al., 2007), and floral color morphs differ in their scent composition (Majetic et al., 2007).

3.3.2 Plant material

In situ plants – In May and June of 2006, I conducted a survey of five wild populations of *H. matronalis* across part of its introduced geographical range in North America: two populations in southern Ontario (“ONT1”: N 44° 01’, W 79° 31’ and “ONT2”: N 43° 32’, W 79° 31’), two

populations in northwestern Pennsylvania (“PA1”: N 41° 36’, W 80° 25’ and “PA2”: N 41° 36’, W 80° 27’), and a population in northern Virginia (“VA”: N 39° 05’, W 78° 04’). These populations were strongly polymorphic (50-80% purple morphs, Appendix D) 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. At each location, the composition of the visitor fauna was observed and was found to be similar (Appendix D).

Common garden plants – In late April and early May of 2006, *H. matronalis* rosettes showing initial signs of bolting were collected from populations PA1 and PA2. Approximately 30 rosettes were transplanted into 4.5” pots using Fafard Middleweight Mix #4 soil (Conrad Fafard, Inc., Agawam, Massachusetts, USA) and moved to a shady area at the University of Pittsburgh’s Pymatuning Laboratory of Ecology (Crawford County, Pennsylvania) within 3 km of the source populations. Plants were watered daily using either a sprinkler system or hand-powered water pump and fertilized once following transplantation with ~ 10 Osmocote pellets (14-14-14 N-P-K, The Scotts Company, Marysville, Ohio, USA). Upon flowering, plants were assessed for flower color and assigned to the common garden experiment ($N = 28$ plants). While I did attempt to include both color morphs in our common garden, it was not possible to determine flower color for plants harvested at the rosette stage. Thus, the resulting garden consisted mostly of purple-flowered plants; therefore, while I discuss both color morphs in wild populations, I focus any comparisons between garden and home reared plants on purple morphs only.

3.3.3 Floral scent collection

Floral scent was collected using dynamic headspace extraction for one hour at dusk (between 1800 and 2100 hours) and one hour at dawn (between 0600 and 0900 hours) following the protocol outlined by Majetic et al. (2007). Briefly, I covered inflorescences (open flowers and buds) with a Reynolds, Inc. nylon resin oven bag attached to a scent trap; scent volatiles were pulled into the trap by vacuum pump, collected by elution with hexane, and stored at -20°C for later chemical analysis as described by Majetic et al. (2007). While floral scent was collected at both dawn and dusk, for the purposes of this paper I chose to focus on scent emitted at dusk, given that it is the time of peak production. Floral scent collections took place over a two to three day period in each wild population, coinciding with peak flowering time at a particular site. Temperatures at time of collection were similar for most populations (~ 16 °C to 23 °C), with the exception of PA2, which was somewhat cooler (~ 13 °C on days of sampling). I measured plant height as a proxy for plant size at flowering, as height is strongly correlated with several vegetative traits (e.g., leaf length: $P < 0.0001$, $r = 0.58$; leaf width: $P = 0.02$, $r = 0.37$; stem diameter: $P = 0.04$, $r = 0.32$; $N = 40$; Appendix A). To calculate emission rates based on the amount of floral tissue sampled, I counted the number of flowers on each inflorescence. Previous study suggests that rates calculated using flower number and rates calculated using fresh biomass are comparable (Majetic et al., 2007), and this non-destructive method allowed us to maintain plants to further assess seed fitness later in their life cycle. I collected *in situ* floral scent from wild plants at flow rates of c. 200 mL air/minute using vacuum pumps powered by portable batteries (xPower Powerpack 1500, Xantrex Technology, Inc., Burnaby, British Columbia, Canada; Husky Portable Power System, Husky Power Products, Ft. Lauderdale, Florida, USA). Plants reared in the common garden environment were moved to a shelter for

scent sampling, where vacuum pumps could be run using outlet power; previous studies of *H. matronalis* suggest that use of a temporary sampling location does not change the number, type, or amount of volatiles emitted (Majetic et al., 2007; C. Majetic, University of Pittsburgh, *unpubl. data*). Ambient air controls were collected concurrently with floral scent samples at all locations.

3.3.4 Gas chromatography-mass spectroscopy characterization of floral volatiles

To determine the chemical composition of our sampled volatiles, I performed gas chromatography-mass spectroscopy (GC-MS) at the University of South Carolina as described in Majetic et al. (2007). Thirty-nine scent compounds (see Appendix D, Table D.3) were identified using computerized mass spectral libraries and retention times, all of which are known terpenoid or aromatic floral volatiles. The compounds identified included 31 volatiles found in my original study of *H. matronalis* floral scent, as well as 8 additional compounds. The compounds fall into 6 subcategories based on their biochemical pathway of origin (as in Knudsen and Tollsten, 1993; Knudsen et al, 2006). Within the terpenoids, compounds were categorized as monoterpenoids, oxygenated monoterpenoids, or irregular and sesquiterpenoids. Within the aromatics, compounds were categorized as benzenoids, phenyl propanoids, or nitrogen-containing benzenoids (see Appendix D, Table D.3). Resulting MS compound peaks for each sample were then integrated using Shimadzu GCMS Solutions Software (version 1.02A, Shimadzu Corporation, Kyoto, Japan). The floral volatile profiles described here have two components: emission rate and composition. I consider the data manipulations and analyses of each of these in turn below.

3.3.5 Scent emission rate calculations

To accurately quantify the amount of each compound emitted, I conducted GC-MS analysis of 7 external standards (1,8 cineole, *E*- β -ocimene, benzaldehyde, linalool, benzyl acetate, 2-phenylethanol, and eugenol), generating dose-response curves and equations. Each floral emitted compound was then matched to an external standard based on knowledge of vaporization rates and structural similarity (see Appendix D, Table D.3) (Debbrecht, 1977, Jennings et al., 1997) and peak area was transformed by equation to μg of compound emitted per flower per hour. When an appropriate external standard was not present, the internal toluene standard was used to mathematically transform the data, as in Majetic et al. (2007). In a few cases, use of external standards led to underestimations when peaks on chromatograms were present but too small to accurately estimate quantity with my equipment. 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 $0.0001 \mu\text{g}$ per flower per hour. By doing so, I could account for the presence of all compounds, including those too small to accurately quantify, as contributors to total scent emission. Compound amounts were summed to obtain total emission rate (μg per flower per hour) within each subcategory. Emission rates for total aromatics and total terpenoids were calculated by pooling the appropriate subcategories, and grand total amount of scent emitted per flower per hour was calculated by summing across all data. Prior to statistical analysis, emission rate data (6 subcategories, 2 categories, and the total emission rate) were natural-log-transformed to improve normality and conform to the assumptions of ANOVA.

3.3.6 Patterns of floral scent composition

In situ *wild populations* – To address whether floral scent composition in wild populations was affected by floral color or population membership, I performed non-metric multidimensional scaling analyses (Borg and Lingoes, 1987). For this technique, I assessed chromatograms and coded all floral scent compounds as either present (“1”) or absent (“0”). This presence-absence data was then divided into two categories (aromatics and terpenoids) and used in two NMDS analyses (PC-ORD, McCune and Mefford, 2006; Borg and Lingoes, 1987), one for each category. I first calculated the aromatic and terpenoid compositional similarities between two plants measured by Sorensen’s dissimilarity index (NMDS using Euclidean distance instead produced similar results). The resulting scores were 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 is 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 2 axes to represent aromatic composition (stress = 16.96) and a model with 3 axes for terpenoid composition (stress = 14.13) (McCune and Mefford, 2006). From these models I could distill the ordination axis scores for each plant. I averaged these scores by source population and flower color to determine the mean position for each color morph within each population and to visualize these graphically. Populations that cluster together are interpreted as more similar in their scent composition than those that are very distant

(Borg and Lingo, 1987). By identifying the source population and floral color for these individuals, I assessed whether clustering is driven by biologically meaningful categories. 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). Thus, clustering of color morphs suggests that pigmentation, a genetic polymorphism within populations, explains part of the variation in floral scent composition, while clustering of purple and white means from the same population suggests that among-population effects play a more important role than flower color in determining floral scent composition (Table 2.1).

Following graphical representation by NMDS, I wished to determine if the clustering patterns found by this technique were statistically different. I thus performed analysis of similarity (ANOSIM) on the presence/absence data used in NMDS for aromatics and terpenoids respectively, using the ANOSIM procedure in the PAST program (PAleontological Statistics, version 1.73: Hammer et al, 2001). ANOSIM is a non-parametric, post hoc permutation analysis to assess the similarity between two or more groups of individuals in terms of a set of independent variables, such as the abundance or presence/absence of a given taxa or species (Clarke, 1993; Hammer et al, 2001). 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). For this analysis, individual plants were assigned to a group corresponding with clustering patterns in NMDS (see Results). 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). I also determined the identity of the major compounds contributing to differences between groups using the Similarity Percentage (SIMPER) procedure in PAST, 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 me to qualitatively compare data sets that contain compounds that are non-normally distributed and/or categorical (i.e., zeros are prevalent). While 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 the more appropriate approach. However, because these techniques use presence-absence data to describe the similarity of individuals, finer-scale differences such as the relative abundance of scent compounds are overlooked. To help further visualize patterns of chemical differences, I generated pie charts of the relative amount of individual terpenoid and aromatic compounds for each color morph in each population. While those compounds with a very small contribution to aromatic or terpenoid scent may not be visible (see Appendix D, Table D.4 for mean values of all compounds), these charts help to determine which compounds may be driving differences between color morphs or among populations.

Similarity of patterns found in floral scent composition may also be explained by geographic location of populations (e.g., Azuma et al., 2001; Knudsen, 2002; Svensson et al., 2005). To assess this possibility, I performed a Mantel test with 5000 permutations (PAST,

Hammer et al., 2001), using distance between population pairs in my first matrix and mean Sorensen's index of either terpenoid or aromatic scent composition for population pairs in my second matrix. A large correlation R value, along with a significant permutation *p*-value, would suggest that the distance between populations has a strong effect on their similarity in floral scent composition.

Common garden vs. in situ home environment comparison – I performed a second set of NMDS analyses using data from PA1 and PA2 purple morphs reared either in their home population or a common garden environment. These analyses resulted in ordinations with 3 axes for both terpenoid and aromatic composition (Terpenoid Stress = 12.11, Aromatic Stress = 10.12). Again, the resulting ordination scores were averaged, this time to find the mean for each population in each rearing environment, and the data was visually examined for patterns based on these two variables. I identified four groups (see Figure 3.4) and examined them for significant differences using ANOSIM/SIMPER, as described above. Significant differences between plants reared *in situ* and in the common garden suggest a role for environment in determining scent floral scent composition.

3.3.7 Floral scent emission rate

In situ wild populations – To determine the effects of floral color and population membership on floral scent emission rates, I conducted individual fixed factors ANOVAs for grand total scent, total aromatics, and total terpenoids emitted (PROC GLM, SAS, 2007). Because a non-random sample of populations along a latitudinal gradient was selected in this study, I treated population as a fixed effect (Gotelli and Ellison, 2004). A significant effect of floral color suggests a role

for within-population effects tied to biochemical pathways and pigment production, while a significant effect of source population suggests that populations are differentiated with respect to scent. When population effects were significant, I performed post-hoc Tukey's tests to determine the pattern of those differences. To further explore which subcategories might be contributing to significant differences in aromatics and terpenoids, I conducted two MANOVA analyses (PROC GLM with MANOVA statement, SAS, 2007), one using emission rates from the 3 terpenoid subcategories as dependent variables and one using rates from the 3 aromatic subcategories.

I also performed correlation analyses (PROC CORR, SAS, 2007) analyses between population mean emission rates (grand total, total aromatics, and total terpenoids) and population latitude to determine if geography influenced floral scent emission rates. A significant correlation would suggest that population location does affect the amount of floral scent emitted.

Common garden population – To determine whether floral scent emission responds to environment or if population differences remain when plants are grown in a common environment, I conducted individual ANCOVAs for grand total scent, total aromatics, and total terpenoids emitted (PROC GLM, SAS, 2007) on common garden purple morphs from populations PA1 and PA2. Source population was designated as a fixed factor; a significant effect would suggest that scent emission depends on population of origin, despite common growing/flowering conditions. I also included plant height at flowering as a covariate to control for the effects of plant vigor on scent production. To further explore which subcategories might be contributing to observed significant differences, I conducted two MANCOVA analyses (PROC GLM with MANOVA statement, SAS, 2007) using emission rates from the terpenoid and aromatic subcategories respectively as dependent variables.

3.4 RESULTS

3.4.1 Question 1: floral color and population membership

Floral scent composition – Mean aromatic compound NMDS values for purple morphs are centrally located and generally cluster together, regardless of population, whereas population means for white morphs are widely divergent (Figure 3.1a). Specifically, three white groups are visible relative to the central purples: whites from PA1 occur near the purples (I - top right quadrant), PA2/ONT2 cluster together and near the purples along axis 2 (II - left quadrant), and lastly VA/ONT1 cluster together (III) in the lower left quadrant of the graph (Figure 1a). Examination of these four clusters by ANOSIM suggests that group III (the VA/ONT1 white morph group), while not significantly different from the cluster of purple morphs, is marginally different from the PA1 white group (I - $R = 0.21$; Bonferroni adjusted $p = 0.08$) and significantly different from the PA2/ONT2 white group (II - $R = 0.11$; Bonferroni adjusted $p = 0.05$). Results from SIMPER suggest that this difference is driven in part by a larger mean abundance of eugenol, methyl eugenol, and 2-phenylethanol in VA/ONT1 white morphs in comparison to other white morph groupings. To further understand this result, I also examined the relative contributions of individual aromatic compounds for each color morph in each population (Figure 3.2a). Benzaldehyde and benzyl alcohol had large contributions to floral scent across all populations and color morphs. However, purple morphs generally had more benzyl propionate and benzyl benzoate than white morphs regardless of population, while white morphs from some

populations (ONT1, ONT2, and VA) were more likely to have nitrogen-containing benzenoids such as benzothiazole and larger amounts of eugenol and methyl eugenol present (Figure 3.2a, Appendix D, Table D.4). Taken together, these patterns suggest that purple morphs show a more conserved aromatic floral scent composition than white morphs.

The composition pattern differs when I consider terpenoids. Mean NMDS values for all purple morphs and for white morphs from 3 of the 5 populations cluster tightly with relatively positive axis 3 values, while whites from 2 populations (VA and ONT1) diverge towards more negative values (Figure 3.1b). Despite this visual pattern, ANOSIM suggests these groupings (VA/ONT1 white morphs vs. all other morphs) are not significantly different in terms of composition ($R = -0.07$; $p = 0.8$). Additionally for terpenoids, color morphs from the same population are often found near one another, suggesting some effect of population affiliation in determining terpenoid floral scent composition. Examination of the mean relative amounts of terpenoids further supports these patterns. Floral scent in all populations is dominated by *E*- β -ocimene and other monoterpenoids (Figure 3.2b). Despite this, scent profiles of the two color morphs are very similar within populations, supporting the general outcome of our NMDS analysis (Figure 3.2b). Populations ONT1 and VA, those with white morphs that diverge, are an exception to this pattern; here, white morphs are more likely to have α -farnesene and unidentified terpenoid 4 than any of the other populations or purple color morphs. In addition, white morphs from VA are more likely to have the furanoid linalool oxides and *E*- β -ocimene epoxide than any other plants. It is intriguing to note that white morphs from these two populations are also highly unusual in terms of their aromatics (Figure 3.1a) and yet they are geographically distant. In fact, Mantel tests comparing Sorensen's index values to a matrix of geographic distances were non-significant for both aromatic and terpenoid composition

(Aromatics: $R = 0.33$, $P = 0.28$, $N = 25$; Terpenoids: $R=0.12$, $P = 0.44$, $N = 25$), suggesting that similarity in floral scent composition is not related to the relative distance between populations.

Floral scent emission – There were no significant differences between color morphs, but highly significant differences among populations in the grand total, total terpenoid, and total aromatic scent emission rates (Table 3.3, Figure 3.3). 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 than terpenoids, these two populations had resulting grand total rates that were also intermediate in value. The interaction between floral color and source population was also not significant for grand total scent and total terpenoids, and only marginally significant for total aromatics (Table 3.3). Analysis of volatile compound subcategories similarly showed significant differences among populations but not between color morphs (Appendix D, Tables D.5 and D.6). No significant relationship was found between the latitude of a population and its grand total emission rate, nor with emission of aromatics or terpenoids (Grand total: $r = -0.66$, $P = 0.23$; Total Aromatics: $r = -0.77$, $P = 0.12$; Total Terpenoids: $r = -0.62$, $P = 0.27$; $N = 5$ for each).

3.4.2 Question 2: common environment

Floral scent composition – Purple morphs from PA1 and PA2 differ in terms of both aromatic and terpenoid scent composition when reared at home (Figure 3.4; ANOSIM: Aromatics – $p = 0.03$, $R = 0.22$; Terpenoids – $p = 0.01$, $R = 0.25$), especially in terms of eugenol and modified

linalool compounds (furanoid linalool oxides and linalool epoxide). However, the scent compositions of both populations are not significantly different when plants are reared in a common-garden environment (Figure 3.4). Together, these results suggest that response to environment plays an important role in determining the scent composition of purple morphs. Perhaps even more interestingly, while both populations show changes, the degree of change differs. For PA1, this composition shift is a result of significant differences between home and garden reared plants in terms of both aromatic and terpenoid scent (Figure 3.4; ANOSIM: Aromatics – $p = 0.01$, $R = 0.30$; Terpenoids – $p = 0.06$; $R = 0.20$); differences between home and garden reared plants for PA2 are not significant. Such an outcome suggests possible genetic variability in responses to rearing environment.

Floral scent emission rate – Common-garden reared plants from populations PA1 and PA2 differ in the amount of total aromatics they emit (Table 3.4, Figure 3.5); here, population PA1 emits less aromatic scent than PA2 while the opposite pattern occurs when scent is examined *in situ* (see Figure 3.3). Analyzing specific compound emission rates indicates that only total benzenoid emission differs between the populations (Appendix D, Table D.7). In contrast, the populations do not differ in their total terpenoid or grand total emission rates (Table 3.4, Figure 3.5; Appendix D, Table D.8). Taken together, these patterns suggest a significant effect of environment on the amount of scent emitted by purple morphs; rearing plants in a common garden either homogenizes the quantity of total scent emitted or changes the direction of differences in emission seen between natural populations.

3.5 DISCUSSION

3.5.1 Floral scent from wild populations: within-population variation

I found mixed evidence for within-population variation in floral scent. While I found no association between floral scent emission rates or terpenoid composition and floral color, I saw a consistent relationship between aromatic composition and pigmentation (Figure 3.1). In particular, composition of purple morphs tended to be conserved, as predicted by shared biochemistry, while that of white morphs was more divergent. This pattern held across a wide introduced range of *H. matronalis* and therefore these results significantly build on previous work on two local populations (Majetic et al., 2007). Few studies have considered the possibility of association between floral scent composition and floral pigmentation. Beyond this work, only Zuker et al. (2002) reports that alterations to anthocyanin-based floral color led to changes in floral scent, namely the production of more methyl benzoate when pigmentation was removed by antisense suppression of the flavanone 3-hydroxylase gene in the anthocyanin pathway. Flamini et al. (2002) also suggest a link between scent and color, showing that purple- and yellow-flowered *Viola etrusca* color morphs differed in terpenoid scent composition; however, they do not describe the pigment biochemistry involved in this system. Pecetti and Tava (2000) suggest that darkly-pigmented flower morphs of *Medicago sativa* emitted more volatiles than light-colored morphs, but fail to report whether these patterns were attributable to different volatile compound categories or specific pigmentation production. This paucity of research suggests that more studies are needed to explicitly evaluate the connections between anthocyanin production and the production of floral volatiles.

Perhaps the most intriguing pattern found in this study is the divergent floral scent composition associated with white color morphs. In *H. matronalis*, white coloration is associated with a lack of anthocyanins (Appendix A). These morphs could arise through a number of mutations in the pigmentation production pathway, as found for other plant species and through studies of transposable elements (e.g., van Houwelingen et al., 1998; Clegg and Durbin, 2000; Nakatsuka et al., 2005). It is possible that white morphs in different populations result from different null alleles for anthocyanin pigmentation, and thus may vary in their potential pleiotropic impact on benzenoid scent emissions. Studies exploring the genetics of aromatic floral scent often find that a single gene product can catalyze the production of several volatile compounds (Negre et al., 2002) and blocking the formation of one volatile product may lead to increased emission of other volatiles in a given pathway (Orlova et al., 2006). Such results suggest that changes to the flux of a biochemical pathway can have profound effects on a variety of end products. Indeed, one group of populations (VA and ONT1) tended to differ from the other white morphs in aromatic composition by the relative greater presence of methyl eugenol and eugenol (phenyl propanoids), as well as 2-phenylethanol (a benzenoid). These compounds are found in the same biochemical pathway, with 2-phenylethanol and (iso)eugenol likely produced in parallel branches from phenylalanine (Orlova et al., 2006). Methyl eugenol is produced in several plants, often through the methylation of eugenol by an *O*-methyltransferase (Lewinsohn et al., 2000). Thus, a mutation early in the metabolic pathway leading from phenylalanine to anthocyanins could change the composition of scent. If any of a number of null mutations (biochemical blockages) can arise to make a flower white (e.g., van Houwelingen et al., 1998; Nakatsuka et al., 2005), then there could also be a large number of possible changes in the production of associated floral scent compounds, including the presence or absence of the 3

compounds described above. For example, if the mutation occurs upstream of the production of scent compounds and anthocyanin pigment, one might get white flowers with no scent due to a lack of flux through the pathway. However, a mutation downstream of the production of scent compounds that blocks pigmentation (e.g., a null chalcone synthase mutation) might result in a white morph that produces many more benzenoid and/or phenyl propanoid odors due to an added flux of precursors, much like the results seen in Zuker et al. (2002). Finally, white pigmentation may be caused by the presence of flavonols in petal tissue (e.g., Frey, 2004); flavonols are also produced by the shikimate pathway and could also potentially alter scent characteristics. Such a possibility has not been assessed for *H. matronalis*. Future studies using a variety of pigment knock-out mutants would allow me to definitively assess these possibilities.

3.5.2 Floral scent from wild populations: among-population variation

In my study, populations differed significantly in both floral scent composition and emission rates (Figure 3.1, Figure 3.3), adding to evidence for such variation across plant species (e.g., Knudsen et al., 2006 and references therein). These results are unique, however, in that they attempt to assess possible causes of population differentiation by using geography as a crude proxy for all potential environmental differences between populations. The use of geography collapses both potential population isolation and environmental differences into a highly simplistic variable. Perhaps not surprisingly, I did not find a significant relationship. In fact, few studies of floral scent variation have documented a relationship between population-specific scent and a geographic pattern. Azuma et al. (2001) found that populations of *Magnolia kobus* are extremely variable in floral scent and that this variation does not reflect any kind of spatial

patterning. Svensson et al. (2005) found no relationship between chemical and geographic distances in *Yucca filamentosa* scent. In contrast, Knudsen (2002) found a negative relationship between geographic distance and quantitative scent similarity in populations of *Geonoma macrostachys*. In all of these studies, while one may be able to eliminate the possibility of similarity in scent among proximal populations, the lack of relationship between geography and floral scent cannot rule out the possibility that floral scent is associated with finer-scale environmental variability, such as soil moisture, plant nutrition, or herbivory levels.

3.5.3 Common environment

I found that floral scent responds significantly to changes in environment. Although I am unable to pinpoint the exact environmental changes associated with this experiment, the outcome suggests that a plant's volatile profile may be plastic and that environmental differences are likely to contribute to among-population variation. While research on floral scent production has concentrated on conserved biochemical pathways, genes, and enzymes in a handful of model species (e.g., Lewinsohn et al., 2000; Barkman, 2003; Dudareva et al., 2003; Negre et al., 2003; Dudareva et al., 2005), many studies have documented changes in vegetative and floral volatile production driven by abiotic factors, including temperature, humidity, and nutrient availability (i.e., Loper and Lapioli, 1971; Jakobsen and Olsen, 1994; Nielsen et al., 1995; Staudt and Bertin, 1998; Gouinguene and Turlings, 2002). While these latter changes are rarely referred to as plastic responses, they clearly fall into this category, suggesting an important role of environment. Moreover, differences in herbivory or pollination can elicit wounding responses or induce post-pollination changes in volatile production (i.e., Theis and Raguso, 2005; as

reviewed in Dudareva et al., 2006). Such patterns suggest that floral scent is indeed highly plastic; future studies should explore the degree of plasticity in floral scent, including the potential for natural selection on the plasticity of floral scent.

I also found that one population greatly changed its floral scent composition in response to growth in a common environment while the other did not. This result is suggestive of genetic variation in environment response (plasticity), specifically a genes-by-environment interaction (if this study effectively incorporates a random genetic sample from each population). Such interactions are rarely considered in studies of floral scent, with most studies that manipulate the environment ignoring genetics (i.e., Jakobsen and Olsen, 1994; Nielsen et al., 1995) and most studies of genetics relegated to only a few individuals in the highly controlled conditions of growth chamber, lab, or greenhouse settings (i.e., Dudareva et al., 2003; Negre et al., 2003, but see studies like Lewinsohn et al., 2000, where multiple chemotypes are used). My study represents an important first step in explicitly exploring the interactions between genes and environment on floral scent production in the wild. This is an area that is wide open for additional research.

3.5.4 Conclusions

The study described here finds both within-population and among-population sources of variation in floral scent; the former is attributable to shared biochemistry between floral color and floral scent, while the latter appears to involve environmental variation and potential genes by environment interactions. This uniquely suggests that a plant's floral scent profile is defined by a combination of both genetic/chemical background and growing location, a perspective often

ignored in studies of floral scent. Given my results, future research must work to disentangle the effects of environment, genetics, and trait associations on floral scent profiles, as well as consider the potential for phenotypic plasticity in this characteristic. Only then will it be possible to truly understand the processes driving variation in floral scent.

Table 3.1 Hypothetical predictions for wild populations if variation in floral scent is driven by factors that differ A) Among Populations or B) Within Populations (namely flower color). Predictions are given for both components of volatile profile: composition (in a “morpho-space” defined by two axes (X and Y)) and quantitative emission rate (as defined in statistical terminology). In the hypothetical graphs pictured, shapes represent different source populations, with purple shapes representing purple morphs and white shapes representing white morphs.

	A) Among Population Variation	B) Within Population Variation (Floral Color)
Composition Expectation		
Emission Rate Expectation	Significant effect of population	Significant effect of floral color

Table 3.2 Hypothetical predictions for population differentiation in floral scent driven by A) Environment, B) Genetics, or C) Genetics and Environment. Predictions are given for the scent composition component of volatile profile (presented in a “morpho-space” defined by X and Y axes). In the hypothetical graphs pictured, shapes represent different source populations, with red shapes representing plants reared in their home environment and blue shapes representing plants reared in a common-garden environment.

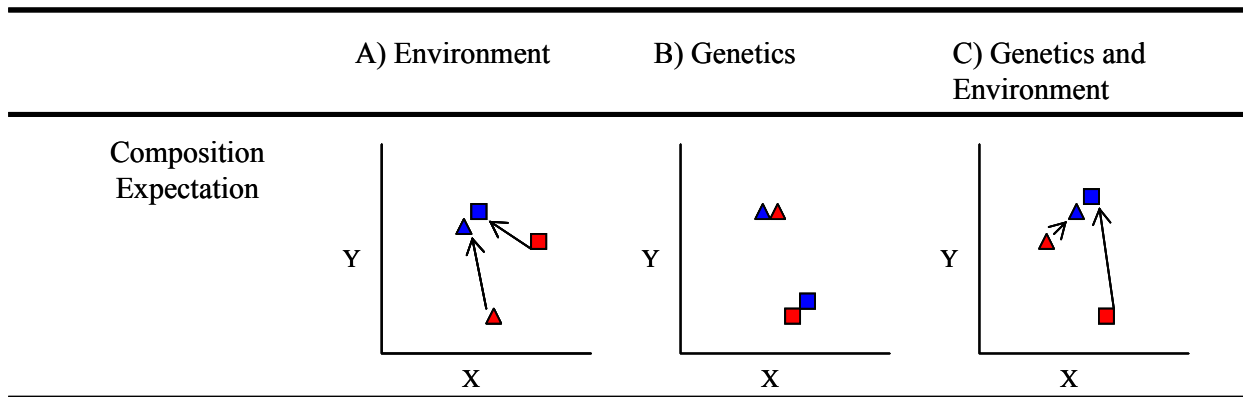


Table 3.3 Individual ANOVAs assessing the effects of floral color and population on three categories of *in situ* floral scent emission rates in *H. matronalis*.

Variable	DF	Total Scent		Total Aromatics		Total Terpenoids	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	9	5.84	0.0001	8.83	0.0001	4.32	0.0001
Color	1	1.32	0.25	0.42	0.52	1.90	0.17
Population	4	10.95	0.0001	17.52	0.0001	8.12	0.0001
Color x Population	4	1.69	0.16	2.10	0.09	0.99	0.42

Table 3.4 Individual ANCOVAs assessing the effects of source population (controlling for plant size) on three categories of floral scent emission rates in common garden reared purple *H. matronalis*.

Variable	DF	Total Scent		Total Aromatics		Total Terpenoids	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	2	0.16	0.85	2.05	0.15	0.05	0.95
Source Population	1	0.30	0.59	4.09	0.05	0.08	0.78
Plant Height	1	0.03	0.87	0.01	0.94	0.03	0.87

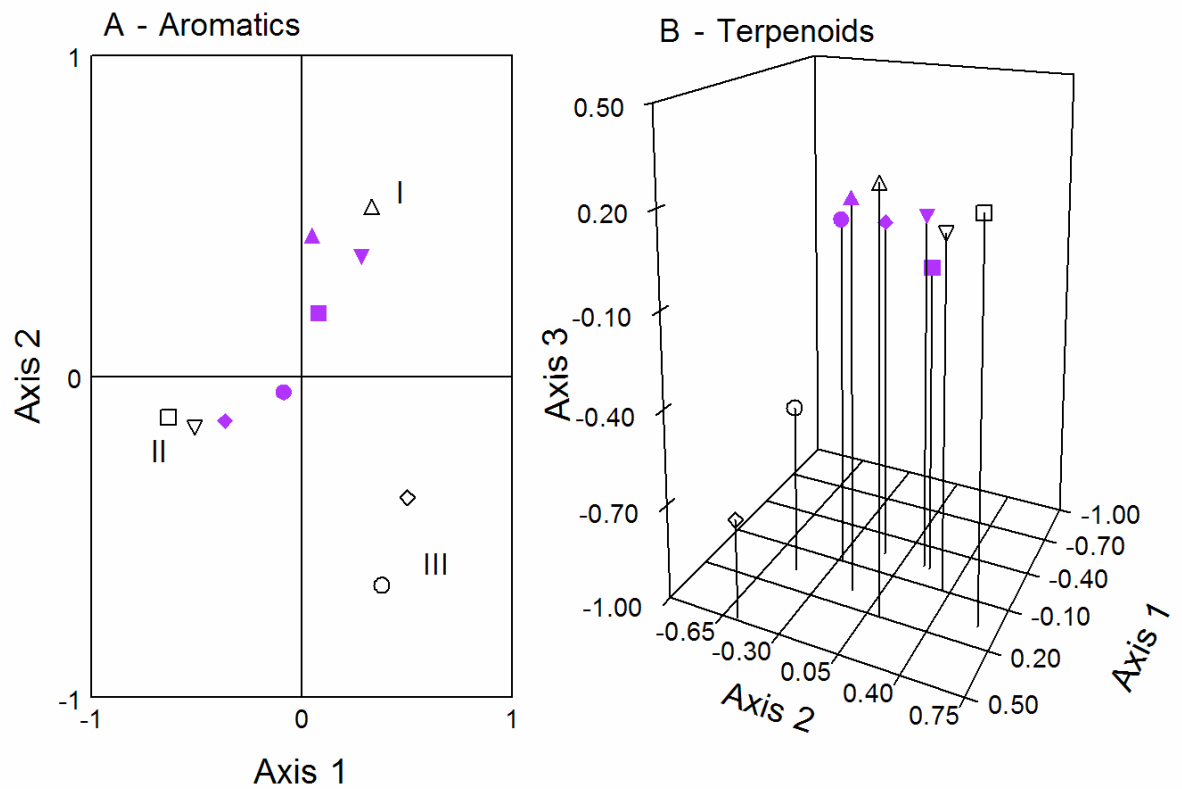


Figure 3.1 NMDS plots of *in situ* scent composition for wild populations of *Hesperis matronalis* in terms of (a) Aromatics and (b) Terpenoids. Purple symbols represent means for purple plants and open symbols represent means for white plants. Populations are represented as follows: triangles=PA1; circles=ONT1; diamonds=VA; squares=PA2; and inverted triangles=ONT2.

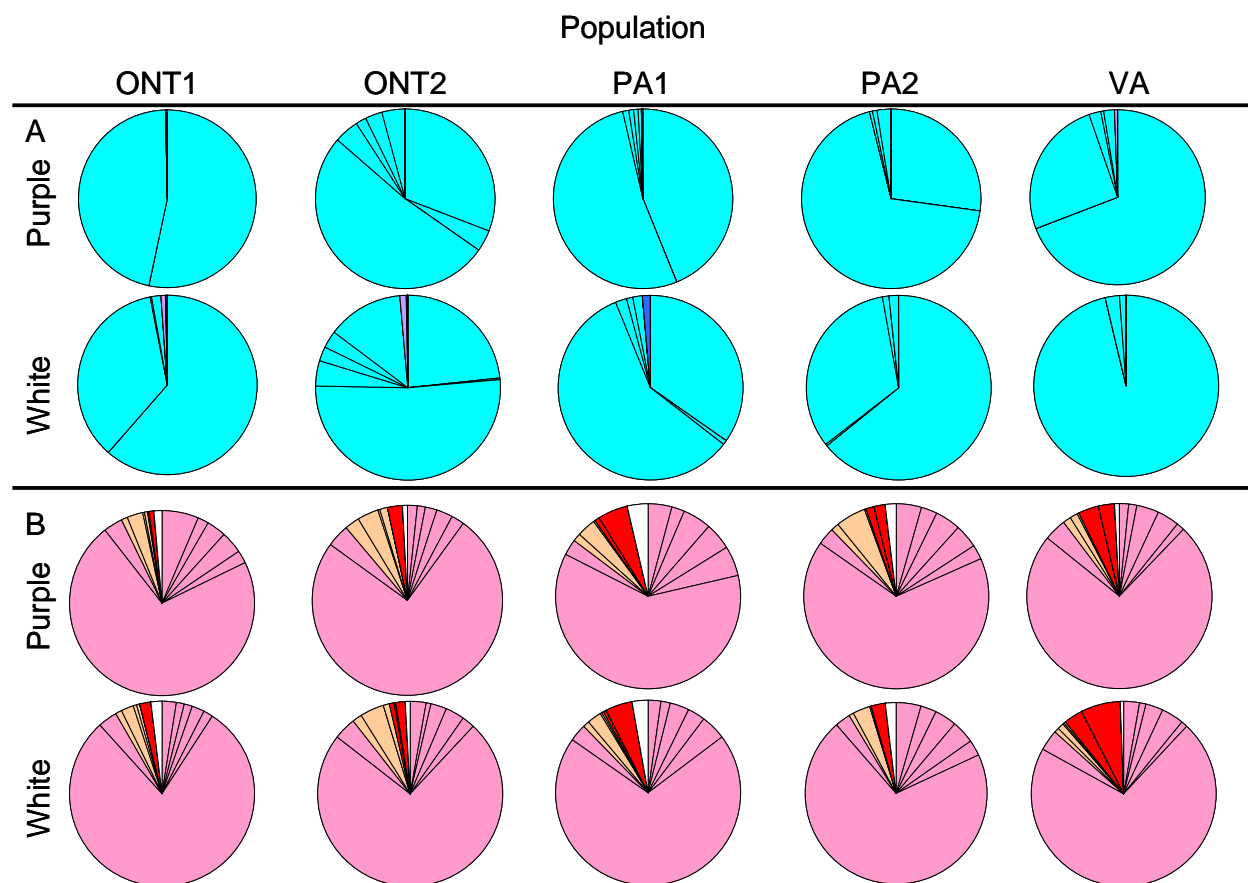


Figure 3.2 Mean relative amount of floral scent volatiles in five populations of *Hesperis matronalis* color morphs in terms of (a) aromatics and (b) terpenoids. Populations are ordered from north to south. Within aromatics, moving clockwise from the top of each graph, are benzenoids 1-9 (teal), phenyl propanoids 1 and 2 (purple), and nitrogen containing benzenoids 1-3 (blue). Within terpenoids are monoterpenoids 1-7 (pink), irregular and sesquiterpenoids 1-4 (peach), oxygenated monoterpenoids 1-12 (red), and unidentified terpenoid 1 (white). Due to the limitations of this visualization method, not all compounds may be visible in each pie diagram; for a complete list of the amount of each compound, in the order presented here, see Appendix D Table D.4.

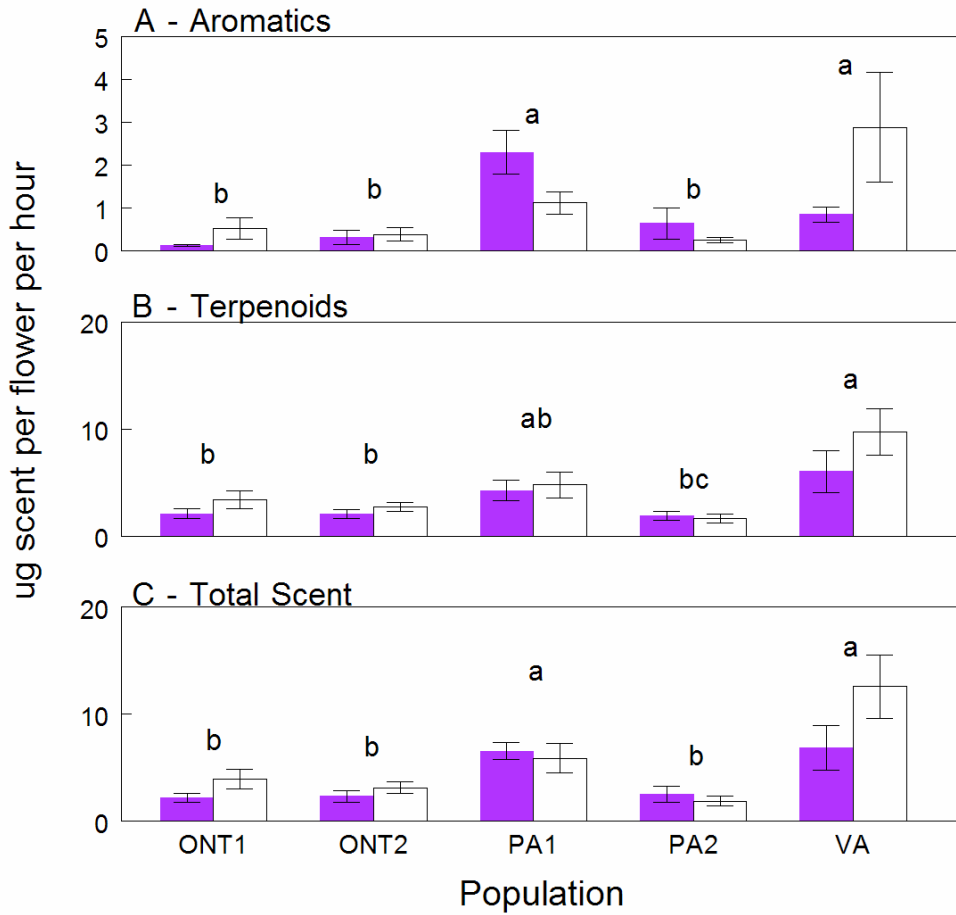


Figure 3.3 *In situ* scent emission rates for wild populations of *Hesperis matronalis* in terms of (a) Aromatics, (b) Terpenoids, and (c) Total Scent. Data has been untransformed for presentation. Purple bars represent purple plants and white bars represent white plants; error bars represent standard error. Letters over bars represent Tukey's test differences between population means.

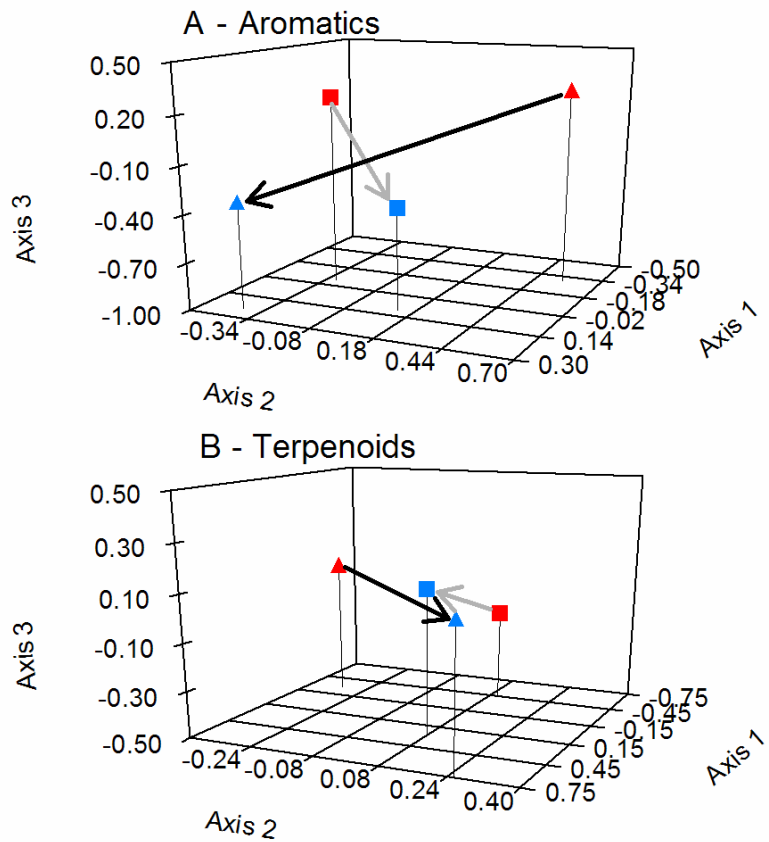


Figure 3.4 NMDS plots of scent composition for purple morphs from two populations of *Hesperis matronalis* – (a) Aromatics and (b) Terpenoids. Red symbols represent scent from home environment *in situ* plants while blue symbols represent scent from plants reared in a common environment. Population PA1 is represented by triangles and population PA2 is represented by squares. Arrows indicate the direction of shift from home environment to common garden floral scent composition; black arrows represent a significant difference between groups determined by ANOSIM and gray arrows represent a non-significant difference.

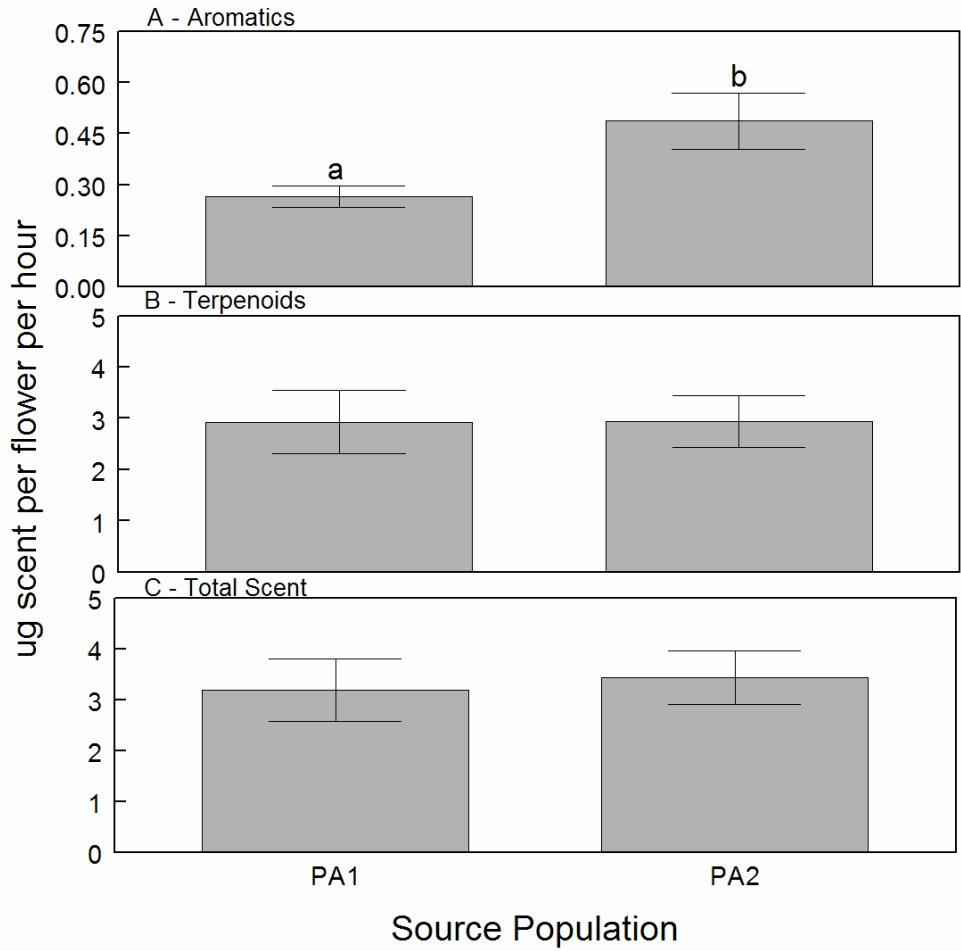


Figure 3.5 Scent emission rates for purple *Hesperis matronalis* plants grown in common garden environments in terms of (a) Aromatics, (b) Terpenoids, and (c) Total Scent. Data has been untransformed for presentation. Error bars represent standard error. Bars not sharing letters are significantly different as determined by ANOVA.

4.0 SMELL OF SUCCESS: FLORAL SCENT AFFECTS POLLINATOR ATTRACTION AND SEED FITNESS IN *HESPERIS MATRONALIS*

4.1 ABSTRACT

Patterns of floral scent are generally assumed to have been shaped by pollinator-mediated natural selection. However, while many studies document behavioral responses of pollinators to floral scent, few document the relationship between floral scent and fitness. In this study, I explore the effect of variation in floral scent emission rate in color polymorphic *Hesperis matronalis* on both pollinator visitation and seed fitness. Using target inflorescences augmented with color-specific floral scent extracts, I find that diurnal floral visitors significantly prefer higher scent emission rates. Such a result suggests that if pollinator visitation is an important component of plant fitness, then plants with greater scent emission rates will have higher fitness, unless there are countervailing forces, i.e., costs of producing excess floral scent, or of attracting enemies. I characterized the relationship between natural variation in floral scent emission rate and seed production for plants under two settings: 1) in small experimental arrays exposed to either day- or night-flying pollinators, and 2) in wild populations exposed to all pollinators. In arrays, I found greater emission rates led to higher seed fitness, but only in plants exposed to day-flying pollinators. In contrast, I found a significant negative quadratic relationship between daytime floral emission rates and seed fitness in wild populations. The difference in results between

arrays and wild populations could be due to differences in pollinators or herbivores, density-effects on scent cues/perception, or pollen limitation of seed production. My results suggest that a more comprehensive approach to the study of floral scent evolution is needed.

4.2 INTRODUCTION

Evolutionary studies of floral characteristics have traditionally focused on visual traits assumed to be attractive to pollinators, including floral display size, shape, and color (e.g., Stanton et al., 1986; Stanton, 1987; Schemske and Bradshaw, 1999; Caruso, 2000; Worley and Barrett, 2000; Ashman, 2003; Irwin and Strauss, 2005). Two key components of these studies are assessment of pollinator response to trait variation, and examination of the relationship between trait variation and plant fitness. A significant trait-fitness relationship is one of the three conditions necessary for evolution by natural selection (Endler, 1986); finding this provides initial support for hypotheses involving pollinator-mediated natural selection. Similar processes are also invoked to explain the evolution of non-visual floral traits, with some recent studies describing and testing pollinator response to floral scent (e.g. Waelti et al. 2008). However, little is known about the relationship between floral scent and plant fitness.

Floral scent is highly variable and often assumed to be an important target of pollinator-mediated selection (Miyake and Yafuso, 2003; Salzman et al., 2007b). Observations of pollinator fauna on plants with known scent characteristics suggest that both day- (e.g., Galen and Kevan, 1980; Pombal and Morellato, 2000; Theis et al., 2007) and night-active floral visitors have distinct olfactory preferences (e.g., Knudsen and Tollsten, 1993; Raguso and Willis, 2005;

Raguso et al., 2003; Hoballah et al., 2005), particularly for aromatic and terpenoid compounds (Dobson, 2006). Likewise, behavioral choice assays show that bees, butterflies, and moths can discriminate in both qualitative (e.g., Heath et al., 1992; Ômura et al., 1999; Cunningham et al., 2004, 2006; Theis, 2006) and quantitative aspects of floral scent composition (e.g., Heath et al., 1992; Andersson, 2003; Andersson and Dobson, 2003; Wright et al., 2005). Floral visitors often prefer scented flowers over unscented ones, translating into increased pollinator approaches, landings or preference (Knudsen et al., 1999; Kunze and Gumbert, 2001; Raguso and Willis, 2002; Schiestl, 2004; Ashman et al., 2005). These findings provide strong evidence for the importance of floral scent across the specialization-generalization spectrum of pollination.

Thus, it is perhaps surprising that the direct relationship between plant fitness and variation in floral scent has rarely been explored. Many studies view the rate of pollinator visitation as a proxy for plant fitness (e.g., Ayasse et al., 2000; Diaz and Kite, 2006), assuming a positive linear relationship between these variables. However, only a few studies specifically document fitness-scent relationships, with mixed results. In some cases, the presence of a less-preferred scent type or lower scent emission led to reduced fitness, through changes in pollinator activity (e.g., Galen, 1985; Galen and Newport, 1988; Miyake and Yafuso, 2003). Other studies found no significant relationship between scent variation and fitness (e.g., Ackerman et al., 1997; Valdivia and Niemeyer, 2006; Salzman et al., 2007a, 2007b). Moreover, no studies have explicitly tested for non-linear relationships between floral scent and fitness, despite several reasons to expect one. Specifically, a negative quadratic relationship might exist between emission rate and fitness if scent production is costly. For example, if scent production is energetically costly (as tested in Grison-Pigé et al., 2001), then seed production may decline at high emission rates, especially when resources are limiting. Similarly, floral scent may incur

ecological costs, such as attraction of florivores or herbivores by increased scent production, resulting in a net fitness decrease (e.g., Raguso, 2004 and references therein; Theis, 2006; Theis et al., 2007). Lastly, high rates of floral scent emission may actually repel the same pollinators that are attracted at low emission rates (e.g., Terry et. al., 2007), potentially leading to intermediate rates being optimal. Given the paucity of empirical evidence for linear or non-linear scent-fitness relationships, we are not yet in a position to evaluate the role of pollinator-mediated selection in the evolution of floral scent.

To address this gap, I explored whether higher floral scent emission leads to increased pollinator visitation and seed fitness in *Hesperis matronalis* (Brassicaceae). This plant is well suited for such studies because its purple or white flowers emit variable amounts of color-specific scent rich in terpenoid and aromatic compounds (Nielsen et al., 1995; Majetic et al., 2007; Chapter 2) known to be attractive to the insect taxa (Dobson 2006) that visit its flowers during day and night (Mitchell and Ankeny, 2001; Appendix B and D). I conducted a three part study focusing on the fitness effects of floral scent emission while controlling for flower color: a scent augmentation experiment, an array experiment where I manipulated pollinator-access, and an observational experiment across several large wild populations. I asked three main questions: 1) How do diurnal floral visitors respond to increased scent emission? 2) Does scent emission rate influence seed fitness when plants are exposed to diurnal vs. nocturnal pollinators? If so, is the relationship due to the emission rates of specific subsets (aromatics or terpenoids) of the scent blend? 3) Can variation in seed fitness in wild populations be explained by floral scent emission rates during day vs. night? If so, is the relationship due to aromatic or terpenoid emission rate? I predicted that increased emission of floral scent would lead to increased pollinator visitation and increased seed fitness, unless costs are associated with high floral

emission rates. I expected stronger scent-fitness relationships for day-exposed plants, given greater abundance of day-flying pollinators observed in wild populations (Majetic, 2008), as well as positive relationships between fitness and both aromatic and terpenoid volatile emission rates, given their demonstrated roles in pollinator attraction.

4.3 METHODS

4.3.1 Study species

Hesperis matronalis (Brassicaceae) is an introduced, potentially invasive (Annen, 2007), herbaceous biennial common to the northern tier of the United States (Mitchell and Ankeny, 2001). Plants over-winter as vegetative rosettes and flower in May. Inflorescences can have as many as 20 flowers open at a time (Mitchell and Ankeny, 2001). *H. matronalis* flowers are hermaphroditic and partially self-compatible in some populations (Majetic, 2008, but see Mitchell and Ankeny, 2001; Weeks and Frey, 2007). Self compatible plants set some seed autonomously, but those that receive pollinator visits have three-fold higher seed set (Appendix B). During the day, flower visitors in the United States are predominately bees (including *Bombus* spp. and *Apis mellifera*) and syrphid flies (a combined ~80% of visitors), with less frequent visits by lepidopterans, including crepuscular moths (Mitchell and Ankeny, 2001; Majetic, 2008).

H. matronalis populations are polymorphic for floral color, with purple or white morphs, although some populations contain a pink intermediate (Dvořák, 1982; Mitchell and Ankeny,

2001; Rothfels et al., 2002). Purple morphs have high levels of anthocyanins in their petals, whereas white morphs have little or no floral pigment (Majetic, 2008). Floral color morphs do not differ in size, shape, pollen or ovule production or vegetative characteristics (Appendix A), but differ in some aspects of scent composition (Majetic et al., 2007; Chapter 2). *H. matronalis* flowers emit a complex volatile blend (39 compounds) that varies diurnally (Nielsen et al., 1995; Majetic et al., 2007). Scent emission rates are two-fold higher at dusk than at dawn (0.041 μg vs. 0.019 μg scent/flower/hour; Majetic et al., 2007), but changes in floral scent composition are subtle (i.e., more aromatics emitted at dusk in some populations; Majetic et al., 2007). The focus of the current study is flower scent emission rate variation; thus, in each component study I control for flower color.

4.3.2 Experimental augmentation of floral scent and response by pollinators

Pentane extraction of whole flower scent and floral scent emitters – I used whole flower extracts to capture the complex floral scent of *H. matronalis* for augmentation of floral targets. I harvested purple and white *H. matronalis* inflorescences during morning (“day”: between 7am and 10am) and evening (“night”: between 6pm and 9pm) from wild populations near the University of Pittsburgh (Allegheny County, PA) and the Pymatuning Laboratory of Ecology (PLE; Crawford County, PA). I extracted volatiles for 20 minutes from these flowers using 2 mL pentane per gram fresh weight (Sigma-Aldrich, 99% purity, GC quality). I stored extracts at -20°C until use. I prepared separate extracts for each color morph at each time period.

I constructed floral scent emitters from 4 mL polypropylene culture tubes and wicks fashioned from coffee filters. I filled treatment emitters with ten flower equivalents of a pentane

extract (~1-2 mL of liquid) that was diluted to 3 mL with mineral oil, whereas I filled control tubes with 1 mL of pentane diluted to 3 mL with mineral oil. I produced five treatments which were used in each trial and are referred to as “control”, “purple day”, “purple night”, “white day”, and “white night” hereafter.

Pentane is commonly used for extraction of scent from floral tissue (e.g., Ashman et al., 2005) and is an appropriate solvent for a broad range of plant volatiles (Prosofski et al., 2007). All of the compounds I have found in the headspace of *H. matronalis* can be extracted with pentane (Kerrola et al., 1994; Antonelli et al., 1997; Gibernau et al., 1997; Gancel et al., 2003; Ashman et al., 2005; Jerković et al., 2006; Teixeira et al., 2007). However, it is possible that compounds in extracts were represented in different amounts than those detected *in situ*. Thus, I evaluated the effectiveness of our pentane extracts at capturing and emitting *H. matronalis* scent by conducting dynamic headspace extraction on 8 emitters – 2 purple day, 2 purple night, 1 white day, 1 white night, and 2 control – following Majetic et al., 2007. I identified 17 of the 39 floral volatile compounds previously recorded for *H. matronalis* flowers. Moreover, scent emission rate (μg scent per flower equivalent per hour) from emitters with night extracts was approximately twice that from emitters with day extracts (night = $0.55 \pm 0.22 \mu\text{g}$ scent/flower equivalent/hour; day = $0.32 \pm 0.09 \mu\text{g}$ scent/flower equivalent/hour; see Chapter 3 for scent quantitation methods). Thus, while my headspace and pentane extractions may not have captured all of the minor blend components, I did succeed in capturing quantitative differences between day and night scent emissions, which was my main goal in this experiment. Therefore, when I added an emitter with morning-produced scent, I effectively doubled the amount of scent, and when I added an emitter with night-produced scent, I tripled the amount of scent emitted from a target.

Experimental arrays – I constructed hexagonal arrays at two sites in an open field at PLE, each consisting of six experimental units spaced with least 1 meter between nearest neighbors. Each experimental unit was composed of a scent emitter plus a floral target. Floral targets were created by haphazardly selecting a white or purple inflorescence harvested from a wild *H. matronalis* population, trimming each to ten open flowers and placing them into individual florist’s pics. On the morning of observation days, each floral target was then randomly assigned to a control, day, or night emitter, taking care to match target and scent color. Emitters were assigned positions in the array at random and targets were arranged such that flower colors alternated. Emitters were given 5 minutes to equilibrate to ambient conditions prior to the onset of data collection.

All pollinator observations were conducted on eight warm sunny days in late May and early June 2007. Two observers simultaneously recorded visitation on each half of an array. Approaches and landings by all pollinator insect taxa by type (bees, syrphid flies, and lepidopterans) to each experimental unit were recorded during a 10-minute replicate. For each replicate within a given array, I rotated each emitter such that each target experienced each level of color-specific scent (i.e., control, day, and night). Observers also switched positions, such that they observed all possible pairs during a 30 minute observation period. New targets and emitters were used for each period. I conducted a total of 50 observation periods for a total of 25 hours of pollinator observation. Observations were conducted at both sites and no significant effect of site was found (data not shown), so sites were pooled in subsequent analyses.

Statistical analysis – I determined visitation rate as the sum of approaches and landings to each experimental unit. I performed an ANOVA (PROC GLM, SAS, 2007) to determine the effect of target color (purple vs. white), color-specific scent treatment (control vs. day emission

rate vs. night emission rate), and their interaction on visitation rates, while controlling for period and replicate. Because initial analysis revealed that replicates within an observation period were not significantly different, I calculated the number of visits per emitter type across all replicates within a period as total number of visits/30 min/target of 10 flowers and reran our ANOVA without replicate.

4.3.3 Floral scent emission rate and seed fitness in arrays with specific pollinator access

Experimental design – In the early spring of 2006, I collected rosettes from four Pennsylvania populations, transplanted them into 1-gallon pots with Farfard™ #4 soil (Conrad Farfard, Agawam, Massachusetts, USA) at PLE, and watered them daily. I constructed eight pollinator enclosures (2m x 2m x 2m) from window screen and PVC pipe. I placed four enclosures at each of two locations at PLE. At each location two enclosures were designated as “day-access” where only diurnal pollinators had access to the flowers, and two as “night-access” where crepuscular and/or nocturnal pollinators had access to the flowers. Pollinator access was controlled by opening and closing the sides of enclosures at specific times of day (i.e., day – 7:00am to 7:00pm; night – 7:00pm to 7:00am). Upon flowering, a total of 98 potted plants were randomly assigned to one of the pollinator-access treatments.

Data collection – For each plant, I recorded inflorescence height at flowering, floral size (as petal length × petal width) of a randomly chosen fully open flower, floral pigmentation and floral scent. Here, I characterized floral pigmentation quantitatively rather than qualitatively (i.e., purple or white). Specifically, I determined anthocyanin content using methanol extraction and spectrophotometry (modified from Harborne, 1998). I read absorbance at 530nm on a

Spectronic 21D spectrophotometer (Model DV #332278, Milton Roy, Rochester, NY); a higher absorbance value reflects darker purple flowers. I sampled floral scent using dynamic headspace extraction and gas chromatography-mass spectroscopy as in Majetic et al. (2007) and Chapter 3 for day-access plants in the morning and night-access plants at night. I calculated floral emission rate ($\mu\text{g}/\text{flower}/\text{hr}$) for each of the 39 identified compounds following Majetic (2008). Individual compounds were classified based on biosynthetic pathway (after Knudsen and Gershenzon, 2006) to form two classes (aromatics and terpenoids). Emission rates for each group were summed and then were natural-log-transformed to improve normality and conform to the assumptions of parametric statistical analyses.

I observed pollinator visitation during several 15-min periods on clear days in late spring 2007. I observed plants in the day-access treatment between 6:30am and 9:00am and 12:00pm and 3:30pm, and plants in the night-access treatments between 7:00pm and 9:00pm. A total of 13 to 16 observation periods were made at each of the three times for a total of 11.25 hrs of observation. I observed a total of 56 visits, 53 of which were by bees and syrphid flies (60% and 40%, respectively) visiting in the afternoon. I calculated visitation rate (visits/flower/hr) for each observation period based on the number of available flowers during each period.

Once plants had been exposed to pollinator access treatments for one month, I counted the number of treated flowers on each individual and fruits on these were allowed to mature. I recorded the number of seeds in each fruit. From these values, I estimated seed fitness as the number of seeds produced standardized by the number of treated flowers per plant.

Statistical analysis – Because my main interest was to determine how variation in floral scent emission rate affects seed fitness when different pollinators were allowed access, rather than to identify the sources of trait variation in our experiment, I conducted an ANOVA (PROC

GLM, SAS, 2007) to remove the effects of replicate and plant source population from all traits. I then regressed residual seed fitness on the residuals of plant traits for each pollinator access treatment separately (i.e., Model: residual seed fitness = $\alpha + \beta_1(\text{residual ln total day emission rate}) + \beta_2(\text{residual floral pigmentation}) + \beta_3(\text{residual floral size}) + \beta_4(\text{residual plant height}) + \gamma_1(\text{residual ln total floral emission rate}^2) + \epsilon_{ij}$). I included the squared term for floral scent emission rate to assess the potential for a quadratic relationship with fitness. When this analysis indicated a significant relationship between emission rate and seed fitness, I further investigated whether aromatic or terpenoid compounds had a tighter relationship with fitness. Because the emission rates of terpenoids and aromatics are highly correlated ($r = 0.73$, $P = 0.0001$), I did this by conducting the regression above, but substituting each class of volatiles for total scent separately.

4.3.4 Seed fitness and floral scent emission rate in wild populations

To determine whether day- or night-produced scent affects seed fitness of *H. matronalis* in the wild, I located four populations that span the latitudinal range of this species in eastern North America: southern Ontario (N 44° 01', W 79° 31'), northwestern Pennsylvania (N 41° 36', W 80° 25'; N 41° 36', W 80° 27'), and northern Virginia (N 39° 05', W 78° 04'). In each population, ten purple morphs and ten white morphs were selected at random and marked for study. For each, I measured floral scent, plant height, and seed production. I collected and analyzed floral scent from *in situ* plants at day and night as in Chapter 3 and I calculated emission rate for aromatics, terpenoids, and total scent as described above (*Floral scent emission rate and seed fitness in arrays...*). When flowering was complete, I enumerated the total number

of flowers and fruits per plant and collected three fruits per plant. To estimate seeds/plant, I multiplied total flowers/plant, proportion fruits/flower, and mean seeds/fruit. Plant height at peak flowering was recorded as an estimate of plant size (as in Majetic 2008). As I were unable to locate some marked individuals, final sample size was 69 plants across four populations.

Statistical analysis – I used ANCOVA (PROC GLM, SAS, 2007) to test for the effects of population, floral color, height, and floral scent emission rate on female fitness. While I found a significant effect of population, there was no significant effect of floral color on fitness, nor were there any significant interactions between population, color, and emission rate. Given this outcome, and the fact that within population replication was low, I sought to look at emission rate-fitness patterns across all populations. Therefore, I removed the effects of population and floral color on seed fitness, scent emission rate, and plant height using individual ANOVAs (PROC GLM, SAS, 2007) and used the resulting residuals to determine if there was a relationship between scent emission rate and seed fitness across populations (PROC REG, SAS, 2007; Model: residual seed fitness = $\alpha + \beta_1(\text{residual ln total day emission rate}) + \beta_2(\text{residual ln total night emission rate}) + \beta_3(\text{residual plant height}) + \gamma_1(\text{residual ln total day emission rate}^2) + \gamma_2(\text{residual ln total night emission rate}^2) + \varepsilon_{ij}$). As above, when I found a significant effect of total emission rate on seed fitness, I investigated whether aromatic or terpenoid components were contributing to the relationship by replacing total emission rate terms in the regression model with aromatics or terpenoids individually.

4.4 RESULTS

4.4.1 Pollinator response to scent augmentation

Approximately 95% of floral visitors were syrphid flies, with the remaining 5% of visits by small bees and lepidopterans. Flower visitors did not discriminate between purple and white target inflorescences ($F_{1, 240} = 0.72$, $P = 0.40$) but did discriminate between scent augmentation treatments ($F_{2, 240} = 4.74$, $P = 0.01$; Figure 1); this occurred regardless of observation period in our experiment ($F_{48, 240} = 3.10$, $P < 0.0001$). Syrphid flies visited targets augmented with night scent emission rate significantly more often (59%) than the control (Figure 1). In contrast, targets augmented with day scent emission rate received slightly but not significantly more (16%) visits than control targets (Figure 1) and this was similar across color morphs (scent-by-color interaction: $F_{2, 240} = 0.75$, $P = 0.48$).

4.4.2 Floral scent emission rate and seed fitness in arrays with specific pollinator access

Plants exposed only to day-flying pollinators experienced little visitation during the morning (0.006 ± 0.006 visits/flower/hour), but greater visitation during the afternoon (0.312 ± 0.167 visits/flower/hour). In this access treatment, seed fitness increased significantly and linearly ($\beta_{\text{totalscent}} = 1.63$; $P = 0.009$) with day floral emission rate (Figure 2). In contrast, none of the other plant traits (floral size, pigmentation and plant height) significantly affected seed fitness (Figure 2 legend). Day emission rates of aromatics and terpenoids had similar positive linear relationships with seed fitness ($\beta_{\text{aromatic}} = 1.26$; $\beta_{\text{terpenoid}} = 1.53$; $P = 0.01$ for both). At night I witnessed a very low level of flower visitation (0.007 ± 0.007 visits/flower/hour), and when

plants were exposed to visitors only at night, the regression model explained little variation ($R^2 = 0.16$) and was not statistically significant (Figure 2 legend).

4.4.3 Seed fitness and floral scent in wild populations

Across wild populations of *H. matronalis*, I found a significant negative quadratic relationship between daytime scent emission rate and seed fitness ($\beta_{\text{dayscent}}^2 = -193.2$, $P = 0.01$), but no significant linear effects of day emission rate nor any significant effects of night emission rate on seed fitness, although plant height had a significant positive effect on seed fitness (Figure 3). Further analysis suggested that daytime aromatic scent emission rate may drive this pattern, as the quadratic term was statistically significant for aromatics ($\beta_{\text{aromatic}}^2 = -134.02$, $P = 0.01$), but not for terpenoids ($\beta_{\text{terpenoid}}^2 = -100.47$, $P = 0.07$).

4.5 DISCUSSION

Increased floral scent emission in *Hesperis matronalis* leads to increased day-time pollinator visitation. When day-flying visitors were the only pollinators allowed access to plants there was a positive linear relationship between emission rate and seed fitness. In contrast, I found little evidence for night visitation, and no relationship between scent emission rate at night and seed fitness when only night visitors were allowed access to plants. In wild populations, I found a negative quadratic relationship between rate of scent emitted at day and seed fitness, but no significant relationship between night scent emission rate and seed fitness. Below I explore the significance of syrphid fly responses to scent, examine potential explanations for the disparity

between the results from my arrays and wild populations and discuss possible causal factors for the negative quadratic relationship between scent emission rate and fitness.

4.5.1 Floral scent and pollinator identity

Syrphid flies responded positively to my scent augmentation treatments, visiting targets augmented with night or day scent more than controls (Figure 1). While this result is consistent with widespread evidence for insect preferences for more strongly scented flowers (i.e., Knudsen et al, 1999; Ashman et al., 2005; Theis and Raguso, 2005), it is particularly novel for syrphid flies, because we have limited knowledge of their preferences and use of scent, especially in the context of generalized pollination systems (e.g., Pombal and Morellato, 2000; Dobson, 2006). Syrphid flies are common pollinators of *Hesperis matronalis* in its introduced range (Conner and Sterling, 1995; Weeks and Frey, 2007; this study) and of several other members of the Brassicaceae (e.g., Conner and Sterling, 1995; Stanton, 1987). Although visual attraction of syrphids has been studied extensively (i.e., Stanton, 1987; Goulson and Wright, 1998), my results indicate that floral scent is an important attractant for these flies. Previous studies have shown that syrphids prefer flowers with high pollen production, and pink rather than yellow or white colored flowers in *Raphanus sativus* (Stanton, 1987; Stanton et al., 1991). It is now known that both pollen (Dobson and Bergström, 2000; Ashman et al., 2005) and anthocyanin-based petal color (Majetic et al., 2007) contribute directly and indirectly to floral scent composition and emission rate. Thus, it is likely that syrphid floral preference is mediated by correlated visual-olfactory floral signals and pollen odors, rather than by visual display. In fact, in my day-access arrays, floral scent emission rate had a significant influence on fitness whereas visual cues, floral

pigmentation or floral size, did not (Figure 2); indeed, floral color was a non-significant factor in all of the experiments described here.

4.5.2 Reconciling disparate scent-fitness relationships

I found strikingly different relationships between floral emission rates and seed fitness in my two experiments. In my restricted pollinator access experiment, increased scent emission rates during the day resulted in a linear increase in seed fitness when plants were visited only by day-pollinators (Figure 2), but no significant relationship between night emission rate and seed fitness when visits were restricted to night-flying pollinators. In contrast, across wild populations there was a negative quadratic relationship between day floral scent emission rate and seed fitness, but no significant effect of night emission rate (Figure 3). While both results suggest that night emitted scent is not a potential target of selection in the introduced range of *Hesperis matronalis*, they offer potentially different views of the type of selection that might act on day-emitted scent. The two experiments differed in both abiotic and biotic features: one involved potted plants in small, sparse arrays with restricted pollinator and herbivore access, whereas the other involved plants in large, dense *in-situ* populations where all pollinators and herbivores had access. Thus, I cannot disentangle potential causes of the different outcomes. However, I can evaluate the likelihood of possible mechanisms of the different scent-seed fitness relationships.

First, the cost of producing floral scent may change with growth conditions. If high emission rates exact a greater energetic cost in dense plant populations, where resource competition is more likely, then the negative quadratic relationship between day-emission rate and seed fitness seen in the wild populations could be the result of greater costs of high emission

rate. However, it is quite difficult to assess the costs of reproduction in plants in general (Obeso, 2002), and estimates of the cost of floral scent are limited to one study in fig trees, which found scent to be a trivial component of total carbon allocation to reproduction (Grison-Pigé et al., 2001). While energetic costs of floral scent may be a larger portion of the reproductive budget for an herbaceous biennial plant, it is still unlikely that these costs are responsible for the large differences between experiments, or for the negative quadratic relationship observed in the wild.

Second, the likelihood of pollen limitation of seed production may have differed between my experiments due to differences in pollinator abundance or density of pollen donors. While diurnal insect visitation rates to my day-pollinator access arrays were equivalent or slightly higher ($1.1\times$) than those of wild populations (0.290 ± 0.048 visits/flower/hour; Appendix B), fewer pollen donor parents were available due to low within-array plant density and isolation of arrays from wild populations. Moreover, wild populations were potentially visited by night pollinators whereas the day-access array excluded these. Thus, seed production of plants in the wild populations was less likely to be pollen limited than those in arrays. Because the intensity of pollen limitation is known to mediate the relationships between attractive traits and seed fitness (Ashman and Morgan, 2004), it is likely that differences in pollen limitation contribute to the differences in the relationship between day emission rate and seed fitness seen here. Further research on the relationship between pollen limitation and the magnitude and direction of selection on floral scent emission rate is needed to determine if this is the case for *H. matronalis* and for scent in general.

Third, the likelihood of herbivore effects on seed production may have differed between my experiments due to differences in herbivore access to the plants. The potential exists for herbivore-mediated effects on seed fitness to oppose pollinator-mediated ones (e.g., Raguso,

2004) and generate the negative quadratic relationship between fitness and floral scent emission rate that I observed in the wild. While I did not record herbivory rates in the present experiments, nor do I have evidence that floral scent attracts herbivores in *H. matronalis*, plants in wild populations are often subject to herbivory (C. Majetic, pers. obs.) whereas the array plants were protected from herbivores to a degree. Thus, the possibility of an herbivore-mediated mechanism cannot be ruled out.

Finally, because plant density differed between my experiments, scent context also differed and this could contribute to the different scent-fitness relationships. My experimental studies, like most behavioral and field studies aimed at evaluating pollinator preferences, took the logical first step of separating floral scent targets spatially and examining insect response to precise scent units. In wild populations, however, insect pollinators are exposed to patches in which many plants emit scent in close proximity. This may make it difficult for insects to distinguish between emission rates of individual plants once they have entered a population (e.g. Valdivia and Niemeyer 2006). Such a situation seems likely for a fragrant species like *H. matronalis*, which can grow in dense patches. Pollinators in my system may initially be attracted to the species-specific scent of the entire population, but may not distinguish between adjacent individuals within the odor plume. Alternatively, plants emitting large quantities of scent may deter floral visitors (e.g., *Osmanthus*; Ômura et. al., 2000) even in a dense scent context. In either case, plants emitting large quantities of scent may be ignored or discriminated against, unless populations are small and/or fragmented, such as those in the colonization phase of a site, not unlike our array experiment. While my study points to a role for scent context in determining floral emission rate-seed fitness relationships, a controlled experiment in which similar plants are subjected to different density treatments would further address this possibility.

4.5.3 Potential for selection in a novel environment

I found no relationship between night-emitted floral scent and seed fitness in either my pollinator access experiment or my wild populations, despite previous evidence for the most floral scent being emitted at dusk (Nielsen et al., 1995; Majetic et al., 2007; Majetic, 2008). Additionally, my work here and elsewhere (Appendices B, D) suggests that crepuscular visitation is rare, whereas day visitation is common. Together, this evidence illustrates a significant mismatch between timing of peak scent production and peak pollinator visitation in North American populations of *H. matronalis*. Thus, it is possible that the relationships between day scent and fitness found here represent the effects of novel pollinator-mediated selective pressures on day floral scent emission. Changes in selective environments often lead to changes in plant phenotypes over time; for example, *Silene latifolia* from its introduced North American range displays weedy life history characteristics and increased native pathogen susceptibility as compared to individuals from native European populations (Wolfe et al., 2004). Little is known about the pollination environment of *H. matronalis* in its native range, but given the timing of scent production, it seems reasonable to suspect previous selection on floral scent emission at dusk or night by a crepuscular or nocturnal pollinator. In North America, it is likely that *H. matronalis* experiences a novel pollination environment, where day-flying pollinators are strong selective agents that target a different aspect of floral scent than native pollinator fauna – day scent emission rates. Comparative studies examining floral scent, pollinator identity and behavior, and reproductive fitness in both introduced and native ranges of *H. matronalis* will be necessary to assess this possibility.

4.5.4 Conclusions

The results presented here suggest that pollinators do respond to increased scent production, but that increased floral emission rates do not always lead to linear increases in seed fitness. My results add to evidence for opposing selection on visual floral traits (e.g., Strauss and Whittall, 2006) and stress the importance of evaluating the putative relationship between pollinator attractants and plant fitness in a variety of selective environments (i.e., Herrera, 1995). Rather than assuming that selection by pollinators has led to the floral scent phenotypes we observe, future studies of floral scent should directly measure seed and pollen fitness in the context of additional hypotheses, including potential energetic costs of scent production, attraction of herbivores and density-dependent effects on pollinator attraction.

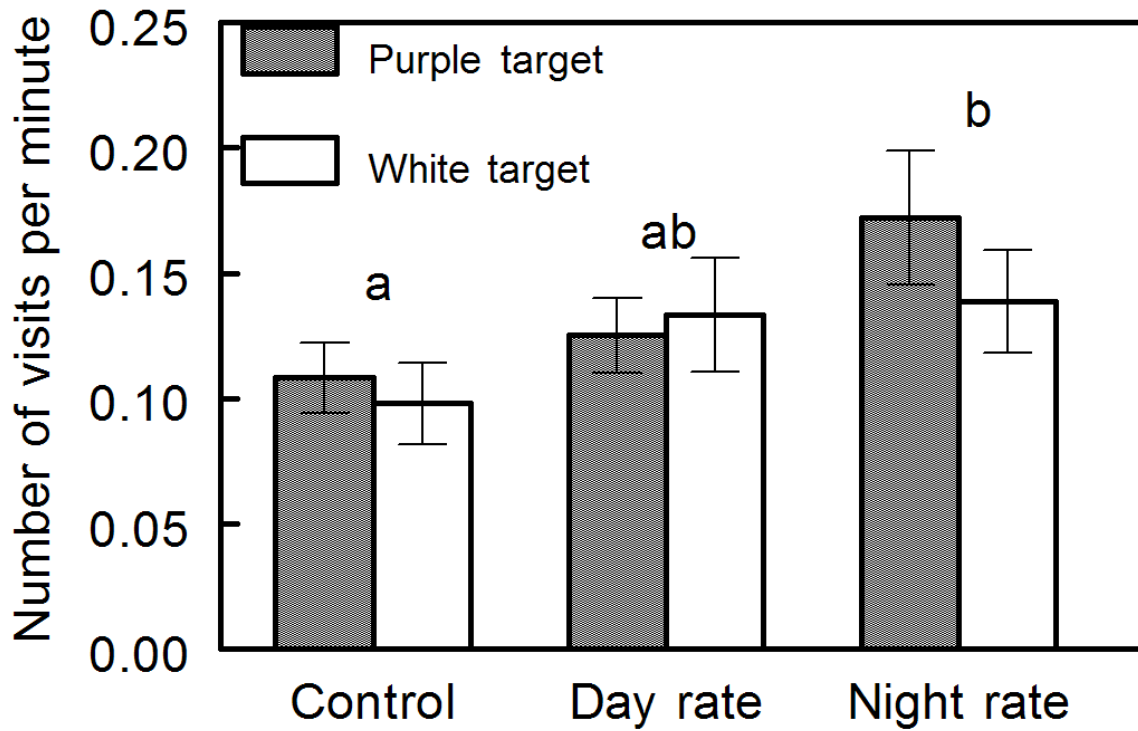


Figure 4.1 Pollinator visits to color-specific scent augmented floral targets (pentane control, day emission rate, and night emission rate). Emission rate is measured in μg scent/flower/hour. Evening emission rate in this experiment was determined to be approximately twofold greater than day emission rate. Letters above bars represent differences between overall treatment means as determined by Tukey's test.

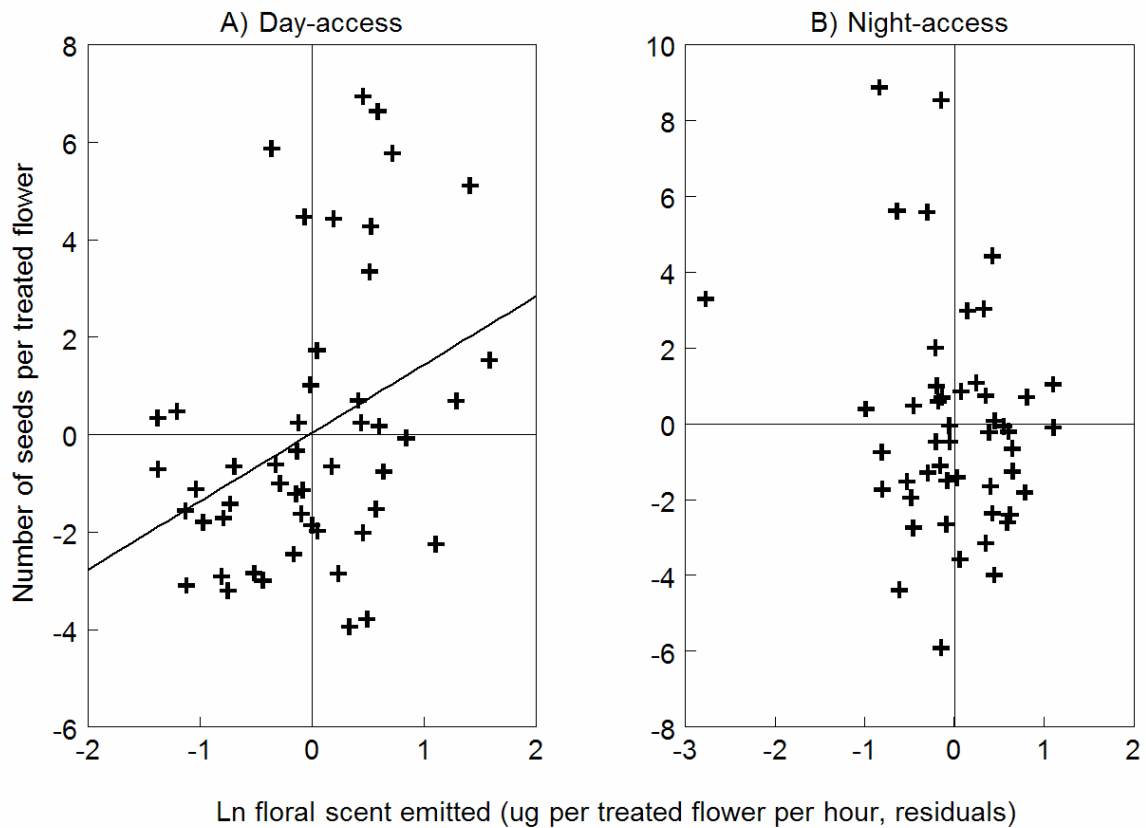


Figure 4.2 Relationship between *H. matronalis* seed fitness and floral scent emission for plants in exclosures that allowed pollinators access only during the day (A) or night (B). Complete models are as follows: Day-access ($R^2 = 0.25$; $P = 0.02$): seeds per treated flower = **1.63**(total day scent) + 0.25(total day scent²) + 0.03(floral size) + 2.23(floral pigmentation) + 0.08(plant height) – 0.08; Night-access ($R^2 = 0.16$; $P = 0.16$): seeds per treated flower = -0.96(total night scent) + 0.25(total night scent²) – 0.02(floral size) – 0.29(floral pigmentation) + **0.12**(plant height) – 0.10. Parameters in bold are significant at $P \leq 0.01$.

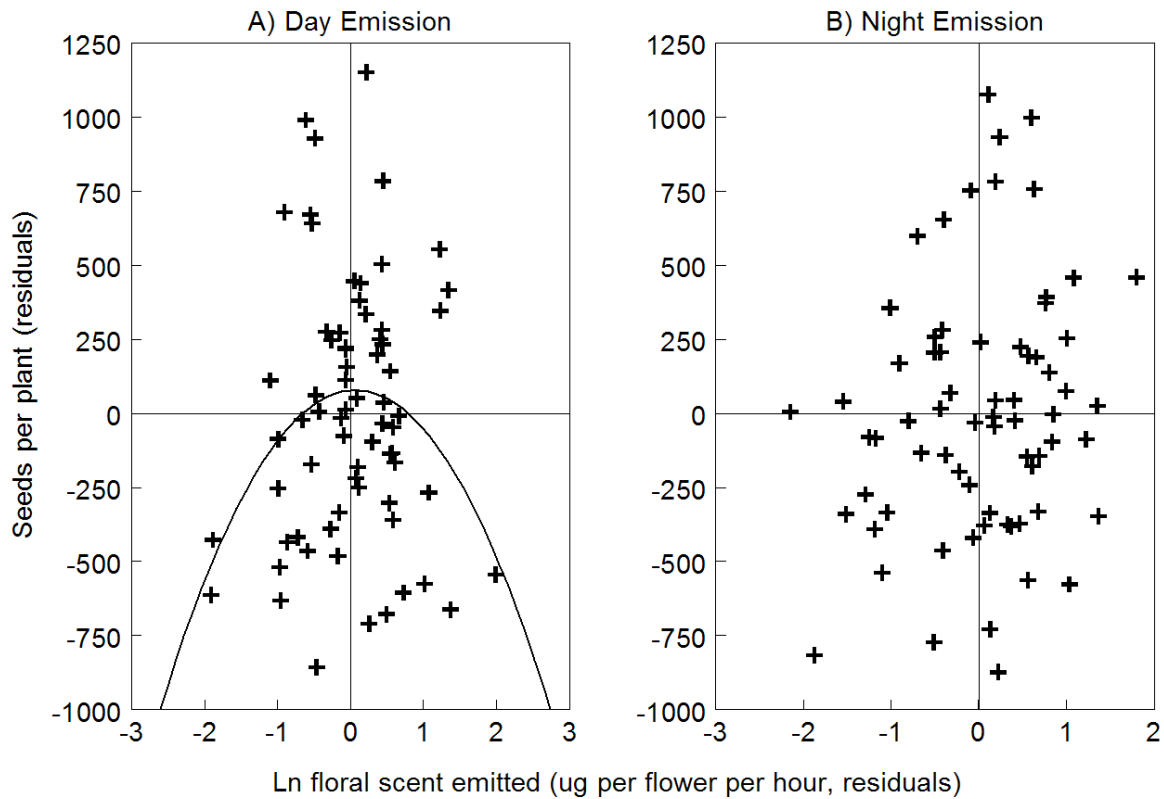


Figure 4.3 Relationship between seed fitness and A) day floral scent emission rate and B) night floral scent emission rate across four wild populations of *H. matronalis*. Complete regression model ($R^2 = 0.38$; $P < 0.0001$): seeds per plant = $12.65(\text{day total scent}) - 193.15(\text{day total scent}^2) + 92.49(\text{night total scent}) - 30.92(\text{night total scent}^2) + 24.27(\text{plant height}) + 116.61$. Parameters in bold are significant at $P \leq 0.01$.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I find that floral scent in *Hesperis matronalis* co-varies with floral color. It is in this context that floral scent meets at least one of Endler's (1986) conditions for natural selection: trait variation, both within and among populations. I also uncover discrimination between floral scent variants by a potential selective agent, syrphid flies. Under conditions limiting access to day-flying pollinators only, I find a positive linear relationship between floral scent and female fitness, as predicted by insect visitor preferences. In contrast, wild populations display a quadratic relationship between daytime floral scent and female fitness. In total, this work contributes significantly to the study of floral scent evolution by stepping beyond the "pollination syndrome" viewpoint and actively assessing the potential for pollinator-mediated natural selection in *H. matronalis*. Few studies specifically promote this type of approach for floral scent (see Galen and Newport, 1988; Ackerman et al., 1997; Salzman et al, 2007b), despite its use in studying a number of other floral traits thought to be under natural selection by pollinators. The research steps advocated here reveal that trait-fitness relationships may be more complex than what is predicted by simply assuming that trait variation and pollinator discrimination lead to natural selection. Below, I review the major results from each empirical chapter in more detail and present several possible directions for future research.

In Chapter 2, I investigated the potential for an association between floral color and floral scent in two populations of *H. matronalis* at two times of day. While scent emission

increased at dusk regardless of pigmentation or source population, floral color and population identity had a significant effect on chemical composition. Purple morphs across both populations tended to have a very similar scent composition, while white morphs had population-specific scent; white flowered individuals in one population had a strong association with terpenoid compounds, while plants in the other population had an association with aromatics. These results uniquely suggested that some variation in scent composition can be explained by floral color, possibly through conserved biochemistry for pigment and scent production.

I extended this research into floral scent variation by conducting a large scale geographical survey of five wild populations of *H. matronalis*, where I examined the potential contribution of within- and among-population variation to overall variation in floral scent profiles (Chapter 3). Floral color contributed significantly to variation within populations, as purple morphs had very similar aromatic volatile compositions regardless of population identity, while white morphs diverged. I also found significant composition and emission rate variation among populations; these patterns were not associated with the geographic locations of populations and so implied some degree of differentiation. These results suggested that floral scent composition and emission rates are highly variable traits across the introduced range of *H. matronalis* and therefore can potentially be targets of natural selection. Additionally, an experiment with common-garden reared plants from two of the surveyed populations suggested that environmental factors can alter the expression of floral scent profiles. Moreover, the degree and direction of changes caused by a novel rearing environment were population-specific. Although this work was not able to disentangle specific genetic or environmental conditions, the results suggested that floral scent has the potential to display phenotypic plasticity. Few studies of this character consider the role that variable response to the environment might have on floral

scent production, and what effects this type of response may have on the potential for natural selection on floral scent (see below).

Finally, I conducted a three-part experiment, assessing potential pollinator response to variation in floral scent emission rates and determining whether relationships between pollinator behavior and scent dictate female fitness-scent relationships under different growing conditions (Chapter 4). I found that syrphid flies preferred to visit target inflorescences augmented to emit more floral scent. As predicted by this pattern of behavior, in environments where pollination was limited to visits by diurnal insects, plants with increased scent emission were found to have increased female fitness; no scent-fitness relationship was found for plants exposed to night pollinators only. When plants in wild populations were assessed, I found a significant quadratic relationship between daytime floral scent emission and female fitness, but no relationship between fitness and night scent. This study is one of a few studies that explicitly assess the relationship between trait variation and fitness differences, a necessary condition for natural selection (Galen, 1985; Galen and Newport, 1988; Ackerman et al., 1997; Miyake and Yafuso, 2003; Valdivia and Niemeyer, 2006; Salzman et al., 2007a, 2007b). This research also surprisingly suggested that although *H. matronalis* produces its largest quantities of scent at dusk, the amount of scent emitted during the day may be subject to diurnal pollinator-mediated natural selection; night scent had no impact on fitness in these experiments.

Together, these findings provide several major unique insights into the study of floral scent evolution. First, floral scent of *H. matronalis* does provide significant trait variation as material for natural selection, particularly when scent is considered in the context of associations with floral color. This relationship between color and scent implies potential biochemical connections, particularly for aromatic compounds which could share production

pathway elements with anthocyanin pigment. More research should consider conservatism in biochemistry as a plausible hypothesis for floral scent variation. Results from my studies of floral scent variation, visitation, and female fitness suggest that syrphid flies (an often overlooked pollinator) are a likely agent of natural selection on floral scent. While many studies make this assumption for other pollinators, few consider the behavior of all possible pollinators and direct relationships between fitness and floral scent before claiming the occurrence of pollinator-mediated natural selection on this trait. Moreover, my research suggests that the relationship between pollinators, floral scent, and fitness may not be as straightforward as assumed. Incorporating these elements into studies of floral scent evolution will provide explicit evidence for the conditions of natural selection and allow researchers to consider increased biological complexity in their questions, a previously difficult task under the restrictions of pollination syndrome theory. From the groundwork described here, it will now be possible to carry out experimental studies measuring natural selection on floral scent for *H. matronalis*.

This dissertation suggests several new directions for research on floral scent evolution, in addition to the two central insights described above. Although my study of within- and among-population variation did not contain the genetic or environmental experimental controls necessary to unequivocally assess phenotypic plasticity, the population-specific changes in floral scent profile in response to a novel rearing environment suggests the potential for floral scent plasticity (Chapter 3). While the effects of environmental factors like atmospheric humidity, light regime, and temperature have been previously explored for the floral or vegetative scent of a few species (e.g., Loper and Lapioli, 1971; Hansted et al., 1994; Jakobsen and Olsen, 1994; Nielsen et al., 1995; Staudt and Bertin, 1998); only a few studies have considered growing condition aspects like soil moisture and nutrient levels (Loper and Berdel,

1978; Gouinguené and Turlings, 2002). If floral scent is indeed plastic, pollinators will be presented with a “moving” selection target, making it difficult to discriminate between individual plants in a species like *H. matronalis*, where flowers are long-lived (C. Majetic, University of Pittsburgh, pers. obs.). Future studies explicitly designed to examine the nature of floral scent phenotypic plasticity and its potential impacts on pollinator fauna will help to present a more realistic picture in this and other plant systems.

In Chapter 4, I present several hypotheses that may explain why I find different relationships between floral scent and female fitness under different conditions. These include differential pollen limitation, a cost of floral scent production, and opposing selective pressures. These possibilities have been well-explored in other systems, but more research focusing on floral scent is needed for all three. Perhaps most intriguing is the final possibility put forth in this chapter: the potential for density effects on floral scent cues to pollinators. We know much about the synergy of olfactory (long-distance attraction) and visual cues (foraging behavior) for attracting pollinators in individual flowers or floral units (e.g., Lunau, 1992; Ômura et al., 1999; Kunze and Gumbert, 2001; Raguso and Willis, 2002). However, individual plants often occur in dense stands where odor plumes overlap, changing the nature of the scent signal provided to pollinators and consequently altering the potential for natural selection on scent variants. Future research should therefore move beyond simple experimental conditions and consider the potential for pollinator-mediated natural selection in a variety of scent environments.

Finally, the work described here uncovers substantial population differentiation for floral scent composition and emission rates across part of *H. matronalis*'s introduced range. There are many possible causes for population differentiation, including differential natural selection in populations and genetic drift (Conner and Hartl, 2004). To truly understand the

evolutionary dynamics of floral scent in this species, future work must include studies that explore the causes of population differentiation, including differential selection pressures across populations and the potential for genetic drift.

APPENDIX A

THE RELATIONSHIP BETWEEN FLORAL COLOR, PIGMENTATION, FLORAL TRAITS, AND VEGETATIVE TRAITS IN WILD POPULATIONS OF *HESPERIS MATRONALIS*

I conducted two field surveys in the summer of 2003 to explore several characteristics associated with *H. matronalis* vegetative growth and floral form. In my first survey, I sought to determine whether anthocyanin content in petal tissue can effectively define floral color (are purple morphs truly purple, i.e, contain more anthocyanins than white morphs); I also tested the anthocyanin content in leaf tissue to determine whether leaves from purple morphs contain more anthocyanin than leaves from white morphs. In each of four populations, I marked 5 purple and 5 white individuals with flagging tape. Tissue was removed from one open flower and one fully developed leaf on each plant for acidified methanol pigment extraction (modified from Harborne, 1998) and spectrophotometry using a Spectronic 21D (Model DV #332278, Milton Roy, Rochester, NY). I then conducted ANOVAs (PROC GLM, SAS, 2007) assessing the effects of floral color, population identity, and their interaction on the light absorbance of leaf and floral tissue extracts.

On the same set of 40 plants, I also measured plant height, number of leaves, stem width, and the length and width of a randomly selected leaf. To determine if these vegetative traits were associated with floral color or population, I conducted individual ANOVAs (PROC GLM, SAS, 2007). I also estimated Pearson correlation coefficients (PROC CORR, SAS, 2007) between the vegetative traits to determine their associations.

I conducted a concurrent survey on a separate set of four *H. matronalis* populations to determine whether floral color was associated with other attractive floral morphological characters. I collected multiple flowers from each of 20 purple and 20 white plants in each of three populations and 20 purple and 16 white in a fourth population for a total of 156 plants sampled. I then measured petal length, petal width, tube length, pistil length, short anther length, and long anther length (as in Conner and Sterling, 1995) on all flowers using digital calipers. Individual flower traits did not differ within a plant (data not shown), so I pooled all measurements to generate means for each plant for each trait. These means were used in individual ANOVAs (PROC GLM, SAS, 2007) to test for effects of floral color, population identity, and their interaction.

Color morphs differed significantly in their petal anthocyanin content; purple morphs displayed higher absorbance than white morphs across all populations examined (Table A.1, Figure A.1), suggesting that this method effectively evaluates pigment content in flowers. Color morphs did not differ in their leaf anthocyanin content (Table A.1, Figure A.1), although we see significant variation in this trait between populations.

Floral color morphs did not differ significantly in any vegetative trait (Table A.2), nor did they differ in terms of any floral morphological trait measured (Table A.3). However, populations tended to differ in terms of leaf size (length and width), as well as stem diameter

(Table A.2). Additionally, I found significant among-population variation in five of the six floral traits (Table A.3). I also found significant correlations between most vegetative traits (Table A.4), suggesting that any one of these traits can be used to as a proxy of vegetative size for *H. matronalis* plants.

Table A.1 Individual ANOVA results assessing the effects of floral color (purple vs. white) and population identity on light absorbance by floral and leaf tissue samples in four populations of wild *H. matronalis* (N=40 plants).

Variable	DF	Petal Absorbance		Leaf Absorbance	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	7	27.61	0.0001	2.62	0.03
Color	1	187.83	0.0001	0.45	0.51
Population	3	1.67	0.19	4.61	0.009
Color*Population	3	0.15	0.93	1.35	0.28

Table A.2 Individual ANOVA results assessing the effects of floral color (purple vs. white) and population identity on vegetative traits in four wild populations of *H. matronalis* (N=40).

Variable	DF	Plant Height (cm)		Number of Leaves		Leaf Length (mm)		Leaf Width (mm)		Stem Width (mm)	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	7	1.26	0.30	0.67	0.69	3.77	0.004	4.92	0.0007	4.86	0.0008
Color	1	0.78	0.38	0.46	0.50	0.14	0.71	0.00	0.99	0.57	0.46
Population	3	1.73	0.18	0.88	0.46	6.86	0.001	10.70	0.0001	8.32	0.0003
Color*Population	3	0.95	0.43	0.53	0.66	1.90	0.15	0.79	0.51	2.82	0.05

Table A.3 Individual ANOVA results assessing the effects of floral color (purple vs. white) and population identity on floral morphological traits in four wild populations of *H. matronalis* (all measurements in mm; N=156 plants)

Variable	DF	Petal Length		Petal Width		Tube Length		Pistil Length		Short Anther Length		Long Anther Length	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Model	7	5.92	0.0001	6.71	0.0001	3.68	0.001	1.66	0.12	2.13	0.04	3.74	0.0009
Color	1	2.75	0.10	1.20	0.27	1.68	0.20	0.51	0.48	0.63	0.43	0.62	0.43
Population	3	11.65	0.0001	13.44	0.0001	7.23	0.0001	1.30	0.28	3.85	0.01	7.89	0.0001
Color*Population	3	1.33	0.27	2.00	0.12	0.92	0.44	2.39	0.07	0.93	0.43	0.78	0.51

Table A.4 Pearson correlation coefficients between vegetative traits in four populations of wild *H. matronalis* (N=40 plants). Plant height is measured in centimeters, while leaf length and width, as well as stem width, are measured in millimeters. Significant correlations are in bold.

	Plant Height		Leaf Number		Leaf Length		Leaf Width		Stem Width	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Plant Height	--	--	0.25	0.13	0.58	0.0001	0.37	0.02	0.32	0.04
Leaf Number	0.25	0.13	--	--	0.38	0.02	0.23	0.15	0.47	0.002
Leaf Length	0.58	0.0001	0.38	0.02	--	--	0.72	0.0001	0.34	0.03
Leaf Width	0.37	0.02	0.23	0.15	0.72	0.0001	--	--	0.15	0.35
Stem Width	0.32	0.04	0.47	0.002	0.34	0.03	0.15	0.35	--	--

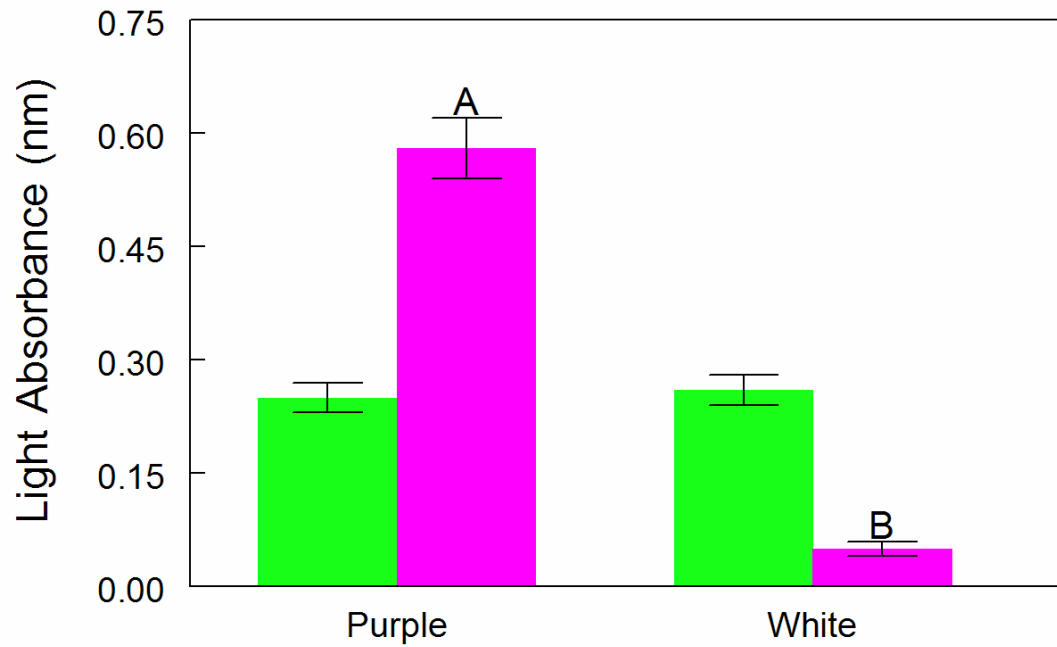


Figure A.1 Light absorbance (nm) of tissue from wild *H. matronalis* color morphs, measured by methanol extraction and spectrophotometry. Pink bars represent petal tissue and green bars represent leaf tissue. Bars with letters above them are significantly different by ANOVA. Error bars indicate standard errors.

APPENDIX B

FLORAL COLOR, FLORAL VISITORS, AND SEED SET IN *HESPERIS MATRONALIS*

To determine the major floral visitors to *H. matronalis*, I observed natural pollination in two wild populations in Crawford County, PA. Observations were conducted on five warm and sunny days in the early summer of 2003, between the hours of 9:00am and 4:30. On each day of observation, I selected a patch of plants within a population, making sure to select an area where visitation to both purple and white color morphs could be observed. I then observed the patch for one hour, recording the number of flowers of each color observed, as well as the identity and number of all visitors to flowers. Visitors were classified in one of four categories: bumblebees, small bees, butterflies/moths, and syrphid and other flies. A new patch of flowers was observed each hour, for a total of 19 hours of observation. From this data, I determined the relative percent of each type of floral visitor. I also summed the number of visits to each flower color during each hour and divided by the number of flowers observed to calculate visitation rate for each color morph (visits per flower per hour). I used this data in an ANOVA analysis (PROC GLM, SAS, 2007) to determine the effects of floral color and population on visitation rate.

Syrphids and other flies made up almost 44% of all floral visits during my observation periods, while 35% of visits were made by small bees. Lepidopterans made 18% of

visits and bumblebees made the remaining 3%. Purple morphs received an average of 0.26 ± 0.07 visits/flower/hour and white morphs received 0.32 ± 0.07 visits/flower/hour; I found no difference between color morphs in terms of visitation rate ($P = 0.4$), nor any significant interaction between floral color and population ($P = 0.94$). However, there was a significant difference in visitation rates between populations ($P < 0.0001$).

I conducted an experiment in the summer of 2005 to determine (1) the nature of self-compatibility in *H. matronalis*, (2) the contribution of day and dusk/night pollinators to seed set, and (3) the effects of floral color on self-compatibility and outcross pollination. Twenty-four *H. matronalis* rosettes were harvested in early spring from a natural population located at the Housing Site of the Pymatuning Laboratory of Ecology (PLE). These rosettes were transplanted into 1-gallon pots with Farfard™ #4 soil (Conrad Farfard, Agawam, Massachusetts, USA), watered daily, and housed in enclosures until flowering. Upon flowering, the plants were moved back to their native population and four plants of each color were assigned to one of three treatments: no pollinators (autonomous pollen only), access by night pollinators, and access by day pollinators. Treatments were imposed by surrounding inflorescences with bags made of small mesh bridal veil; plants in the no pollinator treatment were always bagged, those in the night access treatment were bagged during the day (7am-7pm), and those in the day access treatment were bagged during the night (7pm-7am). Bags on the no pollinator plants were moved periodically to allow space for elongating inflorescences and to simulate manipulations in the other two treatments. Treatments were imposed for 2 weeks, after which the pedicels of all treated flowers on all plants were marked with acrylic paint. Plants were moved to enclosures to protect them from herbivores until fruits had reached maturity. At fruit maturity, all fruits were collected, dissected, and the number of seeds counted to calculate number of seeds per fruit.

This was averaged across all fruits for each plant. I then conducted an ANOVA (PROC GLM, SAS, 2007) to determine the effects of floral color, bagging treatment, and their interaction on seed set.

I found a significant effect of bagging treatment on seed set in *H. matronalis* (Table B.1): plants exposed to daytime pollinators made the most seeds, while plants exposed to no pollinators or dusk/night pollinators made comparatively few seeds (Figure B.1). Additionally, I found a significant interaction between floral color and bagging treatment (Table B.1), where white morphs produce a similar amount of seeds regardless of treatment while purple morphs require exposure to daytime pollinators to set seed (Figure B.1). Such a result suggests differences between color morphs in terms of self-compatibility and contribution of pollinators.

Table B.1 ANOVA results assessing the effects of floral color (purple vs. white) and bagging treatment (always bagged, open at night, or open at day) on seed set in potted *H. matronalis* (N=24 plants, 4 of each color in each of 3 bagging treatments).

Variable	DF	Seeds per Fruit	
		<i>F</i>	<i>P</i>
Model	5	5.12	0.004
Color	1	0.61	0.45
Bagging Treatment	2	8.16	0.003
Color*Bagging Treatment	2	4.34	0.03

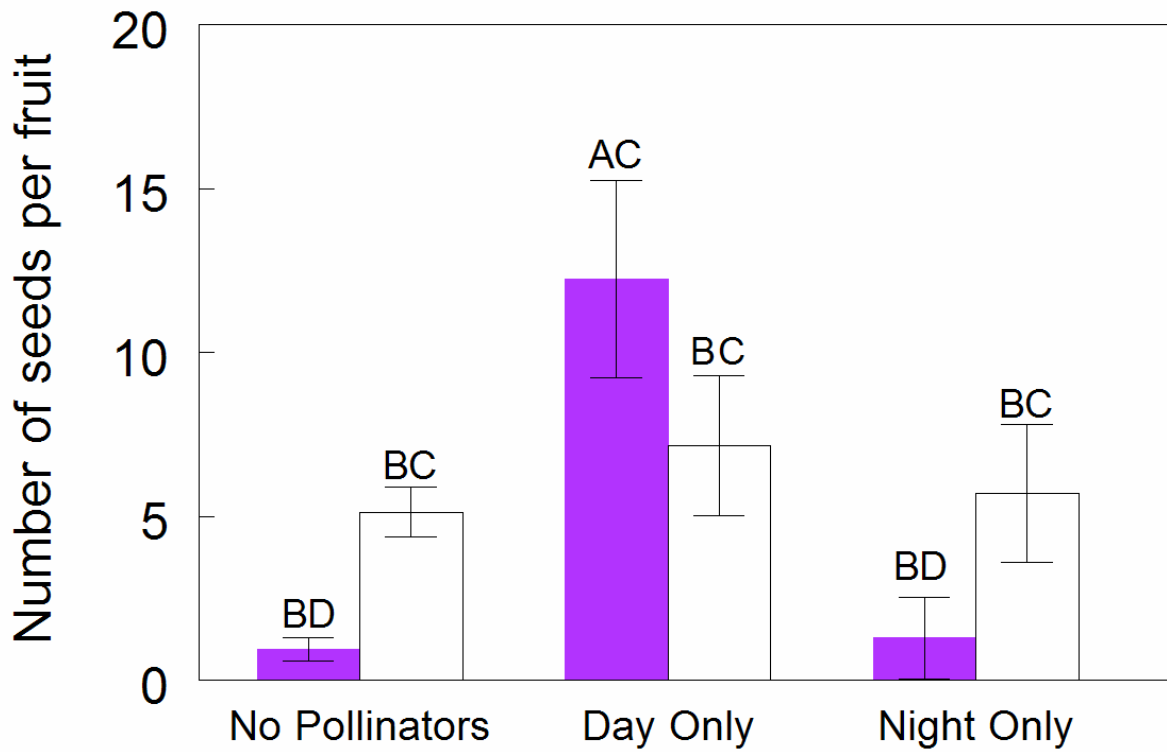


Figure B.1 Seed set (number of seeds per fruit) of *H. matronalis* color morphs by bagging treatment. Purple bars represent purple morphs and white bars represent white morphs. Error bars indicate standard errors. Letters over bars represent differences in means determined by post-hoc Tukey's tests.

APPENDIX C

THE GENETICS OF FLORAL COLOR IN *HESPERIS MATRONALIS*

To determine the genetics of floral color inheritance in *Hesperis matronalis*, I harvested bolting plants from two wild populations in the spring of 2003. These plants were potted into 1-gallon pots with Farfard™ #4 soil (Conrad Farfard, Agawam, Massachusetts, USA) and housed in the University of Pittsburgh greenhouse until flowering. Upon flowering, color was noted on each plant. I randomly selected several fully open flowers on each individual to receive pollen. These flowers were marked with acrylic paint and emasculated to eliminate any opportunity for autonomous autogamy. Half of the marked flowers were assigned to an outcross pollination treatment: using a paintbrush, I applied pollen from a randomly selected plant of opposite color from the same source population. Pollen was obtained from actively dehiscing donor flowers on the day of pollination. The remaining marked flowers were assigned to a self-pollination treatment: pollen was transferred by paintbrush from a pollen donor on the same plant to the emasculated treatment flower. Treated plants were maintained in the greenhouse until fruits had matured. I harvested mature fruit from treated flowers and collected all fully developed seeds. These seeds were then planted in 98-well trays in Farfard™ #4 soil. Crosses making less than 20 seeds had all seeds planted; crosses making more than 20 seeds had only 20 seeds planted.

Plants were reared to rosette stage and transplanted into 3" pots, then reared in the greenhouse for approximately 1 year. Following this growth period, all surviving plants were moved to the Pymatuning Laboratory of Ecology to overwinter. Upon flowering, offspring were scored for floral color. Mortality in the rosette stage due to forced bolting attempts, under-watering, and disease limited the number of flowering individuals to offspring from 8 purple-white crosses, 4 purple self crosses, and 3 white self crosses. The number of purple and white offspring resulting from these crosses is reported in Table D.1.

The number and distribution of these crosses made it quite difficult to determine any genetic patterns for floral color inheritance statistically; all analyses fail to eliminate common patterns of inheritance (data not shown). However, the prevalence of offspring of both colors in most crosses suggested that either many heterozygotes are found in the population or that at least two genes are involved in the inheritance of floral color for *H. matronalis*. Future research focusing on fewer crosses with greater numbers of offspring will provide further insight into the mode of inheritance for floral color of this species

Table C.1 Floral color of offspring from genetic crosses in *Hesperis matronalis*.

Cross ID	Mother Color	# Purple Offspring	# White Offspring
Purple-White Crosses			
EC2	Purple	0	2
HS13	Purple	11	10
RM16	White	1	4
RM17	Purple	8	6
RM1	White	5	2
RM22	Purple	7	4
RM2	Purple	4	3
RM30	Purple	1	3
<i>Sum</i>		37	34
Purple Self Crosses			
RM10	Purple	13	0
RM22	Purple	32	0
RM2	Purple	1	2
RM30	Purple	0	1
<i>Sum</i>		46	3
White Self Crosses			
HS14	White	0	1
RM14	White	2	5
RM3	White	0	2
<i>Sum</i>		2	8

APPENDIX D

CHAPTER 3 SUPPLEMENTAL DATA

To augment the population survey described in Chapter 3, I collected descriptive data on all five populations included in my geographic gradient. This included information on population positions (latitude/longitude), floral color polymorphism distribution, and population density and size estimates (Table D.1), as well the presence or absence of 6 major groups of pollinator fauna in each (Table D.2). Populations across the geographical range of the survey encompassed a wide array of estimated sizes and densities, but all had a larger percentage of purple morphs (Table D.1) and a similar pollinator fauna present (Table D.2).

As part of the work in this chapter, I identified floral scent components, categorized them into six subcategories based on their biochemical origin, and quantified floral scent components using external (or occasionally internal) standards (see sections 3.3.4 and 3.3.5 for details). This information is provided in Table D.3. Additionally, I calculated individual compound means means for each color in each population, as well as for the two garden-reared populations. This information (Table D.4) is presented to supplement Figure 3.2, as some components contributed only a small amount to total scent and do not show up clearly in the pie graphs.

Finally, to further verify whether floral scent emissions differ between color and populations for wild plants or between source populations for garden-reared plants, I conducted two sets of MANOVAs (PROC GLM, SAS, 2007), one on the three subcategories of aromatics (Table D.5, Table D.7) and another on the three subcategories of terpenoids (Table D.6, Table D.8); see section 3.3.7 for statistical details. These tests allowed me to determine which subcategories contributed to within- or among-population variation for wild populations and which subcategories drive differentiation between source populations (see section 3.4 for detailed interpretation).

Table D.1 Descriptive data from five populations of *H. matronalis* across part of its geographic range in North America

Population	Latitude/Longitude	% Purple	% White	Average Density (# flowering stems/m ²)	Size Estimate (# flowering stems)
ONT1	N 44° 01', W 79° 31'	57.8	42.2	24.33	500
ONT2	N 43° 32', W 79° 31'	53.4	46.6	28.33	10,000
PA1	N 41° 36', W 80° 25'	63.8	36.2	29.67	900
PA2	N 41° 36', W 80° 27'	69.44	30.56	34.67	1200
VA	N 39° 05', W 78° 04'	71.67	28.33	46.33	1000

Table D.2 Presence/absence summary of insect visitors to *H. matronalis* across populations in North America.

Population	Large Bees	Small Bees	Syrphid Flies	Day-Flying Lepidopterans	Night-Flying Lepidopterans	Other
ONT1	Yes	Yes	Yes	Yes	No	Beetles, Hummingbird (Rare)
ONT2	Yes	Yes	Yes	Yes	No	No
PA1	Yes	Yes	Yes	Yes	Yes (Rare)	Beetles
PA2	Yes	Yes	Yes	Yes	Yes (Rare)	Beetles, Hummingbird (Rare)
VA	Yes	Yes	Yes	Yes	Yes (Rare)	No

Table D.3 Categorization of volatile compounds identified in *Hesperis matronalis* floral scent and standards used for quantification.

Compound	Retention Time	External/Internal Standard?	Name of Standard
Aromatics			
<i>Benzenoid Compounds</i>			
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 acetate	16.41	External	Benzyl acetate
Benzyl alcohol	17	External	2-phenylethanol
2-phenylethanol	17.43	External	2-phenylethanol
Benzyl benzoate	24.46	External	Eugenol
<i>Phenyl Propanoid Compounds</i>			
Methyl Eugenol	18.48	External	Eugenol
Eugenol	20.06	External	Eugenol
<i>Nitrogen-Containing Benzenoid Compounds</i>			
Phenylacetone	17.67	External	2-phenylethanol
Benzothiazole	18	Internal	Toluene
Methyl anthranilate	21.1	External	Eugenol
Terpenoids			
<i>Monoterpenoids</i>			
α -pinene	4.84	External	<i>E</i> - β -ocimene
β -pinene	6.3	External	<i>E</i> - β -ocimene
Sabinene	6.58	External	<i>E</i> - β -ocimene
β -myrcene	7.36	External	<i>E</i> - β -ocimene
Limonene	7.91	External	<i>E</i> - β -ocimene
<i>Z</i> - β -ocimene	8.55	External	<i>E</i> - β -ocimene
<i>E</i> - β -ocimene	8.82	External	<i>E</i> - β -ocimene
<i>Oxygenated Monoterpenoids</i>			
1,8 cineole	8.03	External	1,8 cineole
<i>Z</i> -furanoid linalool oxide	11.66	External	Linalool
<i>E</i> -furanoid linalool oxide	12.05	External	Linalool
<i>E</i> - 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
<i>Z</i> -pyranoid linalool oxide	15.35	External	Linalool
<i>E</i> -pyranoid linalool oxide	15.59	External	Linalool
<i>Irregular and Sesquiterpenoids</i>			
<i>E</i> -4 dimethyl 1,3,7 nonatriene	9.7	External	<i>E</i> - β -ocimene
6-methyl 5-hepten-2-one	10.16	External	<i>E</i> - β -ocimene
α -farnesene	15.6	Internal	Toluene
<i>E,E</i> -4,8,12-trimethyl- 1,3,7,11- tridecatetane	16.81	Internal	Toluene

Table D.3 Continued

Compound	Retention Time	External/Internal Standard?	Name of Standard
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

Table D.4 Mean floral scent volatile emission rates for wild populations (collected *in situ*) and common garden reared purple morphs of *Hesperis matronalis*.

Values are given in ng per flower per hour. Sample sizes are listed for each population and standard errors are given in parentheses.

Compound	PA1			PA2			VA	
	Purple N=10	White N=10	Common Garden Purple N=13	Purple N=10	White N=10	Common Garden Purple N=15	Purple N=9	White N=10
Aromatics								
Benzenoid Compounds								
Benzaldehyde	1011.7 (470.9)	388.4 (98.8)	151.8 (16.7)	173.2 (53.0)	160.8 (47.2)	290.7 (53.0)	580.5 (181.6)	2779.9 (1287.7)
Phenylacetaldehyde	0.1 (0.0)	0.08 (0.01)	0.04 (0.01)	0.06 (0.02)	0.07 (0.02)	0.06 (0.01)	0.09 (0.01)	0.1 (0.0)
Benzyl acetate	1205.3 (243.1)	654.1 (202.9)	98.3 (29.8)	435.1 (332.6)	82.6 (23.9)	134.9 (56.2)	214.4 (98.6)	72.7 (19.5)
Methyl salicylate	25.8 (8.0)	20.3 (7.3)	4.9 (3.4)	0.0 (0.0)	2.5 (2.5)	24.0 (12.8)	18.5 (6.6)	27.7 (8.6)
Benzyl propionate	20.3 (7.0)	14.1 (7.5)	0.0 (0.0)	4.2 (4.2)	0.0 (0.0)	2.9 (2.9)	4.1 (4.1)	0.0 (0.0)
Phenylethyl acetate	19.9 (8.5)	19.2 (8.5)	0.0 (0.0)	4.6 (4.6)	4.0 (4.0)	5.5 (3.9)	17.9 (10.9)	2.9 (2.9)
Benzyl alcohol	0.1 (0.0)	8.5 (8.4)	0.07 (0.01)	0.07 (0.02)	0.06 (0.02)	0.09 (0.09)	0.1 (0.0)	0.1 (0.0)
2-phenylethanol	0.06 (0.02)	0.04 (0.02)	0.02 (0.01)	0.05 (0.02)	0.02 (0.01)	0.03 (0.01)	0.08 (0.01)	0.08 (0.01)
Benzyl benzoate	11.1 (6.8)	0.07 (0.02)	0.06 (0.01)	16.2 (16.1)	0.09 (0.01)	13.7 (13.7)	0.09 (0.01)	0.09 (0.01)
Phenyl Propanoid Compounds								
Methyl Eugenol	0.01 (0.01)	0.02 (0.01)	0.0 (0.0)	0.02 (0.01)	0.03 (0.02)	0.0 (0.0)	0.07 (0.02)	0.08 (0.01)
Eugenol	0.08 (0.01)	0.05 (0.02)	0.0 (0.0)	0.02 (0.01)	0.05 (0.02)	0.006 (0.006)	3.9 (3.9)	0.07 (0.02)

Table D.4 Continued

Compound	PA1			PA2			VA	
	Purple N=10	White N=10	Common Garden Purple N=13	Purple N=10	White N=10	Common Garden Purple N=15	Purple N=9	White N=10
Aromatics								
<i>Nitrogen-Containing Benzenoid Compounds</i>								
Phenylacetonitrile	0.05 (0.02)	0.04 (0.02)	0.0 (0.0)	0.02 (0.01)	0.02 (0.01)	0.006 (0.006)	0.08 (0.01)	0.08 (0.01)
Benzothiazole	4.9 (3.8)	13.9 (9.0)	10.5 (7.1)	0.0 (0.0)	0.0 (0.0)	15.6 (9.6)	0.0 (0.0)	0.2 (0.2)
Methyl anthranilate	0.04 (0.02)	0.05 (0.02)	0.04 (0.01)	0.0 (0.0)	0.0 (0.0)	0.07 (0.01)	0.01 (0.01)	0.01 (0.01)
Terpenoids								
<i>Monoterpenoids</i>								
α -pinene	175.2 (17.6)	107.7 (31.9)	163.6 (50.6)	80.9 (26.1)	71.0 (11.7)	233.4 (50.5)	103.9 (27.0)	256.3 (101.4)
β -pinene	96.9 (14.9)	78.5 (15.8)	110.9 (34.5)	53.0 (4.6)	46.2 (6.6)	128.3 (30.7)	79.6 (18.7)	111.6 (20.9)
Sabinene	217.5 (36.7)	155.2 (31.1)	28.0 (8.9)	82.1 (14.0)	66.1 (14.6)	111.1 (20.2)	231.4 (57.7)	314.5 (70.1)
β -myrcene	197.1 (33.5)	154.9 (32.0)	49.5 (13.7)	81.0 (12.5)	69.7 (12.5)	151.9 (31.9)	237.9 (53.7)	365.0 (79.1)
Limonene	223.7 (109.2)	208.8 (90.1)	272.6 (53.6)	45.2 (6.2)	45.0 (6.3)	425.1 (122.7)	81.3 (14.8)	106.4 (19.2)
Z- β -ocimene	119.8 (24.6)	144.6 (34.0)	138.5 (58.8)	57.7 (11.0)	44.0 (12.7)	110.2 (18.6)	244.1 (63.7)	358.0 (97.5)
E- β -ocimene	2597.0 (728.1)	3334.5 (940.6)	1406.4 (342.0)	1236.3 (401.7)	1158.3 (356.0)	984.2 (293.1)	4422.2 (1764.3)	6917.2 (1728.9)

Table D.4 Continued

Compound	PA1			PA2			VA	
	Purple N=10	White N=10	Common Garden Purple N=13	Purple N=10	White N=10	Common Garden Purple N=15	Purple N=9	White N=10
Terpenoids								
<i>Oxygenated Monoterpenoids</i>								
1,8 cineole	39.2 (22.6)	19.9 (10.9)	0.06 (0.01)	25.3 (21.8)	0.1 (0.01)	0.08 (0.01)	210.2 (90.2)	320.4 (100.4)
Z-furanoid linalool oxide	0.08 (0.01)	0.03 (0.02)	0.007 (0.007)	0.02 (0.01)	0.01 (0.01)	0.01 (0.009)	0.08 (0.01)	0.08 (0.01)
E-furanoid linalool oxide	0.1 (0.0)	0.09 (0.01)	0.02 (0.01)	0.03 (0.02)	0.03 (0.02)	0.03 (0.01)	0.08 (0.01)	0.08 (0.01)
E-β-ocimene epoxide	0.01 (0.01)	0.02 (0.01)	0.007 (0.007)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.03 (0.02)	0.05 (0.02)
Linalool	226.0 (70.5)	229.5 (110.1)	28.9 (20.0)	35.6 (28.0)	44.0 (17.1)	4.8 (4.8)	166.1 (77.1)	687.5 (293.6)
Linalool epoxide	0.08 (0.01)	0.07 (0.02)	0.0 (0.0)	0.07 (0.02)	0.09 (0.01)	0.006 (0.006)	0.04 (0.02)	0.07 (0.02)
α-terpineol	0.1 (0.0)	0.07 (0.02)	0.04 (0.01)	0.07 (0.02)	0.08 (0.01)	0.08 (0.01)	0.08 (0.01)	0.7 (0.6)
Z-pyranoid linalool oxide	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.01 (0.01)
E-pyranoid linalool oxide	0.0 (0.0)	0.0 (0.0)	0.007 (0.007)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.01 (0.01)
<i>Irregular and Sesquiterpenoids</i>								
E-4 dimethyl 1,3,7 nonatriene	66.1 (5.9)	49.8 (11.1)	98.8 (32.5)	27.0 (6.8)	16.2 (5.8)	94.5 (18.7)	81.3 (16.6)	72.9 (15.0)
6-methyl 5-hepten-2-one	140.7 (23.4)	130.3 (17.1)	236.9 (48.0)	99.9 (24.2)	51.5 (10.0)	359.8 (61.8)	76.8 (9.9)	103.2 (31.8)
α-farnesene	2.1 (1.2)	14.7 (10.9)	18.1 (13.8)	0.001 (0.001)	0.0 (0.0)	12.5 (4.1)	19.0 (7.3)	35.3 (13.0)
E,E-4,8,12-trimethyl- 1,3,7,11- tridecatetrane	6.5 (2.2)	7.6 (3.1)	31.7 (8.8)	5.1 (3.0)	1.3 (0.6)	77.3 (21.2)	4.6 (1.4)	13.4 (4.8)

Table D.4 Continued

	PA1			PA2			VA	
	Purple N=10	White N=10	Common Garden Purple N=13	Purple N=10	White N=10	Common Garden Purple N=15	Purple N=9	White N=10
Compound								
Unknown Compounds								
Unidentified terpenoid 1 (m/z=41, 43, 55, 67, 69, 83, 95, 119, 123, 137)	150.0 (63.3)	134.0 (48.1)	343.1 (196.6)	36.3 (8.8)	30.0 (6.1)	253.1 (62.6)	55.4 (17.5)	47.9 (16.0)
Unidentified terpenoid 2 (m/z=43, 57, 69, 85, 109, 151)	0.09 (0.01)	0.07 (0.02)	0.04 (0.01)	0.07 (0.02)	0.1 (0.0)	0.06 (0.01)	0.08 (0.01)	0.1 (0.0)
Unidentified terpenoid 3 (m/z=43, 57, 69, 85, 109, 151)	0.09 (0.01)	0.08 (0.01)	0.03 (0.01)	0.09 (0.01)	0.1 (0.0)	0.05 (0.01)	0.08 (0.01)	0.1 (0.0)
Unidentified terpenoid 4 (m/z=43, 57, 69, 85, 109, 151)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.02 (0.01)	0.04 (0.02)	0.0 (0.0)	0.03 (0.02)	0.04 (0.02)
Unidentified aromatic (m/z=43, 77, 115, 133)	0.3 (0.3)	0.8 (0.8)	0.0 (0.0)	0.3 (0.3)	0.0 (0.0)	0.0 (0.0)	0.08 (0.08)	0.0 (0.0)

Table D.4 Continued

Compound	ONT1		ONT2	
	Purple N=10	White N=10	Purple N=10	White N=10
Aromatics				
<i>Benzenoid Compounds</i>				
Benzaldehyde	64.8 (16.3)	314.3 (0.194.3)	94.9 (46.4)	88.4 (17.4)
Phenylacetaldehyde	0.09 (0.01)	0.1 (0.0)	12.1 (12.1)	0.8 (0.8)
Benzyl acetate	56.3 (20.3)	181.6 (71.4)	158.6 (72.8)	197.1 (100.5)
Methyl salicylate	0.0 (0.0)	1.8 (1.8)	13.5 (7.4)	16.6 (7.7)
Benzyl propionate	0.0 (0.0)	0.0 (0.0)	6.4 (6.4)	9.0 (6.3)
Phenylethyl acetate	0.0 (0.0)	7.6 (4.2)	8.8 (6.8)	11.7 (7.9)
Benzyl alcohol	0.08 (0.01)	0.09 (0.01)	0.09 (0.01)	0.09 (0.01)
2-phenylethanol	0.02 (0.01)	0.07 (0.02)	0.05 (0.02)	0.05 (0.02)
Benzyl benzoate	0.07 (0.02)	0.09 (0.01)	12.8 (12.8)	51.5 (51.4)
<i>Phenyl Propanoid Compounds</i>				
Methyl Eugenol	0.03 (0.02)	0.04 (0.02)	0.01 (0.01)	0.02 (0.01)
Eugenol	0.07 (0.02)	4.9 (4.9)	0.03 (0.02)	4.4 (4.3)

Table D.4 Continued

Compound	ONT1		ONT2	
	Purple N=10	White N=10	Purple N=10	White N=10
Aromatics				
<i>Nitrogen-Containing Benzenoid</i>				
<i>Compounds</i>				
Phenylacetonitrile	0.02 (0.01)	0.02 (0.01)	0.03 (0.02)	0.01 (0.01)
Benzothiazole	0.0 (0.0)	0.6 (0.4)	0.0 (0.0)	0.5 (0.3)
Methyl anthranilate	0.08 (0.01)	0.04 (0.02)	0.1 (0.0)	0.09 (0.01)
Terpenoids				
<i>Monoterpenoids</i>				
α -pinene	134.8 (53.7)	89.1 (17.4)	35.5 (10.5)	72.9 (23.6)
β -pinene	39.7 (6.1)	38.9 (6.6)	24.5 (6.1)	26.9 (8.0)
Sabinene	68.6 (13.9)	52.8 (10.0)	40.1 (10.0)	72.5 (15.1)
β -myrcene	78.3 (14.7)	75.8 (14.9)	61.1 (10.0)	98.5 (16.4)
Limonene	43.8 (6.6)	55.1 (13.6)	43.0 (4.1)	56.2 (3.7)
Z- β -ocimene	73.3 (16.9)	110.7 (26.2)	83.3 (11.6)	106.3 (16.5)
E- β -ocimene	1483.8 (358.4)	2693.8 (696.8)	1529.7 (422.4)	2025.5 (360.9)

Table D.4 Continued

Compound	ONT1		ONT2	
	Purple N=10	White N=10	Purple N=10	White N=10
Terpenoids				
<i>Oxygenated Monoterpenoids</i>				
1,8 cineole	5.0 (3.3)	0.1 (0.0)	0.5 (0.4)	26.7 (19.3)
Z-furanoid linalool oxide	0.04 (0.02)	0.03 (0.02)	0.1 (0.0)	0.09 (0.01)
E-furanoid linalool oxide	0.08 (0.01)	0.06 (0.02)	0.1 (0.0)	0.09 (0.01)
E- β -ocimene epoxide	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.01 (0.01)
Linalool	16.8 (8.3)	65.5 (33.9)	52.9 (29.0)	0.0 (0.0)
Linalool epoxide	0.02 (0.01)	0.02 (0.01)	0.01 (0.01)	0.0 (0.0)
α -terpineol	0.1 (0.0)	0.09 (0.01)	0.07 (0.02)	0.1 (0.0)
Z-pyranoid linalool oxide	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
E-pyranoid linalool oxide	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
<i>Irregular and Sesquiterpenoids</i>				
E-4 dimethyl 1,3,7 nonatriene	23.7 (6.0)	34.9 (5.3)	48.6 (6.1)	50.9 (8.9)
6-methyl 5-hepten-2-one	53.8 (6.9)	77.6 (15.7)	71.9 (9.5)	109.2 (28.7)
α -farnesene	7.2 (3.0)	22.3 (9.8)	6.0 (3.8)	4.4 (2.4)
E,E-4,8,12-trimethyl- 1,3,7,11- tridecatetrane	9.6 (2.4)	17.6 (4.6)	27.6 (5.9)	28.2 (7.7)

Table D.4 Continued

Compound	ONT1		ONT2	
	Purple N=10	White N=10	Purple N=10	White N=10
Unknown Compounds				
Unidentified terpenoid 1 (m/z=41, 43, 55, 67, 69, 83, 95, 119, 123, 137)	31.0 (6.0)	64.2 (15.7)	16.2 (2.9)	25.4 (7.7)
Unidentified terpenoid 2 (m/z=43, 57, 69, 85, 109, 151)	0.09 (0.01)	0.1 (0.0)	0.09 (0.01)	0.1 (0.0)
Unidentified terpenoid 3 (m/z=43, 57, 69, 85, 109, 151)	0.08 (0.01)	0.09 (0.01)	0.09 (0.01)	0.09 (0.01)
Unidentified terpenoid 4 (m/z=43, 57, 69, 85, 109, 151)	0.01 (0.01)	0.05 (0.02)	0.03 (0.02)	0.02 (0.01)
Unidentified aromatic (m/z=43, 77, 115, 133)	0.0 (0.0)	0.2 (0.2)	0.001 (0.001)	0.0 (0.0)

Table D.5 Individual ANOVA and MANOVA results assessing the effects of floral color and population on 3 aromatic subcategories of *in situ* floral scent emission rates in *H. matronalis*. Type III sums of squares and Wilk's Lambda statistic results presented.

Variable	DF	Individual ANOVA results						MANOVA Results		
		Benzenoids		Phenyl-Propanoids		Nitrogen-Containing Benzenoids		Wilk's Lambda		
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>DF</i>	<i>F</i>	<i>P</i>
Model	9	8.76	0.0001	0.80	0.61	2.48	0.01	-	-	-
Color	1	0.40	0.53	0.40	0.53	1.25	0.27	3	0.68	0.57
Population	4	17.34	0.0001	0.55	0.70	4.75	0.002	12	7.13	0.0001
Color x Population	4	2.13	0.08	1.16	0.33	0.51	0.73	12	1.23	0.26

Table D.6 Individual ANOVA and MANOVA results assessing the effects of floral color and population on 3 terpenoid subcategories of *in situ* floral scent emission rates in *H. matronalis*. Type III sums of squares and Wilk's Lambda statistic results presented.

Variable	DF	Individual ANOVA results						MANOVA Results		
		Monoterpenoids		Oxygenated Monoterpenoids		Irregular and Sequiterpenoids		Wilk's Lambda		
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>DF</i>	<i>F</i>	<i>P</i>
Model	9	3.70	0.0006	5.27	0.0001	4.20	0.0002	-	-	-
Color	1	2.24	0.14	1.47	0.23	0.11	0.74	3	1.91	0.13
Population	4	6.69	0.0001	10.23	0.0001	7.36	0.0001	12	5.37	0.0001
Color x Population	4	0.96	0.43	1.04	0.39	2.05	0.09	12	1.22	0.27

Table D.7 Individual ANCOVA and MANCOVA results assessing the effects of source population (controlling for plant size) on 3 aromatic subcategories of floral scent emission rates in common garden reared purple *H. matronalis*.

Type III sums of squares and Wilk's Lambda statistic results presented.

Variable	DF	Individual ANCOVA results						MANCOVA Results		
		Benzenoids		Phenyl-Propanoids		Nitrogen-Containing Benzenoids		Wilk's Lambda		
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>DF</i>	<i>F</i>	<i>P</i>
Model	2	2.13	0.14	0.42	0.66	0.18	0.83	-	-	-
Source Population	1	4.22	0.05	0.83	0.37	0.25	0.62	3	2.41	0.09
Plant Height	1	0.00	0.99	0.00	0.97	0.15	0.70	-	-	-

Table D.8 Individual ANCOVA and MANCOVA results assessing the effects of source population (controlling for plant size) on 3 terpenoid subcategories of floral scent emission rates in common garden reared purple *H. matronalis*. Type III sums of squares and Wilk's Lambda statistic results presented.

Variable	DF	Individual ANCOVA results						MANCOVA Results		
		Monoterpenoids		Oxygenated Monoterpenoids		Irregular and Sesquiterpenoids		Wilk's Lambda		
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>DF</i>	<i>F</i>	<i>P</i>
Model	2	0.28	0.76	1.02	0.37	3.81	0.04	-	-	-
Source Population	1	0.05	0.83	1.10	0.30	3.37	0.08	3	0.96	0.43
Plant Height	1	0.48	0.50	1.16	0.29	5.02	0.03	-	-	-

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