The Ecology of Floral Signals in *Penstemon digitalis*

ROSALIE BURDON
In this thesis, I combined field observations and lab experiments to explore the ecological significance of floral signals in a North American wildflower, *Penstemon digitalis*. More specifically, to determine the potential mechanisms driving selection on floral scent, I studied how scent mediates interactions with pollinators and antagonists by (1) observing spatiotemporal variation in scent emission (2), floral volatile ability to suppress microbes (3) the honest advertisement of nectar, and (4) if scent could aid pollinator learning by reinforcing visual signals.

Scent sampling of flower development, flower tissues, rewards and inflorescence day/night emission, revealed a complexity in floral scent composition and emission that could reflect several ecological functions. The floral bouquet of *P. digitalis* was strongest when flowers opened, primarily emitted from flower nectaries and was strongest during the day when pollinators are most active, suggesting a role in plant-pollinator interactions.

Because linalool was one of the few floral compounds found in nectar where microbe growth can degrade the pollinator reward, I studied its role in plant-microbe interactions. Bacteria strains isolated from floral and vegetative tissues were exposed to varying concentrations of nectar volatiles: linalool and methyl nicotinate. Linalool inhibited bacteria growth rate from all tissue origins whereas methyl nicotinate had little effect, suggesting that microbes could drive selection on linalool emission strength.

To determine the extent that linalool could honestly signal nectar availability, linalool-nectar associations were measured for inflorescences and flowers. Linalool predicted inflorescence nectar availability but not flower, exposing a limit to its honesty. Pollinator *Bombus impatiens* could use linalool as a foraging signal at varying concentrations, suggesting linalool could be learned and used to choose the most rewarding plants.

Measurement and comparison of signal-reward associations for both olfactory and visual signals/cues of *P. digitalis* displays found display size and linalool honest indicators of nectar. Lab behaviour experiments showed multiple signals correlated with reward could increase bumblebee foraging efficiency and promote learning, providing an explanation for why floral displays are complex and consist of multiple signals.

Together my results show that an integrated approach is required to understand the mechanisms driving the evolution of the floral phenotype.

**Keywords:** Antimicrobial, *Bombus impatiens* learning, indirect signal, multimodal, nectar, protandry, signal-reward association, Volatile Organic Compounds (VOCs)

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"Humble-bees... plants and animals, most remote in the scale of nature, are bound together by a web of complex relations”

Charles Darwin (1859)

For my brother Joseph, who inspired my studies and who never stopped believing in me
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II. **Burdon, R.C.F.,** Junker, R.R. and Parachnowitsch, A.L. Floral volatiles suppress *Penstemon digitalis* microorganisms (Manuscript)


IV. **Burdon, R.C.F.,** Scofield, D.G., Pierce, E., Gegear, R.J. and Parachnowitsch, A.L. Multimodal honesty in *Penstemon digitalis* enhances bumblebee foraging (Manuscript)

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In addition to the thesis chapters, I have contributed to the following paper:

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Abbreviations and Definitions

**ADDITIVE:** The behavioural response to two signals combined is equal to the effect of each signal alone and so do not interact in a direct way, e.g. 2+2=4 (Riffell and Alarcón 2013)

**ANTAGONIST:** interferes with pollination e.g. ants deter pollinators with aggression

**ANTIMICROBIAL:** suppresses or inhibits microbial growth or density

**CUE:** A cue is any feature with regularity that an organism can use as a guide to display a particular behaviour e.g. a mosquito uses CO₂ to locate and feed on a host but the host did not produce CO₂ to attract the mosquito (Ruxton and Schaefer 2011)

**DH:** Dynamic headspace (active ‘pull’ scent trapping: Dudareva and Pichersky 2006)

**HONESTY:** can predict or is correlated with reward and so is useful to the receiver

**IN/DIRECT:** In terms of reward, direct means certainty that scent = reward whereas indirect means there is a decoupling between scent and reward and less certainty

**INFLORESCENCE:** where individual flowers are grouped together on a single plant, spread or clustered (Proctor et al. 1996)

**LARCENIST:** nectar robber

**MULTIMODAL:** communication in the form of different types of sensory stimuli e.g. colour and scent as opposed to colour and pattern (Riffell and Alarcón 2013)

**PHENOTYPE:** the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment and biotic interactions (Martin and Hine 2008)

**POLLINATOR:** In this thesis I often mean bumblebee, but can be any visitor that deposits pollen onto the receptive stigma

**PROTANDROUS:** plants with flowers that each develop from the male-phase into the female-phase (Proctor et al. 1996)
SELECTION: Organisms are under conditions where the survival and reproduction of those with a particular genotype will be favoured or suppressed (Martin and Hine 2008)

SIGNAL: Evolved for communication with the receiver and impacts the receiver response. In general, signals must be honest and reliable, otherwise the signaler will not benefit from emitting/displaying the signals (Ruxton and Schaefer 2011)

SPME: Solid-phase microextraction (static scent trapping: Dudareva and Pichersky 2006)

SYNERGISTIC: The behavioural response to two signals combined is greater than the effect of each signal produced alone, e.g. 2+2 =>4 (Riffell and Alarcón 2013)

TRAIT: a distinguishing quality or characteristic (Martin and Hine 2008)

VOCs: volatile organic compounds, atmospheric chemicals with high vapour pressure (Dudareva and Pichersky 2006)

NB. Definitions without reference are my interpretation for this thesis.
Introduction

The floral phenotype is a distinct, but dynamic, combination of olfactory, visual signals/cues and rewards in the context of a species-specific morphology (Junker and Parachnowitsch, 2015). Floral traits are affected by the immediate environment and the floral phenology (aging, response to pollination (Theis and Raguso, 2005; Farré-Armengol et al. 2014), but are ultimately evolutionarily shaped by complex interactions between plants and their mutualists and antagonists (Raguso, 2009; Armbruster, 2014). For example, floral volatiles are arguably the most dynamic of these traits, having evolved to fill a number of roles in plant–animal interactions ranging from attraction of pollinators and/or repellence of antagonists (Kessler et al. 2008; Wright and Schiestl, 2009), to complex interactions with microorganisms (Junker and Tholl, 2013). Indeed the same compound has the potential to serve many different functions (Raguso, 2016).

From research exploring natural selection on scent (Parachnowitsch et al. 2012; one of only a few studies still at present to do so), we hypothesised a key compound from the floral bouquet of P. digitalis was S-(-)-linalool (Parachnowitsch et al. 2012). My work began by dissecting flowers and tracing where in the flower and when this compound was expressed in relation to other floral volatiles in this system (Parachnowitsch et al. 2013; Paper I). Since then, I have determined how this nectar compound mediates both plant-microbe interactions (Paper II) and plant-pollinator interactions (Paper III). I have tested and challenged hypotheses about nectar scent as an honest signal and its use by pollinators at multiple - increasingly holistic - levels (Paper III, IV). In collaboration with several experts from the field of chemical ecology, pollination biology, plant ecology and evolution, microbe ecology and bumblebee neurobiology, I studied the functional ecology of floral signals in P. digitalis and highlight how the inclusion of floral chemistry into pollination biology can improve our understanding of plant–pollinator interactions at ecological and evolutionary time scales. In particular, I have tried to unravel the potential selection pressures driving linalool emission in P. digitalis. First however, I introduce key concepts highlighting the importance of emitting floral signals and their association with reward.
Spatial and temporal variation in scent: possibilities and limitations

The functional diversity of floral scent is paralleled by the chemical diversity, spatial and temporal variation of the volatile organic compounds emitted by flowers (Knudsen et al. 2006; Muhlemann et al. 2014). Floral scent bouquets comprise, depending on the plant species, a few to more than one hundred individual compounds (Knudsen et al. 2006), and because of recent advancements in scent trapping and analytical techniques (Dudareva and Pichersky, 2006), we are now at a point to explore the activities and potential messages floral scent conveys through emission locality and change over time (Raguso 2008a).

The chemical composition and absolute amounts of floral scent is not static (Paper I). Spatiotemporal variability in the identity and complexity of scent bouquets could provide critical information for the mediation of plant-animal communication because floral visitors can use subtle differences in volatiles to make foraging decisions (Wright and Schiestl 2009). For instance, spatial variation in scent composition between floral tissues may inform visitors about reward location within a flower (Dobson et al. 1996). Whereas temporal variation or rhythmic expression of scent through floral development or in day/night cycles could match pollinator’s activity schedules and inform visitors of a flowers’ current status (Theis et al. 2007; Ruiz-Ramón et al. 2014). Scent emission often marks floral receptivity (Bergström et al. 1995; Raguso et al. 2003; Rodriguez-Saona et al. 2011), while reduced emissions often typify flowers that have been pollinated (Tollsten, 1993; Schiestl et al. 1997). Besides mutualistic interactions, spatial and temporal variation in scent emission may also fill a defensive role. Temporal variation in scent emission may have evolved to avoid attracting antagonists (Borges et al. 2011; Dötterl et al. 2012; Jürgens et al. 2014) and volatile emission by certain tissues or in nectar could be used to repel larcenists, antagonists or herbivores, or function to inhibit pathogenic bacteria (Galen et al. 2011; Huang et al. 2012; Kessler et al. 2015). As reproductive structures, flowers are of great importance for the biological success of plants because they are directly linked to reproductive output (i.e., fitness) (Alleklett et al. 2014). Bacteria at high densities can degrade pollinator reward and subsequently affect pollination (Vannette et al. 2012; Junker et al. 2014), and so volatile-mediated interactions with microbial inhabitants on tissues or in the nectar could play a key role in the reproductive success of plants (Paper II). Therefore, where and when floral volatile compounds are emitted may be an artifact of multiple or opposing selection pressures. In addition, the scale and context of floral scent emission is important in understanding how floral scent contributes to the floral phenotype (Paper III). Context includes aspects of stimulus presentation such as spatio-
temporal variation, receiver condition (forager experience) or stimulus modality (Paper IV). For example, certain compounds, despite being detected as scent, might function more effectively as a flavour. Kessler and Baldwin (2007) showed that volatile components of **Nicotiana attenuata** floral nectar (benzyl acetone and nicotine) could function as an odour to attract and repel hawkmoths and hummingbirds or as a flavor, constrain feeding time. In gustatory trials using varying concentrations of S-(-)-linalool in sugar solutions, I observed that nectar-robbing ants potentially antagonistic to *P. digitalis* and often observed on plants, presented irritation behaviour to 100ng linalool sucrose solutions but overall did not prevent feeding. Pollinator *Bombus impatiens* showed astonishing tolerance of linalool in nectar up to 5000ng, 20 times natural concentrations (data not shown). Thus sensory capabilities and limitations in relation to the strength, chemical composition, and rhythm of floral emission, as a bouquet or individual compounds within, will be driven by interactions with multiple visitors (Raguso, 2008b; Junker, 2016). Understanding how the chemical phenotype evolves is largely unresolved because the same traits that make it functionally diverse are specifically why selective pressures are difficult to determine experimentally.

The importance of honesty

In 350BC, ancient Greek philosopher Aristotle casually noted,

‘*On each expedition the bee does not fly from a flower of one kind to a flower of another, but flies from one violet, say, to another violet, and never meddles with another flower until it has gone back to the hive...’*

Unbeknownst to him, he was the first to describe what we now define as floral constancy. It was 2000 years later that Joseph G. Kolreuter (1733-1806) recognized the significance of ‘bee honey’ or nectar in attracting pollinators. Around the same time founder of ‘pollination biology’, Christian Sprengel (1750-1816), is credited with the discovery that signals such as colour could guide insects to nectar and help drive constancy behavior (Proctor et al. 1996). In plant−pollinator systems, one function of floral phenotype is to ‘advertise’ or signal the presence of a reward to animal pollinators in return for pollination (Raguso 2008b). Because plants hide or protect rewards from direct visual assessment, pollinators must use floral signals such as colour and scent to find and assess reward availability, quality and quantity (Benitez-Vieyra et al. 2010). Furthermore pollinators use signals to compare the most reliable and reward-informative signals within plant populations and among plant species (Petanidou, 2005; Herrera et al. 2006).
Honest floral signals reliably indicate reward quantity or quality, providing pollinators a means by which to distinguish rewarding flowers / inflorescences from less profitable flowers (Schaefer et al. 2004; Wright and Schiestl, 2009). Floral traits such as the number of flowers (display size) (Harder and Cruzan, 1990), flower size (Fenster et al. 2006), colour (Hansen et al. 2007) and scent (Olesen and Knudsen, 1994) have each been recorded as honest signals, learned and used by pollinators as a foraging cue. This ability for pollinators to learn reward associated signals, in turn allows pollinators to learn to avoid unrewarding plants (Thomson, 1981; Wright et al. 2005). For example studies have shown that bumblebees are more likely to visit different types of flowers when presented with flowers of low, infrequent rewards (Fontaine et al. 2008), suggesting that having an unrewarding signal could potentially detract from a plant’s fitness (Salzmann et al. 2007). Thus pollinator-mediated selection for honest signals could be one explanation for why reward-deceptive species commonly mimic rewarding species (Thakar et al. 2003; Schiestl, 2005).

The life history of many pollinators, such as generalist pollinating bees, depends on olfactory communication and so it is unsurprising that they have exceptional olfactory learning abilities and can remember scent as foraging signals for longer than visual signals (Kunze and Gumbert 2001; Menzel, 1999; Giurfa, 2007). Of all the floral traits that comprise a floral display, floral scent may be the most flexible in relation to other floral traits (Junker 2016). For example, scent adaptability through varying strength, concentration (Paper I), or composition of volatile emissions as well as being subject to the same metabolic fluctuations that impact nectar production (Wright and Schiestl, 2009), means that it could have the capacity to vary with current reward status and so provide an honest signal (Wright et al. 2005; Salzmann et al. 2007). Some of this variability can be caused by physical variables such as temperature and air velocity (Raguso, 2008a), meaning that producing a reliable signal can be difficult to maintain. Because pollinators can learn when scent is unrewarding much more efficiently than other floral traits (Kunze and Gumbert 2001), and the reliability and accuracy of the signal drives selective/competitive foraging, there are grounds to question ‘why do plants produce floral scent?’ (Wright and Schiestl, 2009). The answer; using scent as a signal can give honest signalling plants a selective advantage in attracting the attention of potential pollinators. For instance, as a distinct trait, scent can predict reward as a direct or indirect signal (nectar scent) (Paper III), or can increase pollinators foraging efficiency by enhancing the detection or processing of another signal (Paper IV).

‘The secret of life is honesty and fair dealing. If you can fake that, you’ve got it made.’

(Groucho Marx, Comedian)
Aims of Thesis

The general goal of this thesis was to develop a greater understanding of the ecological consequences of producing signals, specifically scent. In particular I aimed to gain insight into how scent and its relationship with nectar could mediate interactions with both pollinators and antagonists in the context of a whole plant. This included determining where and when scent was produced for inflorescences and flowers, it’s ability to suppress microbes, the honest advertisement of reward and how it could aid pollinator learning.

The following questions were addressed

1. Does scent emission of *P. digitalis* differ between day and night? (I)
2. How does the scent profile of *P. digitalis* vary through development and spatially within a flower? (I)
3. Can scent inhibit or facilitate bacteria colonizing *P. digitalis*? (II)
4. Can floral bacteria metabolize floral volatiles to grow? (II)
5. Does scented nectar honestly signal nectar reward availability? (III)
6. Can bumblebees use linalool as a foraging signal? (III)
7. Do inflorescences with honest scent signals have greater pollen deposition? (III)
8. Do floral signals differ in their ability to honestly advertise nectar availability for inflorescences? (IV)
9. Do bumblebees preferentially choose inflorescences with multiple honest signals? (IV)
10. How do multimodal signals affect bumblebee learning? (IV)
Materials and Methods

Study plant

*Penstemon digitalis* Nutt. ex Sims (Plantaginaceae) or common name ‘beard-tongue’, is a native North American perennial found in meadows and prairies (Fig. 1). *Penstemon digitalis* was used as the study system for this thesis because *Penstemon* is a well-established genus in pollination research (Thomson et al. 2000; Castellanos et al. 2002) and because natural selection had been observed on both visual and olfactory components of *P. digitalis* floral displays (Parachnowitsch and Kessler 2010; Parachnowitsch et al. 2012). In particular, phenotypic selection was found for display size and on scent. Selection on scent was found to be stronger than on the visual signals floral color and size, with nectar scent (S)-(+-)linalool identified as a distinct target of selection among the floral bouquet of 23 compounds (Parachnowitsch et al. 2012; Parachnowitsch et al. 2013). The agents driving selection on scent however remained unidentified and so motivated the study on the ecology of floral signals in *P. digitalis*.

Floral phenotype and reproduction

*Penstemon digitalis* has panicle inflorescences with displays of flowers ranging from 1 to >20 flowers. Flower corollas are white with purple striping within the throat of the corolla tube (Fig. 2). The purple colour is probably attributable to delphinidin-based anthocyanin (Scogin and Freeman, 1987) and appears black under UV light, suggesting that it may act as a nectar guide for pollinators (Silberglied, 1979). The lines vary in number and intensity between inflorescences (Parachnowitsch and Kessler 2010). Flowers are protandrous, with the staminate (male) phase transitioning to the pistillate (female) phase in 2-5 days (Fig. 1). Although flowers are self-compatible (Zorn-Arnold and Howe, 2007), bagged flowers fail to reproduce, suggesting that pollinators are necessary for seed set (Parachnowitsch et al. 2012).
Pollinators and herbivores

*Penstemon digitalis* is pollinated by small (*Ceratina, Osmia, Hoplitis* spp; Fig. 2a) to large-bodied bees (*Bombus, Xylocopa, Anthophora* spp; Fig. 2b) (Clinebell and Bernhardt 1998; Mitchell and Ankeny 2001; Dieringer and Cabrera 2002). At our field sites, the large-bodied generalist bumblebee *Bombus impatiens* (Cresson) is a dominant visitor (Parachnowitsch and Kessler 2010). *Bombus impatiens* is a generalist pollinator, also a native of North America (Michener, 2007). Wild *B. impatiens* were used for observations in the field and commercially reared *B. impatiens* were used for lab-based foraging behaviour experiments. Both micro-lepidopterans and dipterans are known pre-dispersal seed predators of this species (Mitchell and Ankeny, 2001; Thomas, 2003), but at our field sites, fruits are attacked primarily by an unidentified micro-lepidopteran (Parachnowitsch and Kessler, 2010).
Study populations and experimental sites

For field observations of floral traits and bumblebee behavior, seven different *P. digitalis* populations were used in Tompkins County, New York, USA (Table 1). When possible, data were collected from three previously studied source populations, Neimi Road (NR), Whipple Farm (WF), and Turkey Hill (TH) in Tompkins County, NY, USA. All scent analysis and plant field observations were conducted at Cornell University, New York, USA (I, III, IV). Studies on antimicrobial effects of volatiles were conducted at Salzburg University, Salzburg, Austria (II), and experiments on bumblebee foraging behaviour were conducted at Worchester Polytechnic Institute, Massachusetts, USA (III, IV).

Figure 2a-b. Example of floral visitors (small-bodied Green Sweat Bee and large-bodied *Bombus*). Photos: Mary Anne Borge 2016, printed with permission.
Table 1. Field site location for seven populations of *Penstemon digitalis* used for floral trait estimates and *Bombus impatiens* foraging observations. *N* indicates how many inflorescences were measured from each population and which year (including floral traits taken for bumblebee observations), (-) identifies which populations were not used.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Year</th>
<th>Latitude (N)</th>
<th>Longitude (W)</th>
<th>Floral Traits</th>
<th>Bumblebee behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR (Casswell Road)</td>
<td>52</td>
<td>2014</td>
<td>42°53'913&quot;</td>
<td>76°37'694&quot;</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>HT (Homestead)</td>
<td>14</td>
<td>2014</td>
<td>42°43'405&quot;</td>
<td>76°47'493&quot;</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>NR (Neimi Road)</td>
<td>54</td>
<td>2012</td>
<td>42°30'092&quot;</td>
<td>76°26'204&quot;</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>2014</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH (Turkey Hill)</td>
<td>43</td>
<td>2012</td>
<td>42°26'428&quot;</td>
<td>76°25'743&quot;</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>2013</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WF (Whipple Farm)</td>
<td>128</td>
<td>2012</td>
<td>42°26'436&quot;</td>
<td>76°25'892&quot;</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>CRX (Casswell Road)</td>
<td>6</td>
<td>2014</td>
<td>42°43'405&quot;</td>
<td>76°47'493&quot;</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>TR (Tower Road)</td>
<td>18</td>
<td>2014</td>
<td>42°44'541&quot;</td>
<td>76°46'556&quot;</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
Spatiotemporal variation in floral scent (I)

To quantify spatiotemporal variation in floral scent emissions, two scent trapping methods were used; dynamic headspace (DH) scent trapping and solid-phase microextraction (SPME) (Knudsen et al. 2006, Fig. 3a-b). Dynamic headspace was used for quantitative analysis of day/night emissions because air sampling with replacement allows for calculation of a standardized rate of scent emission per floral unit (Fig. 3b. The SPME method was used to assess developmental and spatial variation for floral tissues (Fig. 3a). It differs from DH because scent equilibrates in a closed system and is used to assess volatile composition, not emission rate. Together these methods capture the adaptability of scent and are used to develop hypotheses about its ecological functions.

![Diagram of SPME syringe-like device](image)

Figure 3. Example of a SPME syringe-like device to extract volatiles from the headspace of flowers enclosed in a glass vial (a). The SPME fiber is protected by the syringe until manually exposed, and is retracted back into the syringe after scent collection. b) Pull headspace scent collection. Air is pulled through the volatile trap at a specified rate calibrated with the flow meter. Based on figures in Dudareva and Pichersky (2006)

To quantify day/night variation in the floral bouquets of *P. digitalis* inflorescences, scent was collected from 12 plants in 8 h intervals (21:00–05:00, 05:00–13:00, and 13:00–21:00) from two populations over two consecutive 24 h periods; Neimi Road plants were sampled 28–29th June and TH sampled on the 30–31st June 2007. Inflorescences were enclosed in modified plastic drinking cups and connected to pumps that pulled air through volatile-absorbent traps at a standardized flow rate of 200 ml/min. To distinguish floral compounds from background contaminants (plastic cups etc.), two ambient and two vegetative volatile samples were also collected. Scents traps were injected with a tetralin internal standard and eluted with di-
chloromethane (solvent) before gas chromatography-mass spectrometry (GC-MS) analysis. Volatiles were identified from ion fragments and retention indices where known, or were suggested through mass spectral libraries and verified using retention times and mass spectra of authentic standards. Dynamic headspace samples were expressed as internal standard equivalents with the mean air control values subtracted (negative values were zeroed). Two approaches were used to statistically explore day/night variation in scent production. First, plant differences in overall scent emission was visualized using the Random Forest classification algorithm and the likelihood of belonging to either day or night classification was estimated per plant (Ranganathan and Borges 2010). Second, day-night differences for individual compound emissions were assessed using non-linear mixed-effect models with package ‘nlme’ in R (Zuur et al. 2009).

To determine developmental and spatial variation in volatiles emitted by *P. digitalis*, buds, flowers and fruit were dissected from plants and static scent sampled between 10:00 to 16:00 h to match peak hymenopteran pollinator activity and emission. To identify the approximate sources of within-flower volatile production, flowers were dissected into anthers (male reproductive organs), the stigma (female reproductive organ), staminode (fifth infertile stamen), nectary tissue (corolla where nectar is produced) and petal tissue (the rest of the corolla) (Fig. 1). Each tissue sample comprised 6-100 flower parts and were sealed into sterile vials for scent to equilibrate before exposure to SPME fibers. Spatial variation and developmental stage samples of tissue-exposed SPME fibers were injected into a GC-MS and volatiles were identified as above. The chemical composition of SPME samples was presented as the mean relative abundance of volatiles.

**Anti-microbial effects of floral volatiles (II)**

Within-flower spatial variation in floral scent emission could function to inhibit or facilitate microbes dispersed by wind or flower visitors. It was predicted that the growth rate and maximum density of bacteria naturally found colonizing linalool and methyl nicotinate scented nectary tissue would be adapted to/facilitated by volatiles, whereas bacteria isolated from leaf or petal tissues would be inhibited. To test this hypothesis, three distinct bacterial microhabitats were sampled from the flowers and leaves of *Penstemon digitalis* plants (*n* = 3 plants; 2 flowers and 1 leaf per plant, Ithaca, New York, Aug 2014). For each flower, flower corollas were separated into two parts, the scentless flower petals and the volatile-emitting nectary (Paper I). Eight nectary, eight petal and three leaf bacterial strains (*n* = 19) were isolated and treated with low (5ng/ml) and high (100ng/ml) concentrations of volatile in basic nutrient media (SRM + glucose) (Del Giudice et al. 2008),
representing natural variation in linalool emission (Parachnowitsch et al. 2012). For comparison, all strains were tested in control media without volatiles. Bacteria in treatment solutions were transferred into 96-well microwell plates \((n = 7\) replicates/strain, 9-10 strains/plate), with a standardised initial Optical Density (600nm) of 0.01 across strains and treatments (Jousset et al. 2011). The above methods were repeated with SRM minus glucose to assess if any bacteria strain could use linalool or methyl nicotinate as an alternative carbon source.

For analysis, the initial optical density of each strain was set to zero and bacterial growth curves were fit to a modified Gompertz equation (Zwietering et al. 1990) to obtain maximum growth rate and maximum density per strain per replicate. To assess the response of bacteria to linalool and methyl nicotinate relative to the control, we calculated the effect size as the log response ratio \(L = \log_e (\text{volatile treatment} / \text{control})\) of growth rate \((\mu)\) and maximum density \((A)\) for each bacteria strain per volatile and concentration treatment. The antimicrobial properties of linalool and methyl nicotinate at two difference concentrations were compared with mixed-effect models where growth rate or density was the response variable, and volatile as the explanatory variable. Bacteria strain was treated as a random effect to control for variance generated by different bacteria strain responses to volatile treatment. Because we lacked power to test all effects in a single model, to determine a volatile concentration effect or plant tissue effect on bacteria growth rate and density, we performed separate mixed-effect models for linalool and methyl nicotinate.

Determining if nectar scent is an honest signal (III)

Nectar scent is often assumed to be an honest signal of nectar, yet this assumption is rarely tested (Knauer and Schiestl 2015). To assess if linalool emitted by *P. digitalis* nectar could honestly signal nectar quantity, quality, and/or replenishment rate to pollinators for inflorescences, nectar was measured for a minimum of 3 flowers per inflorescence (post pollinator exclusion) and linalool emission was captured using dynamic headspace sampling (as above). For analysis, separate linear mixed-effect models were performed for each response variable; nectar volume \((n = 149)\), sugar amount \((n = 58)\) and replenishment rate \((n = 34)\). Display size was included as a covariate whereas year, population, and pump identity were treated as random effects to segregate variability in nectar trait estimates.

To determine whether linalool acted as a direct (absent when nectar is depleted) or an indirect signal (present before nectar replenishment) at the flower scale, linalool emission of control flowers and flowers with nectar removed, were compared using SPME. In addition because nectar increases
through floral development (from male to female sexual phase) (Castellanos et al. 2002), linalool emission was assessed for paired comparisons of male and female-phase flowers within inflorescences using DH \((n = 36)\). A linear mixed-effect model was used to test for flower-phase differences in linalool, with linalool emission rate (ng/h) phase per inflorescence as the response, flower-phase as the explanatory variable and inflorescence identity and number of flowers per sample as random effects.

A critical assumption of honest signalling is that pollinators have the sensory capacity to detect variation in volatile concentration (a range rather than presence/absence) and can associate it with reward (quality/quantity) (Knau- er and Schiestl 2015). Therefore experiments tested bumblebee *Bombus impatiens* (L.) capacity to use honest scent signals to discriminatingly forage on flowers honestly signalling greater rewards using a range of scent signals paired with varying nectar qualities \((n = 6-10\) bees per assay and minimum of 30 visits). Commercial (naïve) bumblebees were introduced to artificial flowers in the lab with low (5ng) or high-scented (100ng) flowers paired with correspondingly low or high quality or quantity nectar reward (high- and low-scented flowers had the same nectar qualities for the control). Analysis of Variance (ANOVA) was used to determine a signal-reward effect of average bee preference to highly scented flowers and Tukey HSD post hoc tests were performed to compare preference differences between signal-reward scenarios. Binomial tests were used to test if bumblebee preference was significantly different from no preference for each reward scenario.

To assess flower choice of wild *B. impatiens* bees in nature, individual bumblebees were observed visiting inflorescences of *P. digitalis* at four sites, for a minimum of 2 hours/day for two weeks (= 30 h, 62 bees, June 26 - July 10, 2014). Accepted/rejected flowers were defined by whether a bee landed and probed a flower (accept) or approached without probing (reject). For each observation, the sexual-phase of flower visited as well as the proportion of female-phase flowers available to visit per inflorescence was recorded. To test differences in acceptance of flowers based on flower-phase, we used a binomial generalized linear model and a mixed-effect model to assess if the proportion of female-phase flowers per display influenced the number of flowers visited. The response variable (number of flowers visited per inflorescence) was log transformed and bee visitation site and/or plant identity was treated as random.

To determine if bumblebees select more rewarding inflorescences in the field, one inflorescence per block \((n = 20\) blocks) was either supplemented with 9µl high linalool ‘nectar’ (high scent-nectar association), ‘nectar’ only (assumed low linalool treatment) or was untreated (assumed low nectar volume treatment). Pollen deposition was used as a proxy for bumblebee choice. A one-way ANOVA with pollen deposition as the response variable and treatment as the explanatory variable was used to assess choice.
Multimodal signalling enhances bumblebee foraging (IV)

To test if multiple signals are honest within the same species, signal-reward associations were assessed for visual and olfactory components of *P. digitalis* floral displays. Nectar standing crop was measured for inflorescences using a minimum of 3 flowers per inflorescence after flower visitors had been excluded for 8-24 h (*n* = 131). Visual components, display size (number of open flowers), flower size (estimated by averaging the geometric mean of four morphological corolla dimensions) and petal colour (averaged corolla line counts weighted by anthocyanin intensity) were measured for the same inflorescences and/or a minimum of 3 flowers per inflorescence. Linalool emission rate was also measured for inflorescences using dynamic headspace. The comparative honesty of floral signals was assessed using a mixed-effect model with nectar volume as the response variable and standardized display size, flower size, petal colour and linalool emission rate as fixed effects. Population nested in year and pump identity were included as random factors. Thereafter mixed-effect models were performed excluding each significant predictor to compare the $R^2$ of the residuals. In addition, to test the reliability of display size as a visual signal, variation in nectar availability within inflorescences was calculated by measuring nectar from a minimum of 3 flowers per inflorescence (*n* = 58). For analysis a mixed-effect model was used with nectar volume variance within inflorescences as the response variable and display size and mean flower nectar availability as explanatory variables. Random effects were population nested in year and number of flowers measured per inflorescence.

To understand how multiple signal-reward associations could influence bumblebee foraging efficiency, bumblebees were observed in the field and directly tested in the lab. To assess if visual traits influenced the time bumblebees spent foraging on each inflorescence (using the number of flowers visited/inflorescence as a proxy), individual bees were observed foraging on *P. digitalis* inflorescences for a minimum 2 h/day for two weeks over four sites (total = 62 bees and 30hrs, 26 June–10 July 2014). For a general assessment of inflorescence phenotype visited, inflorescence display size, average petal colour and flower size were measured for inflorescence visited. We used linear mixed-effect models to test the response, number of flowers visited, to display size and other floral traits; plant identity was treated as a random effect.

In the lab, bumblebee foraging choice and learning ability was assessed using four experiments where each offered a different multimodal signal (display size and scent) association with nectar quantity. Immediately following training, bumblebees were presented with 9 artificial inflorescences in a randomized design varying whether or not the visual signal of display size and/or olfactory scent signal (linalool) were correlated with total nectar reward available (*n* = 6 bees per assay). It was hypothesized that when both
visual and olfactory signals honestly predicted reward, bumblebee ability to choose the most rewarding inflorescences would increase. In the scenario where display size or scent were reward-informative, or they were uncertain, it was hypothesized that bumblebees should choose inflorescences with the most conspicuous visual signal because of cognitive sensory bias (Schiestl and Johnson 2013). For analysis, bumblebee choice was calculated as the proportion of visits to each type of inflorescence per assay and used Tukey’s post-hoc tests to statistically determine difference in preferences between and within trials. To assess the impact of signals on learning ability, the variance in bumblebee choice was calculated using the frequency of each display type chosen over ~30 visits per trial and compared the variability between trials using $F$-tests.
Results and Discussion

Spatiotemporal variation in floral scent (I)

Detailing the spatiotemporal variation in *P. digitalis* scent was possible through two scent trapping techniques, by using SPME for tissues, over 50 volatiles (including the 23 volatiles previously identified) were identified; encompassing aliphatics, aromatics and nitrogen-containing compounds. Floral scent varied through development for single flowers and diurnally for inflorescences. Furthermore, spatial expression of volatiles varied among floral tissues and rewards within flowers. Volatiles characterizing *P. digitalis* floral bouquets (mainly terpenoids) were predominantly emitted when buds open into flowers (Fig. 3a) and were found to increase in strength during the day for inflorescences. Terpenoid-dominated floral scent bouquets are both associated with attracting bee and lepidopteran pollinators (Das *et al*. 2013) and are known for their defensive functions (Theis and Adler 2012). Therefore increased diel emission of terpenoids in this system could suggest a function to attract day active pollinators (Bergström *et al*. 1995, Robertson *et al*. 1995) and/or repel larcenists (Junker and Bluthgen 2010). Additionally, reducing attractive volatiles at night could be a strategy to avoid attracting florivores or pre-dispersal seed predators (Theis 2006). Although daily variation in scent emission can be a physical consequence of light intensity, day length and temperature (Kesselmeier and Staudt 1999, Hendel-Rahmarim *et al*. 2007, Ibrahim *et al*. 2010), variation of volatiles under strong selection, such as *S-(+)-linalool*, suggests that diel regulation is more likely to have an ecological functions.

Floral tissues and rewards were found to vary in volatile composition. The corolla tube and petals were relatively scentless, whereas the majority of volatiles detected were emitted from the nectary region, the sexual organs or the rewards themselves (Fig. 3b). Tissue- and reward-specific volatile emission could be used to orient pollinators to the flower’s sexual structures, remotely assess reward availability (Dobson *et al*. 1996; Dötterl and Jürgens 2005; Howell and Alarcón 2007) or defend tissues/rewards against antagonists (Dobson *et al*. 1996; Kessler and Baldwin 2007; Junker and Tholl 2013). The spatial distribution of volatile organic compounds may therefore be adaptive, but this will depend on the context of the interaction. This study was part of an emerging trend to characterize not only what volatile compounds flowers produce but also where and when these scents are released.
(e.g. Friberg et al. 2013, Raguso and Weiss 2015, Schiestl 2015). The diversity of spatiotemporal patterns of floral volatile production should not be underestimated because it opens new doors of questions and hypotheses about novel ecological functions and the selective forces that shape them.

Figure 3 (a) Pie charts showing the relative abundance of different VOC classes produced at each stage of *Penstemon digitalis* reproduction. (b) Relative abundance of floral scents produced within tissues of *P. digitalis* flowers (*n*=10). The number of compounds are embedded within each pie. Numbers on the x-axis represent individual volatile organic compounds: for example the relatively abundant monoterpene S-(+)-linalool, is number 19 (See Paper I for full list).

**Anti-microbial effects of floral volatiles (II)**

We identified 14 species of bacteria representing 8 genera commonly found on plant tissues (Effmert et al. 2012). Bacteria cultivated from leaf tissue included strains from the genus *Bacillus*, *Pantoea* and *Pseudomonas*. Petal tissue comprised 5 different genera with *Pantoea*, *Erwinia*, *Serratia*, *Rosenbergiella* and *Pectobacterium*. The strains isolated from nectary tissue were *Pantoea*, *Erwinia* and *Rosenbergiella*, and *Acinetobacter* (Table 2).

On average linalool slowed the growth rate of bacteria significantly more than methyl nicotinate, showing a volatile and concentration-specific effect in keeping bacteria at lower densities for longer. No difference was found
between volatiles in suppressing bacteria maximum density. Thus a major conclusion from this study was that a nectary tissue and nectar-specific volatile under selection (linalool) had a stronger impact on bacteria than one produced in low concentrations (methyl nicotinate). Low linalool concentrations had only a small effect on the growth rate of bacteria whereas in comparison high linalool treatments could significantly slow bacteria growth rate. No significant difference was found in bacteria growth rate suppression among tissues for either high or low concentrations of linalool, but the effect of linalool on individual strains within treatments was variable and concentration specific (Table 2).

Table 2. Listed are bacteria diversity detected among tissues; only samples tested are identified by sample name. The tissue of origin, species identification and BLAST confidence in the identification is given for strains sequenced (n = 47; repeats of untested samples with the same identification are not listed). Strains in bold represent those with a significant growth rate (G) or density (D) for (L) linalool and (MN) methyl nicotinate. The symbol (’’) identifies a faster growth or a higher density response for low concentrations relative to the control, whereas (‘’) symbolises high. Conversely, (’) and (‘’) represent significant growth or density suppression at low or high concentrations.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Tissue</th>
<th>Genus</th>
<th>Species</th>
<th>BLAST ID confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1La_1</td>
<td>Leaf</td>
<td>Pantoea</td>
<td>agglomerans</td>
<td>18/20</td>
</tr>
<tr>
<td>P1Lb_1</td>
<td>Leaf</td>
<td>Pantoea</td>
<td>brenneri</td>
<td>19/20</td>
</tr>
<tr>
<td>P2La_1</td>
<td>Leaf</td>
<td>Bacillus</td>
<td>safensis</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>Pseudomonas</td>
<td>oryzihabitan</td>
<td>19/20</td>
</tr>
<tr>
<td>P1FPa_2</td>
<td>Petal</td>
<td>Pantoea</td>
<td>++GL, ++GMN</td>
<td>18/20</td>
</tr>
<tr>
<td>P2FPb_1</td>
<td>Petal</td>
<td>Pantoae</td>
<td>eucalypti</td>
<td>16/20</td>
</tr>
<tr>
<td>P2FPe_1</td>
<td>Petal</td>
<td>Erwinia</td>
<td>aphidica</td>
<td>18/20</td>
</tr>
<tr>
<td>P2MPa_2</td>
<td>Petal</td>
<td>Erwinia</td>
<td>rhapontici</td>
<td>18/20</td>
</tr>
<tr>
<td>P3Fpa_1</td>
<td>Petal</td>
<td>Serratia</td>
<td>+GL, --DL</td>
<td>20</td>
</tr>
<tr>
<td>P3Fpa_1</td>
<td>Petal</td>
<td>Serratia</td>
<td>+GL, --DL</td>
<td>20</td>
</tr>
<tr>
<td>P3Fpe_1</td>
<td>Petal</td>
<td>Pectobacterium</td>
<td>-</td>
<td>4/20</td>
</tr>
<tr>
<td>P3MPa_1</td>
<td>Petal</td>
<td>Pantoaea</td>
<td>conspicua</td>
<td>18/20</td>
</tr>
<tr>
<td></td>
<td>Petal</td>
<td>Pantoaea</td>
<td>vagans</td>
<td>18/20</td>
</tr>
<tr>
<td></td>
<td>Petal</td>
<td>Rosenbergiella</td>
<td>collisarenosi</td>
<td>18/20</td>
</tr>
<tr>
<td>P1FNb_1</td>
<td>Nectary</td>
<td>Pantoaea</td>
<td>agglomerans</td>
<td>18/20</td>
</tr>
<tr>
<td>P1Fnce_1</td>
<td>Nectary</td>
<td>Pantoaea</td>
<td>agglomerans</td>
<td>18/20</td>
</tr>
<tr>
<td>P1FNd_1</td>
<td>Nectary</td>
<td>Pantoaea</td>
<td>agglomerans</td>
<td>18/20</td>
</tr>
<tr>
<td>P1MNe_1</td>
<td>Nectary</td>
<td>Acinetobacter</td>
<td>bereziniae</td>
<td>19/20</td>
</tr>
<tr>
<td>P2FNa_1</td>
<td>Nectary</td>
<td>Pantoaea</td>
<td>+DL</td>
<td>18/20</td>
</tr>
<tr>
<td>P2FNe_1</td>
<td>Nectary</td>
<td>Erwinia</td>
<td>+GL, --DL, --GMN</td>
<td>18/20</td>
</tr>
<tr>
<td>P3FNa_1</td>
<td>Nectary</td>
<td>Pantoaea</td>
<td>agglomerans</td>
<td>18/20</td>
</tr>
<tr>
<td>P1MNe_1</td>
<td>Nectary</td>
<td>Acinetobacter</td>
<td>++GL, MN</td>
<td>19/20</td>
</tr>
</tbody>
</table>

29
Methyl nicotinate on the other hand had little effect on bacteria growth rate or maximum density but again showed bacteria strain variation. It is not unusual to find strain specific variation in growth rate to different volatiles or high concentrations (Vannette and Fukami 2016) because each genus and strain within the genus can be unique in metabolic capability, nutritional requirements and adaption to environmental stresses including oxidative stress from VOCs (Lindow and Brandl 2003; Lievens et al. 2015). Thus the activities of VOCs have the potential to effect compositions of microbial communities through inhibiting or facilitating the growth of individual strains but that this effect will likely be volatile and/or volatile concentration specific. Through emitting volatile organic compounds from nectary tissues and nectar, plants such as P. digitalis will possess a constitutive defence that can slow the growth of harmful bacteria without the time delay needed for the production of inducible defences.

Determining if nectar scent is an honest signal (III)

Linalool emission rate from P. digitalis inflorescences provided an honest indication of nectar quantity and (marginally) nectar quality but not replenishment rate (Table 3). Individual flowers from which nectar had been removed remained linalool-scented and despite female-phase producing more nectar (Fig 4a), no difference in scent emission was detected between flower sexual phases (Fig 4b). Nectar quality did not differ between flower sexual phases. Therefore linalool likely diffuses into the nectar from the nectary tissue, independently from nectar production. This suggests linalool can only function as an indirect signal of nectar availability, contrary to the hypothesis.

Bombus impatiens bees were able to use honest linalool emissions to make foraging decisions in controlled laboratory experiments. More specifically, bumblebees used differences in linalool emission to preferentially forage on high nectar quality flowers. However bumblebees did not show a preference for high or low-linalool scented flowers when nectar quality was the same. In the field, bumblebees showed no preference for more rewarding female-phase flowers and visited a similar amount of flowers per inflorescence, irrespective of the proportion of female flowers comprising a display. In addition, pollen deposition among inflorescences supplemented with linalool and/or nectar was no different to the control inflorescences, supporting lab experiments showing bumblebees will not select inflorescences based on linalool-reward volume associations. Together lab and field studies suggest that as an indirect signal of nectar availability, linalool could function to encourage pollinator constancy among inflorescences whilst manipulating visitors to probe both rewarding and unrewarding flowers (Thomson 1981; Gegear and Laverty 1998). Results also suggest that a direct association with reward did not drive selection on linalool.
Table 3. Summary of three mixed-effect linear models with Nectar volume ($n = 149$), Replenishment rate (µl/ h) ($n = 34$) and Sugar amount (mg) ($n = 68$) predicted by explanatory variable linalool emission rate (ng/ h) and covariant display size. Results are given for models fitting trait means per inflorescence. Population nested in year and, pump identity are included as random effects. Significant explanatory variables are given in bold.

<table>
<thead>
<tr>
<th>Nectar trait</th>
<th>Linalool emission rate</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log (Nectar volume)</td>
<td>0.172 ± 0.06</td>
<td>3.08</td>
<td>0.002</td>
</tr>
<tr>
<td>Square root (Sugar amount)</td>
<td>0.002 ± 0.001</td>
<td>1.90</td>
<td>0.057</td>
</tr>
<tr>
<td>Log (Replenishment rate)</td>
<td>-0.035 ± 0.07</td>
<td>-0.50</td>
<td>0.617</td>
</tr>
</tbody>
</table>

Figure 4 a) Floral sexual phase comparison of average nectar quantity (reward) and b), The scent difference between paired flower sexual phase flowers within an inflorescence. Each point is one inflorescence (olfactory signal). Boxplots show the median (line), 25–75% quartiles (boxes), ranges (whiskers) and extreme values (filled circles).
Multimodal signalling enhances bumblebee foraging (IV)

Nectar volume was predicted by both display size and linalool emission rate but not by flower size or petal colour (Table 4). The distinction between whether a floral signal is plastic or constant within a display could determine its function in attracting pollinators. Floral signals that can vary through time (e.g. scent, display size) can correspondingly vary with reward and so may be more likely to function as honest signals that reinforce pollinator behaviour during intraspecific visits (Paper I; Junker and Parachnowitsch et al. 2015). In contrast, relatively constant and distinct/conspicuous floral signals (e.g. flower colour and flower size) could allow pollinators to distinguish among rewarding and less profitable species, thus contribute to pollinator preference for one plant species over another (Wright and Schiestl 2009).

Table 4. Summary of a mixed-effect model with nectar volume predicted by display size, scent flower petal colour and flower size ($n = 131$). The models fit trait averages produced per inflorescence, with population nested within year, and pump identity as random effects. Significant explanatory variables are given in bold.

<table>
<thead>
<tr>
<th>Floral signal</th>
<th>Log (Nectar volume) $\beta \pm SE$</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Display size</td>
<td>0.225 $\pm$ 0.059</td>
<td>3.722</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Linalool emission rate</td>
<td>0.169 $\pm$ 0.059</td>
<td>2.888</td>
<td>0.005</td>
</tr>
<tr>
<td>Flower size</td>
<td>0.043 $\pm$ 0.086</td>
<td>0.479</td>
<td>0.620</td>
</tr>
<tr>
<td>Colour</td>
<td>-0.081 $\pm$ 0.056</td>
<td>-1.441</td>
<td>0.152</td>
</tr>
</tbody>
</table>

Larger displays contained more nectar on average, however the distribution of reward was variable so that a smaller proportion of flowers within a display contained the majority of the nectar. This is important because nectar variability weakens the correlation between signal and reward. However, in the field, bumblebees visited more flowers of inflorescences with larger display sizes, suggesting that display size impacts pollinator attraction through an expectation of reward and not actual availability. This concept was supported in the lab. Here, bumblebees preferentially visited plants producing at least one honest signal (Fig. 5b-d), and were most efficient when display size correlated with reward availability and was reinforced by scent (Fig. 6d). Through risk-sensitive foraging and associative learning, over
time, bumblebees adapt initial preferences to forage using signals most accurately predicting reward (Makino and Sakai 2007; Beritez-Vieryra et al. 2010). Therefore plants producing honest signals will benefit from a greater or increased proportion of visits within a population driving selection of reward informative signals. Plants could balance the risk of self-pollination through greater attraction by varying the availability of reward.

Figure 5 a-d. Proportion of visits to 1, 3 or 6 flower displays where total amount of reward is correlated (b, d) or uncorrelated (a, c) with display size and/or scent. Bars represent the mean proportion and standard error of visits to each display size ($n = 6$ bees per treatment combination). The dashed line represents no preference.
Figure 6 a-d. Frequency of visits to 1, 3 or 6 flower displays over a sequence of visits to displays where total amount of reward is correlated (b, d) or uncorrelated (a, c) with display size and/or scent. Filled circles represent mean bee choice and standard error per visit within treatment combination (n = 6 bees/treatment). The dashed line represents average display size preference over time and \( \sigma^2 \) states the variance in bumblebee choice per treatment combination.
Concluding remarks

In animal-pollinated plants, floral scent plays a vital role in floral displays, specifically because of its ability to fulfill multiple roles. In particular, the adaptability and diversity of floral scent allows flowers to mediate interactions with both mutualists and antagonists, as suggested by Paper I and shown by Papers II and III. We show that scent can function with visual floral traits to enhance pollinator attraction (Paper IV), but ultimately how influential scent is in mediating interactions, will depend on its association with reward and the scale at which pollinators forage. Thus, floral scents can have crucial functions in the reproductive biology of flowering plants, which often cannot be accomplished by other flower traits such as morphology, floral colour or rewards. Combined, our work suggests that floral scent is best understood in the context of other floral traits and, conversely that such information is incomplete without scent. Therefore it is essential to integrate the chemical ecology of flowers into pollination ecology in order to comprehensively understand the complex interactions that occur within plants, between plants and their biotic/abiotic environment. Future work should compare the relative impact of pollinators and antagonists on *Penstemon digitalis* fitness in order to determine the force driving selection on linalool emission.
Summary in Swedish

Samverkar linalool med andra signaler för att bistå pollinatörerna i deras nektarsök? (uppsats IV).


Vita lögner kan ta dig långt: ärlig till en gräns: Som en av de dofter som utsöndras av nektar skulle man kunna förvänta sig att linaloolproduktion skulle vara kopplad till med nektarproduktion, och att avsaknad av linalooldoft skulle vara en ärlig signal till pollinatörer att blomman är tom. Vi undersökte detta genom att samla dofter från tomma blommor, blommor med nektar och hela växter. Ett överraskande fynd vi gjorde är att tomma blommor fortfarande producerar linalool, vilket visar på en gräns för växtens är-
ighet. Dock producerade växter med många blommor mer doft och hade också mer nektar att erbjuda. Pollinatörer kan alltså lära sig att använda linalooldoften som en ärlig signal, även om de inte helt kan undvika tomma blommor. Detta kan också vara ett sätt för växten att påverka pollinatörer så att de lämnar växten och undviker att sprida pollen till sina egna blommor - samspelet mellan blomma och pollinatör kan vara mer komplicerat än ens Sprengel kunde föreställa sig!


"Humlor... växter och djur, mest avlägsna i naturens storlekskala, är sammanbundna genom ett nät av komplexa samband"

Sammantaget visar min forskning att dofter kan signalera nektartillgång men också ha flera andra funktioner.
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First I would like to thank my supervisor: Amy L. Parachnowitsch and co-supervisor Jon Ågren for granting me this opportunity. I’ve had many adventures, met some amazing people and got paid to do something I love. Amy has been a fantastic supervisor, coauthor and academic role model. I would also like to extend my utmost gratitude to Douglas G. Scofield who has actively taken a supportive mentor role in the last year (as well as for being my R programming guru). Many thanks to Magne Friberg for many reasons but mainly because discussions on evolutionary pollination biology are never as fun without him.

Second, thank you to my committee members (Douglas Scofield, Robert Gegear, Robert Junker), coauthors, teaching and researching colleagues (Brita and Bengt), administrators, technicians (Kirsten), and fellow PhD students at Plant Ecology and Evolution, at Cornell and at WPI who have all in someway contributed to the successful fulfillment of this thesis through activities, help and encouragement. Thank you also to Wittko Francke for supplying the illusive volatile my thesis is based on. I would especially like to thank my office mates Charlie and Matt, and friends from home and in Uppsala for the last four years of banter, fika breaks, advice, encouragement, distraction and support.

Lastly, thank you to my family (Mum, Dad, Joe, Katy), extended family (Janet and Chris) and my future husband, Mark Ramsden, as I couldn’t have succeeded without you.


Robertson, G.W., Griffiths, D.W., Woodford, J.A.T. and Birch, A.N.E. (1995) Changes in the chemical composition of volatiles released by the flowers and


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)