### Improving our Chemistry: Challenges and Opportunities in the Interdisciplinary Study of Floral Volatiles

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<td>Raguso, Robert; Cornell University, Neurobiology and Behavior Thompson, John; University of California, Ecology and Evolutionary Biology Campbell, Diane; University of California, Ecology and Evolutionary Biology</td>
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Improving our Chemistry: Challenges and Opportunities in the Interdisciplinary Study of Floral Volatiles.

R.A. Raguso, J.N. Thompson and D.R. Campbell

The field of chemical ecology was established, in large part, through collaborative studies between biologists and chemists with common interests in the mechanisms that mediate chemical communication in ecological and evolutionary contexts. Pollination is one highly diverse and important category of such interactions, and there is growing evidence that floral volatiles play important roles in mediating pollinator behaviour and its consequences for plant reproductive ecology and evolution. Here we outline next-generation questions emerging in the study of plants and pollinators, and discuss the potential for strengthening collaboration between biologists and chemists in answering such questions.

1 Introduction

Shortly after its birth as a field, Chemical Ecology captured the interest of a generation of biologists seeking to understand the mechanisms behind complex ecological interactions, and natural product chemists eager to characterize the key molecules mediating such interactions. Among the most exciting studies were those attempting to understand the role of chemistry as a driver of reciprocal evolution between interacting enemies or mutualists, that is, coevolution. Accordingly, the “secondary compounds” of plants came to be considered as potentially evolved defences against herbivores and pathogens, attractants for pollinators, or subtle mediators of yet other interactions. In turn, these compounds became a potential focus for natural selection on herbivores, pathogens, and mutualists.

The possibility of “chemical arms races” helped to fuel the growth of Chemical Ecology as a field, and stimulated decades of fruitful collaborative research between chemists and ecologists. This process has stalled to some effect in recent years, as many analytical chemists and industrial laboratories have shifted their attentions from the discovery of novel, ecologically-relevant small molecules to high throughput health-related proteomics analyses, whereas many biologists studying plant-animal interactions now focus more on questions such as the network mathematical properties of food webs and the molecular underpinnings of morphological traits under natural selection.

This essay explores the prospects and opportunities for renewing and strengthening collaborative interactions between chemists and ecologists in addressing the next generation of chemical ecological questions. We will use the complexity of volatile organic compounds (VOCs) in flowers, and their importance to plant reproductive ecology, as a context in which to discuss these ideas, reviewing several case studies as key examples. Our goal is to highlight topics and approaches that require better communication between chemists and ecologists so that we can work together more effectively and also simultaneously broaden and refine the kinds of answers we seek to major questions. The next stages of the dialogue will require ecologists to think more rigorously about VOC identification, chirality and dosage, and will require their chemist collaborators to understand the necessity of statistical replication, multivariate approaches to visualizing complex chemical phenotypes, and to appreciate that mundane compounds (in the sense of their not being novel) may still have subtle, complex and pivotally important ecological functions whose elucidation is worthy of their investment.

2 Next-generation questions about the ecology of floral volatiles

2.1 Natural selection on volatiles and how it depends on animal behaviour

Much of the formative literature on floral VOCs developed from the study of specialized model systems, in which floral scent was noteworthy for its chemical mimicry of non-floral substances (e.g. insect sex pheromones and carrion) or its elicitation of unusual pollinator behaviours (e.g. harvesting of essential oils by male euglossine bees). These studies broke new ground by demonstrating how to integrate sensitive chemical analyses in the...
laboratory with behavioural assays in the field, often guided by electrophysiological screening of bioactive volatiles\textsuperscript{7,18}. More recent studies following this tradition have demonstrated the relevance of floral VOCs to the larger spectrum of plant-pollinator interactions, including nectar- and pollen-based floral markets with more generalized floral visitors and less unusual scent composition\textsuperscript{9,20}. It is now well established that floral VOCs evoke pollinator responses at many spatial scales – from distance attraction to landing and probing\textsuperscript{21} – and that such responses can be either innate or learned\textsuperscript{22}. Less well established is how such phenomena contribute to outcrossing, gene flow and reproductive isolation through floral constant\textsuperscript{23,24}. Most studies linking floral scent composition and pollinator behaviour lack explicit measurements of fitness, in other words, the reproductive consequences of chemically mediated plant-pollinator interactions that would allow us to measure natural selection\textsuperscript{25,26}. Very few of the growing number of published studies of phenotypic selection on floral form, colour or display include VOCs as a factor\textsuperscript{27}. Below we outline emerging questions addressing this gap, and the experimental approaches needed to fill it.

The few studies of selection on floral VOCs have focused largely on detecting directional selection on individual volatiles such as 2-phenylethanol\textsuperscript{28} or on total scent from whole flowers\textsuperscript{25,29}. One of the challenges in understanding natural selection on scent is that the blend may contain dozens of compounds, a large number of which have the potential to influence pollinator or herbivore responses\textsuperscript{26}. This large set of compounds with the potential for multiple functions opens new questions about how selection might work to favour specific combinations of volatiles in specific ratios. We will need to move beyond measuring directional selection to consider stabilizing selection for particular emission rates, perhaps because one compound has both positive effects on fitness mediated by pollinators and negative effects mediated by herbivores, as suggested for methyl benzoate in Petunia\textsuperscript{30}. Furthermore, we need to consider that the effect of one volatile on fitness might depend on the emission rate of another volatile, generating correlational selection (synergistic or non-additive effects on fitness), as has been demonstrated for some morphological traits. For example, increasing spur length increases fruit set to a greater extent for plants with more flowers in the orchid Dactylorhiza laponica\textsuperscript{31}, whereas in Nicotiana glauca the fitness effect of style length depends on corolla length\textsuperscript{32}. Such non-additive effects could be driven through pollinator attraction, as attraction to a particular VOC may vary depending on how that compound functions within blends of other compounds. Because different volatiles are sometimes produced by different floral parts\textsuperscript{33,34}, natural selection on floral morphology could also indirectly affect the evolution of floral blends, just as selection favouring or disfavouring particular VOCs could, in turn, affect the evolution of morphology\textsuperscript{35,40}. Evolution either of volatiles or floral morphology could therefore simultaneously affect the attraction of pollinators and their efficiency as pollinators once they reach a flower.

### 2.2 The bridge to macroevolution

One of the most difficult problems to understand at the interface of chemistry and biology is the extent to which both natural selection and evolutionary constraints on biosynthetic pathways shape the directions of macroevolutionary change. Population-level variation in VOC composition has the potential to be a major driver of diversification of plant lineages, through its effects on reproductive isolation between divergent plant populations\textsuperscript{31,34}. We already know that floral chemistry affects reproductive isolation among some closely related species\textsuperscript{35,36}, and it has the potential to affect adaptive radiations of plants both directly and indirectly.

We lack studies, however, that directly evaluate how the diversification of floral VOCs has shaped patterns of speciation within any plant lineage, as Hodges and Arnold\textsuperscript{41} have done for the effects of floral morphology (nectar spurs) as key innovations that accelerate rates of evolutionary diversification. More generally, there are few systematic studies of how qualitative or quantitative patterns in VOC emissions have diverged within major lineages of flowering plants\textsuperscript{42}. For example, one recent study tracks sulphides and other VOCs indicative of microbial decay across Angiosperm phylogeny in an analysis of the convergent evolution of brood-site deceptive flowers\textsuperscript{43}, shedding light on how such compounds contribute to niche diversification. Detailed studies of plant genera are beginning to increase but remain uncommon\textsuperscript{44}. Even if we had a synoptic sense of such patterns across major lineages, we would not yet grasp the extent to which natural selection has shaped the deepest diversification of volatile organic compounds as components of floral phenotypes. Is the fact that some plant groups (e.g. Lamiaceae) tend to produce terpene-based VOCs whereas other groups (e.g. Araceae) tend to produce sulphides or other compounds an indication that selection has acted directly on the deep divisions within biosynthetic pathways\textsuperscript{45}? Or rather, are the major phylogenetic differences in floral chemistry evidence of indirect selection acting on other traits, such as constitutive plant defences (e.g. glucosinolates in the Cruciferae, terpenoid latex in the Moraceae), pleiotropic relationships between VOCs and floral pigments\textsuperscript{46} or induced plant defences that interact in complex ways with reproductive ecology and life history strategies\textsuperscript{47,48}? Pollinator shifts do not occur in an ecological vacuum, and placing studies of floral chemistry into a whole-plant context is likely to reveal novel selective forces and constraints\textsuperscript{48,49}.

Resolving those questions would get us closer to understanding the conditions under which evolutionary transitions among branches of the same biosynthetic pathway should be more common than transitions between pathways. Although it would seem to be a good assumption that transitions within pathways are more common than between pathways, that assumption is turning out to be unsafe. In the North American genus Lithophragma (Saxifragaceae), for example, some populations of the same species are dominated by different carbon-based pathways and others by nitrogen-based pathways\textsuperscript{38,50}. Similarly, there is a sharp geographical cline in cone scent chemistry among populations of the Southern African cycad Encephalartos villosus, in which unsaturated hydrocarbons dominate the scent of northern cycad populations and nitrogenous pyrazines dominate the scent of southern ones\textsuperscript{31}. Both of these examples illustrate chemical aspects of the geographic mosaic of coevolution, as the pollinators in each case are different populations of the same moth and weevil species, respectively, and both represent nursery pollination systems that vary geographically in degree of mutual dependence. More difficult yet is to understand the extent to which the presence or absence of different VOC blends are the result of selection acting semi-independently on multiple genetic loci or other genetic processes. Masking of whole biosynthetic pathways could result in major biochemical- and ecological- shifts among populations or species through one or a few genetic changes. These points are amply illustrated in the genus Petunia (Solanaeae), for which recent transgenic and breeding studies provide some salient lessons. The floral emissions of Petunia x hybrida and its parent species are dominated by volatile phenylpropanoids and benzenoids produced by the shikimate pathway, and do not express the essential enzymes
or substrates required for monoterpene emission in petal tissues. The major VOCs responsible for pollinator (hawkmoth) attraction to one of these parent species (P. axillaris) are related benzoid products (methyl benzoate, benzaldehyde and benzyl alcohol) whose biosynthetic enzymes are well characterized for petunia. However, the emission rates of these compounds are under the epistatic control of ORODANT-1, a MYB-like transcription factor known to up-regulate nocturnal emissions of benzoid VOCs by controlling the flux of their non-volatile precursors in the shikimate pathway. Furthermore, co-regulated phenylpropanoid VOCs such as isoegenol, which are emitted in relatively low amounts and do not attract hawkmoths, were shown to be essential for repelling non-pollinating insects that eat floral tissues. It remains unclear why some floral VOCs (e.g. methyl eugenol) appear erratically in many different plant lineages worldwide, whereas other compounds (e.g. carvone oxide) are restricted to a few genera of euglossine-bee pollinated Neotropical orchids and Dalechampia vines.

Hybridization among populations also has the potential to shift blends and pollinator visits quickly. For example, two sexually deceptive orchid species (Ophrys lupercaulis and O. iricolor) bloom synchronously on Sardinia, producing hybrids with intermediate VOC composition and floral morphology, resulting in gene flow via breakdown of pollinator specificity by male bees of two Andrena species. Studies of hybrids between O. lupercaulis and another species, O. arachniformis in southern France, reveal that F1 hybrids are less attractive to the pollinators of each parent species, but produce novel VOCs absent in both parental species, and are more attractive to Andrena vaga, a novel bee pollinator. Although the hybrids in this case are triploid and sterile, the authors outline how similar scenarios might give rise to rapid hybrid speciation and reproductive isolation through recruitment of a novel (and constant) pollinator.

Hybridization accompanied by genome duplication (allopolyploidy) or simply whole genome duplication of parental chromosome sets (autopolyploidy) also could have strong effects, because individuals will carry four sets of some alleles involved in floral VOC production. In the genus Henchera (Saxifragaceae), autotetraploid plants (i.e., those with two chromosome sets from the same parental species) attract different suites of pollinators from each parent species, but produce novel VOCs absent in both parental species, and are more attractive to Andrena vaga, a novel bee pollinator. Although the hybrids in this case are triploid and sterile, the authors outline how similar scenarios might give rise to rapid hybrid speciation and reproductive isolation through recruitment of a novel (and constant) pollinator.

Genome-wide analyses of the genetic architecture of some biosynthetic pathways are further enriching our understanding of the evolution of VOCs. Recent studies have indicated that terpene production is distributed in some species across multiple chromosomes, but much variation in expression of terpene diversity can arise from relatively few genes. In soybeans, terpene synthase genes (GmTPSs) are distributed over ten of the twenty chromosomes and can be grouped into six types based on their genetic architecture and DNA sequences. Most of these genes have a combination of conservative and altered motifs, and most are expressed especially in reproductive organs.

Findings like these allow us to answer important evolutionary questions about floral scent. For example, certain combinations of VOCs are more commonly encountered in floral bouquets than others. Do these patterns result from their key biosynthetic enzymes being encoded by closely linked structural genes, comparable to metabolic gene clusters related to plant defense, or from multiple products of single biosynthetic enzymes with unusual biochemical properties? Or are such patterns more likely to reflect shared regulatory networks, such as the single regulatory locus that controls the expression of three floral pigment genes in Mimulus, or from correlational selection favouring combinations of VOCs even if they are not genetically linked? Data from petunia suggest a shared regulatory network, as transcripts for 7 VOC biosynthetic structural genes accumulate in coordinated fashion but are not closely linked. With these examples of regulatory pleiotropy in mind, how might selection act upon allelic variation in regulatory loci, given that petunia flowers attract pollinators and repel florivores with different components of the same VOC blend?

Taken further, what are the patterns of phenotypic and genotypic correlations between floral volatiles and other phenotypic traits? Are there subsets of VOCs that are phenotypically integrated with each other or with morphological traits, and how do these levels of
integration compare with that observed for floral morphology. A recent review of phenotypic integration for morphological traits found that morphological traits in flowers were less tightly correlated than morphological traits in animals, with the lowest correlations between floral and vegetative traits. Is this also true for floral volatile traits? Furthermore, are correlations between traits, whether between two floral volatiles or between floral volatiles and other traits, maintained by correlational selection? Kárpati et al. addressed this question using behavioral bioassays of Manduca sexta moths, which utilize Nicotiana and Datura plants as both larval hosts and adult nectar sources in western North America. Experiments in which floral and vegetative VOCs from N. attenuata and D. wrightii were decoupled and recombined resulted in greater moth attraction to floral blends when coupled with vegetative blends from the same species, but not from the opposite species. This finding reinforces the idea that plant reproductive and defence traits are not independent, and that selection might act in complex ways to integrate floral and vegetative VOC phenotypes.

In addition to genetic associations for volatile production, the distribution and magnitude of variation in scent composition and emission rates remain poorly understood. To date, most studies of floral VOCs (as well as other metabolic products) have focused on characterizing an average value for a species. Yet, there are very few studies that have tracked VOC variation at lower scales, from populations within species, plants within populations, and flowers within plants. Most chemical ecological studies of floral scent have based on the assumption that variation is greater among species than within species, but careful analysis of multiple populations demonstrates this is not always the case, and most studies with expanded population-level sampling have revealed variation. Quantifying the distribution and magnitude of actual biological variation (rather than methodological artefact) in VOCs within and among populations, species, and higher lineages is important, because it affects how we should proceed with phylogenetic analyses and with sampling in the field.

Given the potential for cryptic variation in floral VOCs at the population scale, what are the genetic causes of such variation? A response to natural selection depends on genetic variance in the trait and on the patterns of genetic correlation between traits. In contrast with other floral traits, we know little about the heritabilities of floral VOCs in natural populations, or even how repeatable phenotypic measurements are across multiple flowers produced by the same individual. The flip side of heritability is plasticity – how environmentally plastic is floral VOC production, and is this plasticity adaptive? Volatile emissions vary with environmental conditions and over time as well as among individuals. For example, the diel patterns of fragrance emission match patterns of pollinator activity in thistles, snapdragons and petunias, and emissions plummet in response to ethylene pulses that accompany pollination or senescence. We already know that some plants are able to alter production of VOCs during diurnal cycles to coincide with the activity patterns of their pollinators. Abiotic influences, such as increased temperatures, also affect VOC emissions from plants. Will increased temperatures with global change cause adaptive or maladaptive phenotypic shifts in VOC emissions? To answer such questions, we need to know more about the fitness consequences of dosage-dependent responses to floral VOCs. In a rare example, moderate emission of 2-phenylethanol by alpine skypilot (Polemonium viscosum) reduces flower damage by ants at no cost to pollination, whereas high emission of 2-phenylethanol has a net negative impact due to sharp reduction in bumblebee visitation. In this example, dosage-dependent effects of floral VOCs modulate the strength of bumblebee-mediated selection on floral shape. Prior studies of these plants identified an altitudinal gradient in floral chemistry, transitioning from plants with skunky-scented calyx trichomes at timberline to sweet (2-phenylethanol) scented plants lacking skunky trichomes above timberline, reflecting alternative strategies to balance ant repellence and pollinator attraction.

For volatile attractants and defences alike, we need to know whether certain VOC blends are more effective in some ecological communities (e.g. terpene-rich pine forests or sagebrush steppe) than others, a question that lies at the heart of efficacy-based theory of signal evolution. Such questions represent the next frontier for community ecologists working with floral VOCs, because they affect not only interactions with pollinators but also with herbivores, pathogens, and other plants. Herbivory can affect the production of floral volatiles, which can affect attraction of pollinators and parasitoids. Changes in volatile production in one plant, resulting from herbivory, can affect levels of volatile production in neighbouring plants and the effects may be greatest among genetically related individuals. Studies of the direct and indirect effects of community-wide effects of plant signalling will likely show further ways by which VOC variation in plants shapes patterns of plant competition, herbivory, pollination, and broader interspecific patterns of convergence and divergence of traits.

3 Opportunities for renewed collaboration between biologists and chemists

3.1 What biologists need from chemists in collaborative studies on floral volatiles

Biologists generally lack the training and expertise to identify ecologically important chemicals, often lack access to appropriate equipment or shared-user facilities, and few are able to produce usable samples through fractionation or organic synthesis. There is a long tradition of fruitful collaboration between biologists and chemists in the identification of biologically active chemical compounds that have novel and fascinating structures. Yet, many compounds of interest to biologists are structurally mundane to natural product chemists, and thus may not appear to be worthy of their investment. Also, the need for statistical replication requires biologists to analyse numerous replicates from a given species, which may be unlikely to produce novel structures once that species is characterized, whereas the qualitative and quantitative variation among those replicates is of crucial interest to biologists. Below we discuss the mismatched interests of biologists and chemists and outline ways to find common ground in collaborative studies.

3.1.1 A chemical tool kit.

A fundamental requirement for laboratory analyses and manipulative field studies alike is access to high purity, authentic VOCs with the appropriate isomeric or enantiomeric configuration. These criteria are crucial not only to the study of specialized pollination systems driven by novel compounds, but also to the study of ubiquitous VOCs like limonene (which is chiral) and β-ocimene (which has geometric isomers). It can be difficult or prohibitively expensive for biologists to acquire sufficient amounts and purities of such compounds from commercial sources. For example, trans-β-ocimene is one of the most widespread and multifunctional floral VOCs, which is usually encountered in great excess (> 10:1 ratios) over the cis isomer. This compound is thought to play pivotal roles in...
pollinator attraction, from the obligate weevil-pollinated dwarf European palm 90 to bee-pollinated Mimulus lewisii, a model system for the study of floral isolation 91, to the more generalized community of terpene-scented shrubs in the Spanish maquis 92,93. However, the lone commercial option available from Sigma-Aldrich, Inc. (W353901) for manipulative experiments is an ocimene blend dominated by the cis isomer. How might biologists more effectively engage chemists – or their students – as collaborators to study such common and potentially important VOCs, when their structures are not particularly novel or interesting?

Expanding on this theme, access to biologically relevant chemical libraries remains a problem. Identification of unknown components of VOC blends requires (at minimum) access to sensitive GC-MS equipment and a battery of authentic standards with which to confirm (or reject) tentative MS matches through co-retention on different stationary phases 101. For most biologists studying floral VOCs, the acquisition of reference libraries for compound identification is opportunistic at best, and often includes commercial essential oils for which (presumably trustworthy) published analyses allow direct comparisons with unknowns. A common approach in proteomics and natural product screening is the use of combinatorial, solid phase synthetic chemistry to create compound libraries 102,103. A similar approach to library creation is that of diversity oriented synthesis 104. The application of these approaches to generating batteries of monoterpenes or phenylpropanoid compounds, for example, would create transformative tools that would allow more comprehensive screening of floral VOCs at community scales by biologists lacking training in organic synthesis or access to collaborators with such training.

Even so, most studies of floral VOCs continue to generate unknowns that cannot be identified through co-chromatography and MS comparison. Many of these compounds are novel and would generate interest among chemists. This potential is exemplified by collaborative studies over the past decade on sexually deceptive orchids in Australia. Schiestl, Peallk, Francke and colleagues 106 identified novel alkylated cyclohexanediones, named “chloroglottones” in honor of Chiloglottis, the genus of deceptive orchid from which they were identified. The combined approaches of electrophysiology, organic synthesis and behavioural assays made it possible to identify the key volatiles and to produce enough of them to test their biological activity with the thynnine wasp pollinators 107,108, leading to the most thorough study to-date on population-level VOC variation and its evolutionary consequences 109. Extending studies of this system geographically to Drakaea orchids in Western Australia pollinated by related thynnine wasps revealed yet another set of novel VOCs that mediate sexual deception, this time a series of alkylated pyrazines 110. This multinational collaborative approach continues to yield conceptual breakthroughs. One compelling reason for writing this review is to point out the rarity of such collaborations, despite their remarkable success.

Expanding tool kits also require conceptual, as well as technological innovation. A current challenge for biologists studying floral volatiles is therefore to move beyond para-taxonomic identification of VOCs to a more predictive science of volatile ecology that incorporates the full range of selection pressures acting on production of VOCs. For example, an important paradigm shift in pollination biology has been the realization that flowers simultaneously attract mutualists (pollinators, beneficial microbes, floral predators that enhance pollination success) while repelling antagonists (florivores, nectar or pollen thieves, harmful microbes).

This complex “filtering” of floral visitors is accomplished in part by multifunctional floral VOCs that show dosage effects and synergistic interactions with other compounds 111,112. This may require deeper knowledge of metabolic processes, well beyond the scope of floral biology. If biologists had a deeper understanding of the kinds of VOCs and related metabolites most likely to shape the ecology and evolution of interactions – for example, those that typify oxidative stress 113 or are perceived through unique neural representations 114 – they could focus on identifying and targeting those classes of compounds, and measuring how selection acts upon them.

3.1.2 Updated and question-appropriate sampling methods

Many next-generation questions will require large sample sizes taken under field conditions. For example, quantitative genetic studies are notorious for requiring sample sizes of several hundred, although this requirement is loosening with next-generation sequencing methods 115. Phenotypic selection analyses require not only large samples to characterize stabilizing and correlational selection 115, but also measurement under those field conditions in which pollinators and herbivores encounter VOC emissions. So, biologists will need to measure floral VOCs in ways that can be done quickly and reliably under field conditions away from the lab or sources of power or refrigeration.

The need for rapid extraction for later analysis via GC-MS or real time measurement (e.g. using portable sensors or PTR-MS) is amplified for ecological interactions that occur only during short periods of time each day or night 101. For example, in Ipomopsis tenuiutuba plants, indole is emitted from flowers only for a few hours after dark when hawkmoths are active. A suitable field sampling method needs to concentrate quickly the low abundance signal, which in this species is about 1 ng per hour. No solvent-based method provides a feasible solution to this problem, but dynamic headspace adsorption on Tenax followed by thermal desorption-GC-MS has now made these analyses possible 11,116.

Because biologists often need large sample sizes to study how variation is distributed among individuals within populations and even within individuals over time, continuous access to a GC-MS for weeks or even months and optimization of run times is becoming increasingly critical for experiments with high sample replication. The problem to solve, however, is not just high-throughput sampling and processing methods. These procedures must be accomplished in a way that adheres to a major axiom of chemical ecology: minor products (i.e. VOCs emitted in small quantities) can have important biological functions. The major products or most abundant metabolites are not always biologically active 117, nor are they necessarily the targets of selection 118. How can biologists and chemists collectively work toward discerning meaningful signal from noise in complex volatile blends? This is challenging, because analytical methods often produce large numbers of compounds in very small quantities. Screening protocols (such as electroantennography or bioassay-guided fractionation) have proven to be very helpful in this process. Many studies of floral chemical ecology continue to take a targeted approach, either focusing on specific compounds (e.g. indole 119) or classes thereof (e.g. monoterpenes 120) through selective analyses appropriate to that target variable. Some studies, however, have adopted unbiased approaches that, while creating many more treatments and replicates, have revealed unexpected insights 121,122. One solution is metabolomics, which represents the natural extension of high throughput, untargeted approaches, and more studies of VOCs are adopting omics protocols and statistical methods 123. Methods
developed in the rapidly emerging fields of bioinformatics have the potential to help investigators make better decisions between targeted and untargeted approaches in the study of VOCs\textsuperscript{122}, with the caveat that they remain correlative in nature.

### 3.1.3 Statistical Aspects and Experimental Design

Many of the ecological and evolutionary questions about the distribution and diversification of plant VOCs concern variation in relative proportions of compounds, proportions of individuals harbouring particular compounds of blends, distributions of compounds or blends across life history stages or among populations relative to ecological variables, and distributions within and among phylogenetic lineages. Biology is inherently about the processes that shape variation into metabolically, ecologically, and evolutionarily meaningful patterns across space and time. The major questions are therefore inherently statistical questions, and these present challenges because the multivariate statistical tools needed for many important analyses are still in development. These statistical challenges are not unique to biology or the application of chemistry to biology. They also occur in other scientific fields that must analyse patterns of variation as combinations of qualitative data and quantitative data that are correlated to varying degrees, sometimes organized temporally and sometimes spatially. That in itself is useful, because it means that the search for better statistical tools is continuing among researchers studying a wide range of scientific questions.

The potential complexity of the analysis can become obvious even after collection of just a few samples. For example, initial analyses of a few individuals of \textit{Lithophragma} (Saxifragaceae) from different geographic regions suggested that individuals of this genus were capable of producing dozens of VOCs\textsuperscript{91}. Those results suggested that the chemical blends differ among populations, but that immediately created the question of just how many populations, and individuals per population, need to be sampled? Subsequent studies using plants grown under multiple standardized conditions showed that a combination of genetic differences and plasticity in expression of compounds contributed to the observed differences, with most of the differences likely due to genetic differences among populations\textsuperscript{92,93}. Other studies have found considerable plasticity in production of plant volatiles growing under various environmental conditions or following interactions with herbivores or pathogens\textsuperscript{94}. For example, the chiloglottones described above are produced only in the presence of UV-B radiation\textsuperscript{95}.

Variation in floral scent itself, however, may be favoured by natural selection and hence may be adaptive under some ecological conditions\textsuperscript{123}. Natural selection on plants often reflects the evolution of networks of interacting plant and pollinating species, rather than pairwise interactions\textsuperscript{124,125}. Variation in blend production itself may therefore represent an evolutionary result of natural selection\textsuperscript{126}. Studies such as these indicate that we need a better understanding of which classes of VOCs are more variable in expression than others so that we can develop better sampling strategies for ecological and evolutionary studies. Only through studies that sample large number of individuals within and among populations will we start to understand these patterns and be able to refine sampling efforts for future studies.

Once adequate samples are in place, then a wide range of statistical analyses are available, but no current techniques can accommodate all the major ecological and evolutionary questions that need to be addressed. Traditional techniques such as multidimensional scaling (MDS), principal components analysis (PCA), and canonical discriminant analysis (CDA) are useful in showing the multidimensional correlation structure of the data, the combinations of compounds by which populations or species differ, and in the case of canonical discriminant or canonical coordinate analysis, which compounds are most responsible for the observed differences\textsuperscript{127}. But these techniques are inadequate for probing how complex blends vary hierarchically among populations within meta-populations or within phylogenetic lineages\textsuperscript{128}.

New methods that rely on machine-learning, Bayesian statistics, or network theory methods are providing potential new approaches to analysing complex chemical data sets. However, these developments suggest that future collaborations between biologists and chemists may require additional collaboration with multivariate statisticians and probability theorists. Network theory, for example, has in recent years provided important new insights into many scientific fields, including analyses of the neural networks, how species assemble within biological communities, and traffic flow within the internet\textsuperscript{129,130}. The techniques and range of questions that network theory can address, however, are evolving rapidly. Taking full advantage of them will require collaboration with those whose expertise is more mathematical and statistical rather than chemical or biological. As studies of adequately sampled populations continue, the potential to use these techniques to identify complex blends that co-occur repeatedly among taxa or communities will also increase. Network analyses also have the potential to help in evaluating which plant compounds are important and which are not for particular interactions with other species, thereby allowing chemists and biologists to focus more efficiently on the subsets of compounds likely to be truly important for any specific biological question.

### 3.2 Understanding what biologists must learn to collaborate more effectively with chemists

#### 3.2.1 How to describe chemical data and understand the appropriate units.

As we have described throughout this essay, floral VOCs present biologists with opportunities to explore a major - albeit invisible - driving force of many biotic interactions, but they also present the challenge of understanding the most appropriate ways to collect, describe and think about chemical data. How do chemical interpretations and biological interpretations of chemical data differ, and under what conditions can those lead to biological misinterpretation? Chemists often are critical of what they perceive as biologists’ superficial understanding of structural chemical principles (nomenclature, the importance of absolute configuration, isomerism, chirality), which surfaces in the way that biologists present chemical data in their publications. For example, most floral studies treat linalool as a single compound, despite the experimental demonstration that its enantiomers can have different phytogenetic distributions and ecological functions\textsuperscript{131}. In fact, the chiral specificity of linalool is critical not only in determining feeding vs. oviposition in a nectar-based pollination system (\textit{Datura} plants pollinated by \textit{Manduca} moths\textsuperscript{132}), but also in the context of a highly specialized nursery pollination system between a fig (\textit{Ficus hispida}) and its obligate wasp pollinator (\textit{Ceratosolen solmsii})\textsuperscript{133}.

However, the chemical ignorance of biologists often is exacerbated by lack of access to the most updated or sensitive analytical
equipment, such as 2D-GC-TOF-MS, chiral GC columns, preparative GC, 2D-NMR and derivatisation techniques that facilitate determination of structural chemical properties. Such equipment is more likely to be found in a core departmental facility than in the lab of a single biological investigator. Access to such facilities through per-sample fees, along with proper instruction, would provide biologists with the experience necessary to understand how to discuss chemical data when they seek collaboration with chemists. The challenge for chemists interested in working with biologists is to accept the need for statistical replication and resist the temptation to dismiss the potential biological importance of a compound because its structure is mundane. Exciting discoveries can result, showing, for example, that common VOCs can sometimes work in uncommon ways such as a pollinator attractant becoming a repellent as dosage increases. This is precisely what was found by Terry and collaborators for β-myrcene as a mediator of pollinator behaviour in Australian cycads, which they communicated with very high impact. These kinds of discoveries, resulting from biologists and chemists working outside their normal protocols and comfort zones, make collaboration particularly worthwhile.

3.2.2 How to better understand the costs and limitations of sampling methods.

Different but complementary methods are often used for VOC analysis, including dynamic or static headspace, and solvent or thermal desorption GC-MS, and each has advantages and disadvantages for biological analyses. For example, solid phase microextraction (SPME) has been used as a low-cost form of thermal desorption for nearly two decades, with great benefits for identifying highly volatile constituents or tissue-specific emissions, but due to its nature as an equilibrium-based method, cannot be used to calculate emission rates. Consultation with analytical chemists before a biological project begins, rather than after the data are collected, can therefore be of immense help in designing a study that best addresses any particular biological question. Because the quantity of a volatile compound can be very sensitive to collection conditions, one of the goals must be to standardize the data as much as possible. A clear understanding of the error intrinsic to the methods used to measure chemical traits is as important as a clear understanding of the limits of statistical methods. This becomes very important when attempting to measure phenotypic selection gradients using chemical data, in which it is crucial to eliminate as many sources of non-biological variance as possible. Some basic level of understanding is needed in order to compare results between complementary methods (e.g. using different kinds of detectors), which allows for greater rigor in meta-analyses between different studies, or phylogenetic mapping of VOCs. For example, when two studies of the same species differ in the presence of a given compound, we need to know with confidence when such variation represents biological differences, rather than methodological artefacts due to different thresholds of analytical detection. At present, comparative studies are rife with assumptions based on heterogeneous approaches to data quantitation.

We also need a better understanding of which new chemical approaches biologists are missing in their repertoire, and why these approaches would be useful for biological studies. In a world of restricted funding for research, it is also important to know which techniques are simply impractical within most budgetary and infrastructural constraints and what are reasonable per-sample costs for each proposed new method. If a new protocol is very expensive to acquire or operate, how might those expenses be defrayed? These are the kinds of questions that biologists must answer before launching into time consuming, remote field experiments. Constructive dialogue between biologists and chemists on these questions requires agreement on what constitutes a worthwhile question, and then how much replication is needed to answer the biological questions. Short courses and workshops have the potential to stimulate such dialogue, especially when hosted at sites that inspire wonder and opportunity through the shared experience of biological, chemical and cultural diversity.

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5 References