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Review

Insect pheromones: An overview of function, form, and discovery

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ABSTRACT

For many species of insects, lipid pheromones profoundly influence survival, reproduction, and social organization. Unravelling the chemical language of insects has been the subject of intense research in the field of chemical ecology for the past five decades. Characterizing the forms, functions, and biosynthesis of lipid pheromones has led not only to the development of strategies for controlling agricultural pests but has also provided insights into fundamental questions in evolutionary biology. Despite the enormous variety of chemical structures that are used as pheromones, some common themes in function and biosynthetic pathways have emerged across studies of diverse taxa. This review will offer a general overview of insect lipid pheromone function and biochemical synthesis, describe analytical methods for pheromone discovery, and provide perspectives on the contribution of chemical ecology to pest control and understanding evolutionary processes.

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Abbreviations: CHC, cuticular hydrocarbon; QMP, queen mandibular pheromone; DART, direct analysis in real time; ESI, electrospray ionization; GC–MS, gas chromatography–mass spectrometry; GC EAD, gas chromatography electroantennogram detection; LDI, laser desorption ionization; NMR, nuclear magnetic resonance.

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Trivial and systematic pheromone names

Blattellaquinone: (3,6-dioxocyclohexa-1,4-dien-1-yl)methyl-3-methylbutanoate
Bombykol: (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol
CH503: (3*R*,11*Z*,19*Z*)-3-acetoxy-11,19-octacosadien-1-ol
cis-Vaccenyl Acetate (cVA): (11*Z*)-octadec-11-en-1-yl acetate
Crematoenone: (*E*)-1-((1R*,2R*,4aS*,8aR*)-2-(hept-6-enyl)-1,2,4a,5,6,7,8,8a-octahydro-naphthalene-1-yl)-but-2-en-1-one
Disparlure: (7*RS*, 8*SR*)-7,8-epoxy-2-methyloctadecane
Exo-brevicomin: (1*R*,5*S*,7*R*)-7-ethyl-5-methyl-6,8-dioxabicylo[3.2.1]octane
(*E*)-β-farnesene: (6*E*)-7,11-dimethyl-3-methylenedodeca-1,6,10-triene
Frontalin: (1*S*, 5*R*)-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane
HDA (QMP component): (2*E*, 9*RS*)-9-hydroxy-2-decenoic acid
HOB (QMP component): methyl 4-hydroxybenzoate
HVA (homovanillyl alcohol, QMP component): 4-(2-hydroxyethyl)-2-methoxyphenol
Ipsdienol: (4*S*)-2-methyl-6-methyleneocta-2,7-dien-4-ol
Ipsenol: (4*S*)-2-methyl-6-methylene-7-octen-4-ol
Japonilure: (5*R*)-5-[(*Z*)-dec-1-enyl] oxolan-2-one
ODA (QMP component): (*E*)-9-oxodec-2-enoic acid
Sulcatol: 6-methylhept-5-en-2-ol

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1. Introduction

Pheromones are chemical signals used for communication between members of the same species. Some of the most important decisions made by organisms are mediated by pheromones. Many of these signals, particularly those produced by insects, are lipid molecules. Among the numerous roles that have been elucidated for pheromones include attraction, aggression, aphrodisiacs, anti-aphrodisiacs, aggregation, kin recognition, and alarm signaling. So pervasive are these molecules that a number of organisms mimic the chemical language of insects in order to lure prey or unwitting pollinators. For example, predatory bolas spiders emit the same sex attractant signals used by moths to ensnare the moths at close range by swinging a bola of silk [1]. In addition, some varieties of orchids both look and smell like different species of moths to attract pollinators [2]. Since the discovery of the first pheromone in 1959, the field of chemical ecology has rapidly progressed with the incorporation of methods from multiple scientific fields including analytical chemistry, neurophysiology, and genetics. This interdisciplinary approach has allowed our understanding of pheromone detection and behavior to be distilled down to the level of discrete neural circuits [3–5]. Additionally, discoveries in chemical ecology are now routinely applied to manipulate the behavior of agricultural pests and disease-bearing insects [6]. This review will offer a general overview and summarize recent findings on lipid pheromone function, structural diversity, biochemical synthesis, as well as the methods used for pheromone discovery. We will also provide perspective on the utility of pheromone biology in agricultural pest management and show examples of how comparative studies of pheromone systems have provided insights into the broad field of evolutionary biology.

2. Behavioral functions of pheromones

The term "pheromone" was originally proposed by Karlson and Lüscher in 1959 as "substances which are secreted to the outside by an individual and received by a second individual, in which they release a specific reaction". It is derived from two Greek words, *pherin* (to transfer) and *hormōn* (to excite) [7]. In the same year, Adolf Butenandt, a German biochemist who was awarded the

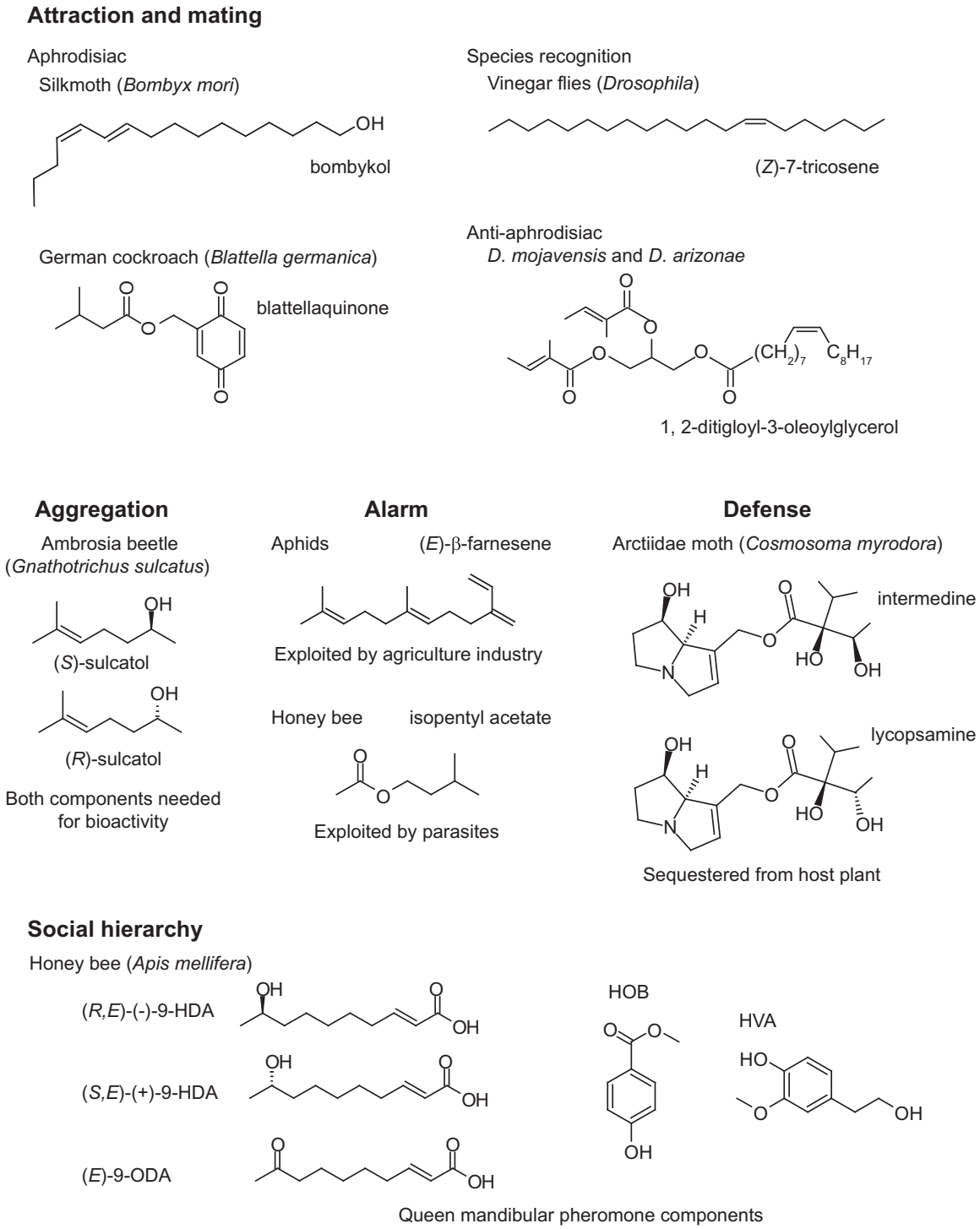

Fig. 1. Examples of insect pheromones and their function in social behaviors. A pheromone can be comprised of multiple components, as shown for sulcatol and the queen mandibular pheromone. HDA: hydroxyl-2-enoic acid; ODA: oxodec-2-enoic acid; HOB: methyl 4-hydroxybenzoate; HVA: homovanillyl alcohol.

Nobel Prize in Chemistry in 1939 (for the chemical synthesis of sex hormones), identified the first pheromone chemically [8]. Since then, numerous advances have been made in our understanding of the functional properties of pheromones.

2.1. Aphrodisiacs, attractants, and anti-aphrodisiacs

Pheromones play an important role in the reproductive behaviors of many insects. Chemical signals are used to recognize conspecifics (members of the same species), attract potential mates, indicate reproductive status, and advertise fitness. The first active pheromone was chemically identified in 1959 from the silkworm moth, *Bombyx mori* [8]. Named bombykol (Fig. 1), the attractant pheromone is emitted by females from a gland at the tip of the abdomen and advertises female availability and location. Remarkably, concentrations as low as 200 molecules/cm^3 (in the air) are capable of attracting males [9]. Initial characterization of bombykol required isolation from 500,000 female abdominal

glands. Following fractional distillation, a diluted portion of each fraction was tested for its ability to induce a "flutter dance" response in the male. Current analytical instrumentation requires much less starting material for chemical characterization. However, the pairing of fractionation with a behavioral assay remains a common strategy for the identification of new pheromones.

In many dipteran species (e.g., house flies, fruit flies, vinegar flies, and mosquitoes), long chain dienes and monoene hydrocarbons found on the cuticular surface (cuticular hydrocarbons, CHCs) serve as attractants and aphrodisiacs that influence mate choice and induce courtship [10,11]. The first CHC pheromone identified from a dipteran species was (*Z*)-9-tricosene from the housefly, *Musca domestica*. This compound is found in the feces and cuticle of females and attracts males [12]. Synthetic (*Z*)-9-tricosene is commonly used as a bait in commercially available housefly traps [13]. Some of the same CHC signals used in mate selection are also important in species recognition. For example, cross species experiments using *Drosophila simulans* and *Drosophila sechellia* show that *D. sechellia* females "perfumed" with *D. simulans* CHCs induce courtship from *D. simulans* males [14].

CHCs can act as anti-aphrodisiacs as well and play an important role in preventing interspecies attraction. Experiments by Billeter et al. elegantly showed that female *Drosophila* which have been genetically manipulated to express very low levels of CHCs become attractive to males of other species [15]. This atypical cross-attraction is partly attributed to the absence of CHCs which normally inhibit courtship from other species but function as aphrodisiacs within the same species [16]. Interestingly, the linear alkene (*Z*)-7-tricosene (Fig. 1) has been identified as one of the signals which prevents interspecies courtship and establishes a species barrier between *Drosophila melanogaster* and other drosophilids. When the sensory receptor for (*Z*)-7-tricosene is genetically ablated in *D. melanogaster*, male flies are willing to court females of other species, despite obvious disparities in size and pigmentation. In this case, the absence of inhibitory signals overrides all other sensory cues in the decision to court [17].

In several species of bees, butterflies, and vinegar flies, anti-aphrodisiacs and courtship inhibitors are used by males to manipulate the behavior of other conspecific males [18–20]. For example, in *D. melanogaster*, the male-specific lipids *cis*-Vaccenyl Acetate (cVA) and (3*R*,11*Z*,19*Z*)-3-acetoxy-11,19-octacosadien-1-ol (CH503) are transferred from males to females during mating and inhibit courtship from subsequent courting males [21–23]. In other species of *Drosophila*, a complex mixture of male-produced triacylglycerides play a similar role (Fig. 1) [24]. This strategy benefits both males and females since potential competitors are dissuaded from inseminating mated females and females also spend less energy fending off unwanted mates.

Other than serving as "on" or "off" switches for mating, attractive pheromones can also function as nuptial gifts and signals that convey information about the quality of the sender. For example, pyrrolizidine compounds, which are toxic substances for many animals, are used by Arctiid moths in some cases as attractants for females and also as defensive compounds [25,26]. Males ingest alkaloids such as intermedine and lycopsamine (Fig. 1) from host plants and pass the compounds to females through direct contact with pheromone-infused abdominal brushes [27] and seminal infusion [28]. Females are attracted to males with higher titers since the pheromone offers the benefit of protection from predators not only for the females but also for her eggs. In addition to direct benefits, age and fertility are two other types of information that are conveyed by cuticular lipid pheromones. Kuo et al. showed that male *D. melanogaster* prefer younger females to older females and this decision is largely discerned through distinct age-related cuticular lipid profiles [29]. Similarly, females of the butterfly species *Bicyclus anynana*, also select mates on the basis of pheromone composition but prefer profiles correlated with mid-aged rather than younger males [30].

2.2. Kin recognition and aggression

Kin recognition for eusocial insects such as ants, wasps, bees, and termites is largely mediated by CHCs. The signals are qualitatively identical between colonies but quantitatively distinct [31–33]. Detection of foreign pheromone profiles produces aggressive behaviors such as increased antennal and mandibular movements. Studies of the paper wasp, *Polistes biglumis*, provide some of the most convincing evidence for the direct role of CHCs in kin recognition. Female *P. biglumis* wasps fail to recognize nestmates after a pentane wash of the individuals but regain recognition upon re-application of the extract [34]. As with courtship signals, the absence of an inhibitory signal disinhibits aggressive behavior. Aggression can also be elicited by disrupting the relative ratios of components in the endogenous CHC blend by artificially increasing quantities of a single component [31,35].

How are multi-component pheromone blends detected? Recent studies of carpenter ants (*Camponotus japonicus*) identified a population of sensory cells in the antennae that are specialized for the detection of non-nestmates. Ozaki et al. recorded increased electrical activity in antennal sensory neurons upon exposure to a glass rod coated with CHC extract from non-nestmates, but not from nestmates [36]. Interestingly, kin recognition is not necessarily innate – wasps learn to associate nestmate signals (a blend of linear and methyl-branched alkenes and alkanes) with their home nests during an early developmental stage [37–41]. Honeybees also acquire a "template" of the chemical profiles of nestmates, consisting of alkanes, alkenes, and possibly free fatty acids and hydrocarbons from the comb wax [42]. Since cuticular lipid profiles can be altered by diet, temperature, and physiological state, the template by which honeybee nestmates are recognized requires constant updating [43]. Thus, both endogenous signals as well as signals acquired from the environment are important for colony identification.

Kin recognition signals offer ample opportunities for exploitation by parasites. For example, the parasitic wasp *Polistes atrimandibularis* infiltrates the colony of *P. biglumis* by camouflaging itself with the hydrocarbons of the host colony. Despite initial aggression from the host queen upon first ingression, the parasitic queen wasp is able situate herself in a few hours by rapidly changing her normal alkene-rich profile to one containing more alkanes, a profile characteristic of the host species [44,45].

In *Drosophila*, males display heightened aggression in the presence of a limited resource such as access to mates or food [46,47]. Exposure to the volatile male lipids cVA (which also serves as an aphrodisiac for females) and (*Z*)-7-tricosene increases agonistic encounters between males [48,49]. Recent experiments combined genetic manipulation of *Drosophila* pheromones with behavioral analysis to examine the contribution of the pheromonal bouquet to the decision to mate or fight. Transgenic females that smell and taste like males but look female induced aggression from wild type flies [50]. In contrast, males that smell and taste like females elicited courtship from males. These results illustrate the primacy of smell and taste signals in sex recognition and behavioral decisions.

2.3. Social hierarchy

Eusocial insect societies are characterized by cooperative broodcare and a division of labor between a queen and a primarily sterile worker caste. Pheromones exuded by the queen maintain social hierarchy by advertising the fecundity of the queen and

suppressing reproduction in workers. A broad analysis of 64 different species of bumblebees, ants, and wasps showed that saturated linear and branched hydrocarbons are conserved as a chemical class of queen pheromones. Surprisingly, the same combination of linear alkanes (n-C_{27}, n-C_{29}, and 3-MeC_{29}) are capable of inhibiting ovary development in wasps, bumblebees, and ants, three independently evolved insect lineages. The results indicate that queen pheromones likely derived from common fertility signals used by an ancient ancestor of all social insects [51].

Amongst eusocial insects, the chemical ecology of honey bees (genus *Apis*) has been well-studied because of their agricultural importance. The pheromone blend of *Apis mellifera* consists of at least nine different compounds secreted from multiple glands and controls the reproductive behavior, social cohesion, and physiology of the colony inhabitants [52–54]. For instance, a collection of five synergistic components secreted from the queen mandible (collectively named the queen mandibular pheromone, QMP; Fig. 1) serves to attract workers to groom and feed the queen, encourage swarming behavior, and prevent reproduction of workers by inhibiting ovarian development. Removing the *A. mellifera* queen from a colony resulted in 5–24% of workers developing ovaries and potentially becoming fertile [55]. Levels of hormones [56], fat body lipids [57], and gene expression patterns in the brain [58] are also altered by queen pheromone activity. The chemical complexity of the queen pheromone provides functional redundancy and lowers the possibility of workers becoming desensitized to individual components.

2.4. Aggregation

As with attractants, aggregation pheromones attract members of the same species to the site of release; however, both sexes are affected. Beetles, mites, cockroaches, flies, wasps, bees, thrips, and locusts are all known to use aggregation pheromones [59]. These molecules, many of which are low molecular weight esters, acids, alcohols, and isoprenoids (Fig. 1), are usually secreted in a blend of odors and are paired with other attractive stimuli such as food or breeding substrates. Aggregation to a food source provides benefits such as promoting communal feeding [60–63], facilitating mate-finding [64], and offering protection from environmental conditions and predators [65–68]. Cooperative feeding is particularly important for bark beetles where multiple individuals are needed to overcome tree defense mechanisms such as toxins and sap [69]. *Drosophila* larvae use a similar strategy to promote communal feeding: (*Z*)-5-tetradecenoic acid and (*Z*)-7-tetradecenoic acid are deposited by *D. melanogaster* larvae and are used as attractants for other larvae, including those of other species [70]. Interestingly, *D. simulans* larvae do not produce these pheromones but are still attracted to these compounds, perhaps benefitting from chemical eavesdropping of a semiochemical produced by another species (known as a kairomone).

2.5. Alarm

Alarm pheromones can cause dispersal of conspecifics and predators or increase recruitment and aggression towards an antagonist [71]. Alarm pheromones tend to be less specialized than other kinds of pheromones and can serve as general communication cues capable of being received by other species. One of the best studied alarm pheromone is (*E*)-β-farnesene (Fig. 1), a sesquiterpene that is released by many aphid species upon predation and signals conspecifics to stop feeding and disperse [72]. (*E*)-β-farnesene has been targeted as a means to prevent aphids from feeding on plants [73]. A second well-characterized alarm pheromone is isopentyl acetate, a banana-like odor that is released by stressed honey bees (Fig. 1). Together with 2-heptanone and methyl benzoate, the chemicals promote worker recruitment and aggression [74,75]. Smoke, which is used by bee-keepers to calm hives, inhibits receptors involved in the detection of isopentyl acetate [76].

Natural enemies can eavesdrop on alarm pheromones of other species in order to find prey. Lady beetles [77], hover flies [78], parasitic wasps [79], and *Carabidae* ground beetles [80] exploit alarm pheromones as kairomones, chemical cues which benefit individuals of another species rather than the emitter. One of the most sophisticated and diabolical examples of this exploitation was recently proposed as a cause contributing to honey bee colony collapse disorder, a phenomenon in which worker bees abruptly disappear from colonies. Torto et al. described a multi-trophic manipulation whereby parasitic small hive beetles (*Aethina tumida*) are drawn to bee hives by bee alarm pheromones, including isopentyl acetate [81]. Interestingly, *Kodamaea ohmeri* yeast, which use the beetles as a vector, also produce isopentyl acetate when grown on pollen in hives. Mimicking of the bee alarm pheromones by yeast attracts even more beetles. Eventually, the hive is overrun with beetles and their larvae, causing the bees to abandon the hive.

3. Diversity of chemical structures

The complexity of social functions mediated by insect lipid pheromones is matched by the chemical diversity of both volatile and non-volatile signaling signals. The former are likely to be detected by olfaction whereas the latter are usually gustatory or contact signals. Studies of numerous taxa have identified pheromones from diverse chemical classes including hydrocarbons (linear or branched), fatty acetate esters, alcohols, acid, epoxides, and ketones, isoprenoids [82,83], and triacylglycerides [84–86]. More comprehensive descriptions and examples of chemical structures can be found in [87] and the on-line database Pherobase (http://www.pherobase.com/) [88].

Many insect pheromones are derived from the same pool of biochemical precursors such as fatty acids and isoprenes, thus allowing a diversity of chemical structures to be generated by slight modifications to a main backbone. Common changes include the addition of functional groups to form alcohols, aldehydes, acetates, epoxides, or ketones [89], incorporation of branched fatty acids or amino acids [90], or a change in stereochemistry [86,91]. In order for chemical communication to be effective, the signal needs to be detected amidst substantial chemical noise. To ensure specificity, insects have evolved highly specialized channels of communication, both on the sending as well as the receiving side. For some species, behavior is elicited only by a single stereoisomer. For other species, the ratios of components in chemical blends serve to identify not only species but distinct populations within the species such as colonies, kin groups, castes, or regional races. Here, we highlight a few examples of how chemical diversity contributes to functional specificity.

3.1. Double bond placement

Considerable variety in double bond position can be found amongst closely related species, and this placement is often crucial to the species specificity and function of a pheromone. For example, the green-headed leaf roller *Planotortrix excessana* uses (*Z*)-5-and (*Z*)-7-tetradecenyl acetate while its sister species *P. octo* uses (*Z*)-8- and (*Z*)-10-tetradecenyl acetate [92,93]. Further examples of double bond regioisomerism have been observed in numerous other leaf roller species pairs [94–96]. Sex pheromones of other lepidopteran species also encode specificity through complex blends of polyenes with between 2 and 4 sites of unsaturation [89]. The number, position, and modification of these bonds into

epoxides allows for combinatorial variation. In *D. melanogaster*, positional isomers are found for the female aphrodisiac heptacosadiene: cosmopolitan populations use (*Z*, *Z*)-7,11-heptacosadiene while African and Caribbean populations produce primarily (*Z*, *Z*)-5,9-heptacosadiene [97]. In each of these examples, subtle shifts in double bond position are able to impart functional specificity and to strongly influence mate discrimination.

3.2. Chirality

There are many examples illustrating the specificity of a single enantiomer and inactivity of its mirror image [91]. In leaf cutter ants, the (*S*)-isomer of the alarm pheromone 4-methyl-3-heptanone was shown to be 400 times more effective than the (*R*)-isomer [98]. For the gypsy moth, the (7*R*, 8*S*)-enantiomer of disparlure, the female sex pheromone, was 10^6 times more effective than the (7*S*, 8*R*) antipode [99]. Interestingly, the natural configuration of the pheromone is not necessarily the most bioactive. In some cases, one or more of the non-natural stereoisomers may be more active than the insect-produced stereoisomer. This phenomenon was observed for the German cockroach (*Blattella germanica*) where four synthesized stereoisomers of the sex pheromone blattellaquinone (Fig. 1) [100] elicited greater biological activity compared to the insect-produced stereoisomer [101,102]. A similar phenomenon was observed in *D. melanogaster* where the insect-produced version of the sex pheromone CH503 was functionally weaker compared to synthetic stereoisomers not found in nature [103,104]. From these unexpected results we can conclude that recapitulation of biological activity is not necessarily the most rigorous test for determining the natural configuration. Moreover, these examples illustrate the importance of determining the absolute structure of pheromone components when designing lures for traps. Lastly, it may be worthwhile to test non-natural stereoisomers of natural pheromones as potential lures.

3.3. Combinatorial complexity

Biological activity can also be encoded by a species-specific blend of compounds in a particular ratio. For example, exo-brevicomin, the aggregation pheromone of the Western pine beetle (*Dendroctonus brevicomis*), showed highest bioactivity when the (1*R*, 5*S*, 7*R*) version was combined with (1*S*, 5*R*)-frontalin, both of which are bicyclic acetals [105]. However, mixtures of the antipodes were significantly less effective. Similarly, sulcatol, the aggregation pheromone of the ambrosia beetle *Gnathotrichus sulcatus*, is most effective as a racemic mixture of (*R*) and (*S*)-sulcatol (Fig. 1). The pure enantiomers yielded little or no response. Notably, a 50:50 racemic mixture of the enantiomers elicited an even stronger aggregation effect than a 35:65 (*R*/*S*) combination, which is the ratio that is naturally produced [106].

In moths, a shift in the ratio of stereoisomers has been identified as one mechanism underlying the generation of new species. Regional races of the European corn borer moth *Ostrinia nubilalis* are distinguished by race-specific blends of the female sex pheromone (*E*)-11- and (*Z*)-11-tetradecenyl acetate. One race is characterized by a 98:2 *E*/*Z* molar mixture [107,108] while the other is distinguished by a 3:97 *E*/*Z* molar mixture [108,109]. Males exhibit strong attraction to the ratio expressed by females of their own pheromone race although both populations are genetically compatible and produce fertile offspring. Expectedly, hybrids express a 65:35 *E*/*Z* molar mixture of the pheromone [110]. Pheromone races have also been observed for the pine engraver beetles, *Ips pini* – an East Coast race uses a blend of 33:66 (*R*)-(−)- and (S)-(+)-ipsdienol whereas the West Coast race produces a 90:10 *R*/*S* ratio [111]. A combination of geographical separation and predatory pressure (from wasps and beetles that use the chemical signal to locate prey) is postulated to underlie this divergence [112,113].

Not all pheromone blends are synergistic or additive. For example, the (*R*)-enantiomer of japonilure, a sex pheromone released by female Japanese beetles *Popillia japonica*, is behaviorally antagonized by its antipode, (*S*)-japonilure. A blend of the (*R*)- and (*S*)-japonilure enantiomers is significantly less effective at attracting males to traps placed in the field [114]. It was later discovered that the (*S*)-japonilure antagonist is the sex pheromone for another beetle species, *Anomala osakana* (Osaka beetle) [115]. Notably, (*R*)-japonilure is the behavioral antagonist for *A. osakana*. This reciprocal system whereby each stereoisomer is inhibited by its antipode is thought to prevent males from pursuing females of the wrong species.

3.4. Length

Slight differences in pheromone length can also affect the specificity of the behavior it induces. In *D. melanogaster*, the 23 carbon monoene (*Z*)-7-tricosene has been shown to inhibit male–male-courtship and is necessary for male–male aggression but the slightly longer 25 carbon monoene, (*Z*)-7-pentacosene does not produce this effect [49].

3.5. Use of similar compounds in different taxa

Similar compounds are sometimes used in different ways in different taxa of insects. Higher levels of a methyl-branched CHC 2Me-C_{26} on *D. serrata* males increase mating success in this fly species [116]. In contrast, the same molecule together with another methyl-branched CHC, 2Me-C_{28}, functions as a sex pheromone produced by females of the long-horned beetle, *Mallodon dasystomus* [117]. Similarly, cVA is secreted by male *D. melanogaster* and used as an aphrodisiac for female flies. However, this compound is also produced in females of the cerambycid beetle *Ortholeptura valida* as a female-specific sex pheromone [118].

4. Biochemistry of pheromone biosynthesis

The synthesis of pheromones has been well-studied in numerous orders of insects and shares many similarities with the biochemical pathways known for lipid metabolism [119]. Precursors for pheromones are derived from three main sources: *de novo* synthesis, conversion of precursors provided by the host plant or substrate, and direct incorporation of molecules found from the host. The specificity of pheromone chemical structures is encoded by biosynthetic enzymes, some of which are found expressed in sites of pheromone synthesis such as glands and oenocytes, specialized epidermal cells. Characterizing the enzymes and precursors in the pathway has been a major research endeavor because of the potential application to pest control. In addition, genes encoding biochemical enzymes serve as useful markers for assessing how pheromone profiles evolve across evolutionary time (see Section 6). This section is divided into three parts. First, we briefly survey the different sites of pheromone synthesis. Second, we describe the different classes of species-specific enzymes that modify common precursors and produce specialized molecules. Third, we describe some of the new approaches used to identify pheromone biosynthesis enzymes.

4.1. Site of pheromone synthesis

Pheromones and components of pheromone blends are produced in a wide variety of organs located in species-specific

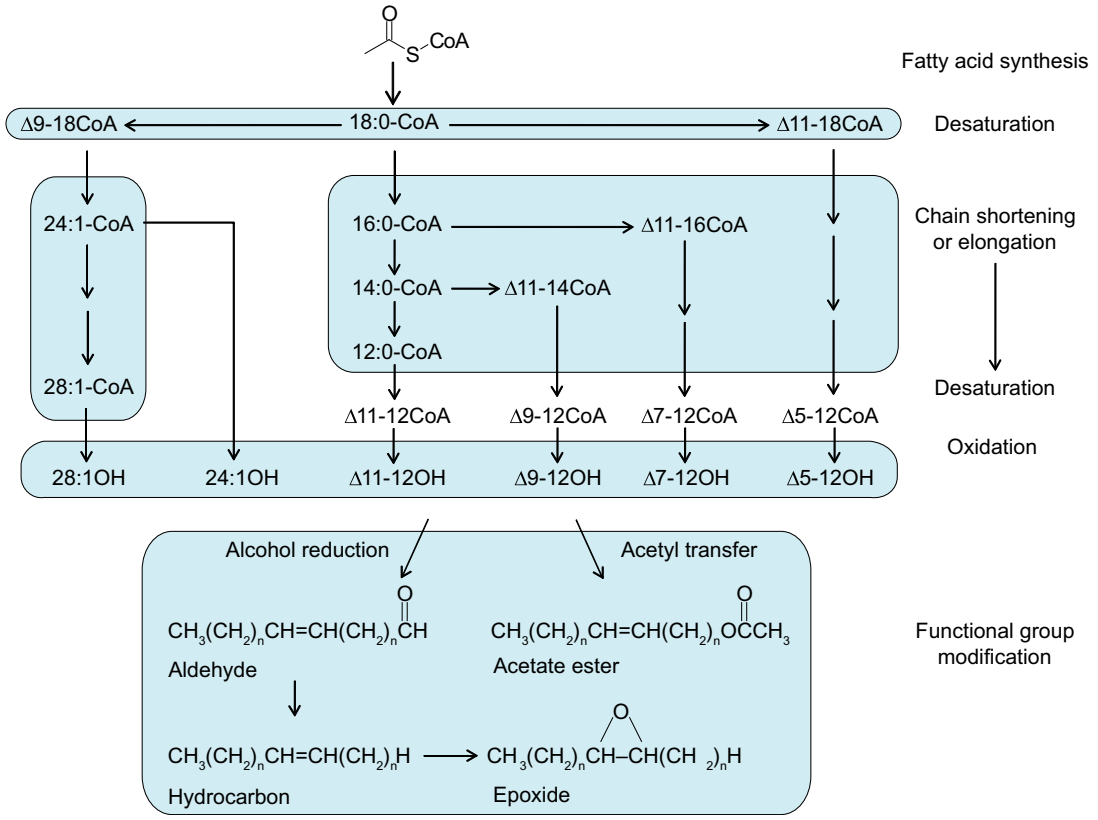

Fig. 2. Biochemical pathways underlying the synthesis of pheromones in Diptera and Lepidoptera. Houseflies and other dipteran fatty acyl CoA precursors undergo elongation whereas lepidopteran precursors are shortened. Desaturation can occur before or after shortening. Desaturases with different carbon length specificities generate molecules with varying double bond positions, using the same starting material. Functionalization enzymes also contribute to chemical diversity. Figure adapted from [179,240].

structures. Commonly found amongst many insects are specialized secretory cells known as oenocytes. The shape, size, distribution, and number of oenocytes varies significantly across species and can reside beneath the epidermis in clusters or be individually distributed across the fat body [120]. In addition to producing cuticular hydrocarbons, oenocytes are also needed for general lipid metabolism and storage [121]. Other tissues that produce pheromones are more specialized. For example, females of most moth species produce sex-pheromones in glands found within the terminal abdominal segments [122]. Male *Drosophila* produce sex pheromones in the ejaculatory bulb, a secretory site at the terminus of the anogenital region that is linked to the male reproductive organs [22,123]. Ants use multiple glands such as the Dufour, poison, pygidial and mandibular glands [124]. Remarkably, honey bee queens are thought to have at least 15 different pheromone glands [125].

4.2. Enzymes in pheromone biosynthesis

Fatty acids, originating both from *de novo* synthesis and dietary sources, are the basic building block for many pheromones. A majority of the enzymes involved in pheromone synthesis modify fatty acids. We highlight some of the enzymatic classes and provide examples of the known genes encoding these enzymes.

4.2.1. Fatty acid synthases

Many insects are capable of synthesizing fatty acids *de novo* from acetyl-CoA precursors (Fig. 2) via the activity of acetyl-CoA carboxylase and fatty acid synthase. It is generally assumed that the process of generating fatty acid precursors for pheromone product uses the same pathways for fatty acid synthesis in general metabolism. Experiments from Lepidoptera, houseflies, fruit flies, and cockroaches using labeled precursors have shown elongation of fatty acyl-CoAs to long chain fatty acids. For example, in the pheromone glands of moths, labeled acetate precursors are incorporated into common fatty acids with 16 and 18 carbon chain lengths [126–130].

Work by Blomquist and co-workers indicate that insects have two forms of fatty acid synthases, a cytosolic fatty acid synthase and a microsomal fatty acid synthase [131]. The former is used to synthesize linear hydrocarbons while the latter is needed for synthesizing branched hydrocarbons [132]. Branched CHCs require amino acids such as valine, methionine, and isoleucine as the initial biosynthetic precursor [133] and incorporation of methylmalonyl during elongation [133–135]. A second route of synthesis incorporates propionate, succinate, and butyrate and has been shown in beetles, houseflies [136], American cockroaches [137], some species of moths [138], and *Zootermopsis angusticollis* termites [139]. A probable candidate for the microsomal fatty acid synthase in *Drosophila* is *CG3524*/*mFAS*. Oenocyte-specific knockdown of *CG3524* gene expression in both *D. melanogaster* and *D. serrata* removed almost all methyl branched CHCs while leaving the other CHCs intact [116].

4.2.2. Desaturases

Desaturase enzymes add a double bond to fatty acid substrates and can be highly specific for different lengths or class of precursor. Genes encoding desaturases that play roles in pheromone synthesis have been identified in many insects. In Lepidoptera, a large family of acyl-CoA desaturases is known to modify saturated, singly, or double unsaturated fatty acyl intermediates (Fig. 2). In particular, Δ11 desaturases, which place a double bond at the C11 position, appear to play a major role in moth and butterfly species [140–145]. Numerous other enzymes have been identified for

placement of double bonds at other positions in saturated fatty acyl precursors [146,147]. Notably, differential expression of a Δ10-desaturase, *desat5*, has been suggested to underlie interspecific sex pheromone differences in moths belonging to the genera *Ctenpseustis* and *Planotortix* [148]. Several desaturases exhibit specificity for monounsaturated substrates [149–154]. Lastly, several desaturases in Lepidoptera are known to produce triene pheromone components, also using linoleic acid as a substrate [89,155].

In *Drosophila*, much work has been devoted to elucidating the function and conserved use of desaturases *desat1*, *desat2*, *desatF* because of their putative roles in contributing to reproductive isolation and species formation. *desat1* transforms palmitic and stearic acid to palmitoleic acid and oleic acids, both of which are known to serve as the precursors for mono- and di-unsaturated hydrocarbons 23–29 carbon atoms in length [156,157]. Mutations of *desat1* cause a large decrease in male and female unsaturated hydrocarbons and a concomitant increase in saturated hydrocarbons [156], illustrating that a single gene product is capable of dramatically shifting the overall hydrocarbon profile. The desaturase is expressed in the oenocytes and ejaculatory bulb, two major sites of pheromone production in *D. melanogaster*. Additionally, other splice isoforms are expressed in parts of the nervous system, abdomen, and fat bodies, indicating probable roles in behavior and basic metabolic processes [158]. Another desaturase, *desat2*, is specific for the substrate myristic acid (C14:0) [159]. A difference in the activity of *desat1* vs. *desat2* is hypothesized to contribute to distinct cuticular hydrocarbon profiles which distinguish cosmopolitan *Drosophila* species from those originating from sub-Saharan Africa [159,160]. Differences in cuticular profile between males and females are also derived from sex-specific desaturase expression. Sexually dimorphic CHC profiles in several *Drosophila* species can be attributed to expression of the *desatF* enzyme [161], which adds a second double bond to monounsaturated fatty acyl precursors [162–164].

We note that different groups of researchers use their own nomenclature and systems for naming desaturases. The *desat1* from the Albre et al. paper is not orthologous to the *desat1* gene in *Drosophila*.

4.2.3. Elongases

Fatty acid elongases are one class of enzyme that control the length of very long chain fatty acids. Elongation reactions are known to take place in American cockroach and the housefly *M. domestica* based on in vitro assays with microsomes isolated from integument tissue. In preparations from cockroach, stearyl-CoA was elongated up to 26 carbons while linoleoyl-CoA was elongated to 28 carbons [165]. The elongated products likely serve as the precursors to pentacosane and heptacosadiene, two of the major cockroach CHCs. In house flies, C18:1-CoA and C24:1-CoA were elongated up to 28 carbons using a similar preparation (Fig. 2) [166]. The genes encoding these elongases have yet to be identified in these species.

In *Drosophila*, only two elongases have been characterized although 17 others are predicted from the genome based on homology [119]. The female-specific elongase *eloF* shows higher activity in females than males, and is capable of elongating saturated and monounsaturated fatty acid substrates up to 30 carbons in length [167]. Knockdown of *eloF* expression results in a shortening of unsaturated dienes (from (*Z*, *Z*)-7,11-nonacosadiene to (Z, *Z*)-7,11-pentacosadiene) but not elimination of very long chain hydrocarbons, indicating the involvement of other elongases [168]. A second elongase, *elo68α*, is found in the male ejaculatory bulb and testes and is thought to contribute to elongation of male sex pheromone, cVA [169]. Based on heterologous yeast expression, this elongase uses myristoleic and palmitoleic acids as substrates, extending each by 2 carbons each. To achieve the final product, a reductase will likely be needed to remove the carbonyl as well as an acetyl transferase in order to form the acetate.

4.2.4. Reductases

Fatty acid reductases (FARs) convert fatty-acyl pheromone precursors to alcohol (Fig. 2). A large family of FARs has been identified for Lepidoptera and their substrate specificity is thought to underlie species-specific pheromone production [143,170]. Some FARs preferentially reduce either *Z* or *E* configuration acetates or acids, as found in different populations of European corn borer moth [171]. Other *Ostrina* species exhibit reductases with broader specificity [172]. Acetate esters can also be produced by acetyl transferases converting fatty alcohols to acetates [173–175]. Other functional group-related enzymes include aldehyde-producing oxidases. These have been identified from the pheromone glands of *H. zea* [176] and Manduca hawkmoths [177].

4.2.5. Cytochrome P450s

Cytochrome P450s form a large multi-gene family that catalyze an extremely diverse range of chemical reactions, although one of the most common reactions catalyzed by cytochromes P450 is a monooxygenase reaction [178]. In many species of insects, the final step of CHC synthesis requires the cleavage of long-chain aldehyde precursor to a hydrocarbon [179]. Recently, using a combination of genetics and biochemistry, the product of the cytochrome P450 gene *Cyp4g1*, was shown to catalyze the oxidative carbonylation step in pheromone-producing oenocytes of *Drosophila* [180]. Silencing expression of the *Cyp4g1* gene in the oenocytes dramatically reduced CHC levels. The Cyp4g1 enzyme subfamily is also conserved in honey bees and aphids and its innovation is thought to have allowed insects to adapt to non-water environments by providing a waxy, waterproof coating in the form of CHCs. Besides *Cyp4g1*, other cytochrome P450s have also been implicated in pheromone synthesis in houseflies [181] and Lepidoptera. For example, in the fall webworm *Hyphantria cunea*, the epoxidase *Cyp341b14* catalyzes the formation of the epoxy ring in two of the four sex pheromone components: *cis*-9,10-epoxy-(3*Z*,6*Z*)-3, 6-henicosadiene and *cis*-9,10-epoxy-(3*Z*,6*Z*)-1,3,6-henicosatriene [182].

4.2.6. HMG-CoA related enzymes

Isoprenoids (which are formed from 1 to 6 five-carbon isoprene units) are another common chemical class of pheromones used by species of bark and pine beetles. Many of the monoterpenoid components are produced *de novo* from acetate, mevalonate, and glucose or modified from host pine tree terpenes such as myrcene vapors [183]. Incorporation of injected radiolabeled acetate and mevalonate into pheromone products provided definitive evidence of *de novo* isoprenoid synthesis (reviewed in [184]). Two major regulatory enzymes contribute to the initial phase of monoterpenoid production: HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) synthase and HMG-CoA reductase. Three enzymes were already well-characterized in vertebrate systems as the major rate controlling enzymes involved in the mevalonate pathway and cholesterol synthesis. The corresponding homologues were identified from various species of bark and pine beetles using molecular biology to screen an expressed sequence tag library [185–187]. HMG-CoA reductase expression was subsequently localized to the midgut of males [188].

4.3. Strategies for identifying pheromone synthesis genes

Although numerous categories of fatty acid modification enzymes involved in pheromone synthesis have been identified, not all of the encoding genes have been annotated in the

ever-increasing number of sequenced insect genomes. The majority of such enzymes are likely to be used in the synthesis of fatty acid-derived compounds involved in other aspects of metabolism and physiology. However, several strategies can be used to identify the enzymes that are involved in the synthesis of pheromones. First, *in situ* hybridization is useful for visualizing cellular sites of gene expression in pheromone-producing tissues. Second, next generation sequencing (NGS) technology is routinely applied to describe the transcriptome of pheromone glands [189–191]. The combination of NGS with bioinformatic analysis of an increasing number of published genomes has allowed rapid identification of putative enzyme candidates in many "non-model" species. Ultimately, however, the function and *in vivo* expression of the candidate enzyme requires validation. Increasing use of methods such as RNA interference, transgenics and genome-editing tools will help in this regard [191–195].

5. Methods for pheromone discovery and detection

Chromatographic separation combined with mass spectrometry (MS) is the main analytical method used for the detection and structural elucidation of pheromones. The mass of the intact molecule (i.e., that of the molecular ion) provides a preliminary assignment of the elemental composition and often enables assignment of features such as the degree of saturation. Further structural information can be derived from the pattern of fragmentation upon dissociation of the molecular ions. Characteristic fragmentation patterns may be produced either within the initial ionization process [e.g., by using electron ionization (EI)] or by subjecting selected ions to tandem MS. Below we discuss the most commonly used MS and tandem methods for pheromone detection and characterization in addition to some recent innovations that have widened analytical possibilities, particularly with respect to polar and higher molecular weight pheromones.

5.1. Gas chromatography

Gas chromatography is the most widely used method for detection, quantification, and structural characterization of volatile pheromones. Extracts of cuticular lipids from whole body washes with solvent or volatile molecules collected from headspace of an enclosed volume are introduced into the system and column through injection or desorption, respectively. The compounds are separated based on their vapor pressure and affinity for the column stationary phase. Once eluted from the column, the compounds are commonly detected by flame ionization detection (FID) or by mass spectrometry. With FID, combustion of the compounds and ionization in a hydrogen flame produces a change in conductivity measured by detector electrodes. The change in electrical conductivity is proportional to the concentration of the analyte. The identity of the compound is based on known retention times (how quickly the compound arrives at the detector), previously determined using synthetic compounds.

Compounds eluted from the column can also be detected by MS following ionization by EI. EI typically produces intact molecular ions and distinct patterns of fragmentation which can be used to search against a database of spectra such as the Wiley Registry of Mass Spectral Data [196] and the National Institute of Standards and Technology database [197]. GC–MS analysis is particularly useful for differentiating between branched vs. linear alkanes since each class of molecule has a characteristic retention time index and produces distinct patterns of fragmentation. However, determination of structural features such as the position and stereochemistry of functional groups and double bonds often requires chemical derivatization prior to GC–MS analysis. As with FID analysis, the availability of synthetic standards is useful for structural confirmation. Synthetic versions of natural compounds are expected to exhibit identical chromatographic behavior and fragmentation patterns.

While commonly used for quantitation and structural characterization of volatile pheromones, GC analysis has several drawbacks. First, under the standard conditions used for the analysis of hydrocarbons, GC is limited to the detection of low molecular weight apolar lipids. However, the use of high temperature columns and different temperature and ionization parameters can allow heavier molecules such as triacylglycerides to be analyzed [198]. Second, the high amount of internal energy that is imparted onto the analyte during EI can cause rapid fragmentation of the radical parent ion thus making it difficult to identify the intact molecular mass. Recently introduced cold EI-based GC–MS provides an improvement in this regard by enhancing the detection of intact molecular ions through the use of lower elution temperatures [199]. Third, sample preparation can be time-consuming since it requires collection and extraction from multiple individuals. In addition, sample analysis requires, on average, between 30 and 45 min. Lastly, whole body elution obviates spatial information and prevents identification of specialized secretory sites such as glands.

To achieve spatially-resolved analysis with GC, solid phase microextraction fibers (SPME) have been combined with GC analysis to enable sampling from discrete anatomical regions. A thin fiber coated with an adsorbent material is used to adsorb molecules from headspace or directly from insect cuticles by physical contact. A number of stationary phase coatings are available depending on the polarity of the compounds of interest. Compounds are vaporized directly from the fiber after placement in a GC inlet. In addition to allowing for spatially resolved sampling, SPME has several other advantages in terms of sample preparation. For example, pheromone emission can be measured in real time from live insects [200], sample collection can be performed in the field, and there is less risk of extracting lipids from internal sources. In terms of quantitation, recent analysis of *D. melanogaster* CHCs showed that samples obtained from SPME exhibit the same quantitative proportions as samples prepared by extraction [201]. Despite these advantages, sampling by SPME has several drawbacks: first, samples collected on the fiber are completely used up by injection into the GC. Second, it is difficult to derivatize samples adsorbed on a SPME fiber. Third, different fiber compositions are selective for different chemical classes.

5.2. Direct analysis in real time mass spectrometry

Direct analysis in real time (DART) MS is a recently introduced ambient mass spectrometry method [202]. Ionization of analytes takes place under atmospheric pressure conditions. A high electrical potential is applied to a stream of helium gas, generating ions (which are filtered out for DART analysis) and excited state helium atoms (Fig. 3A). In one proposed ionization pathway, the excited helium atoms interact directly with water in the atmosphere, facilitating the formation of protonated water clusters. The charged water clusters readily transfer a proton to many types of analyte molecules, generating protonated molecules ($[M+H]^+$). Heating the gas stream further facilitates the desorption/ionization, process.

Several features of DART MS are advantageous for chemical ecology studies. First, samples can be placed directly into an open-air ion source and require minimal preparation. The source configuration accommodates a variety of sample shapes and sizes including intact insects, dissected body parts, SPME or other probes, and capillary tubes dipped into liquid extracts. For insect studies, intact individuals are handled with forceps and placed directly into the source, producing near-instantaneous measurements of the chemical profile. In terms of analytical capability,

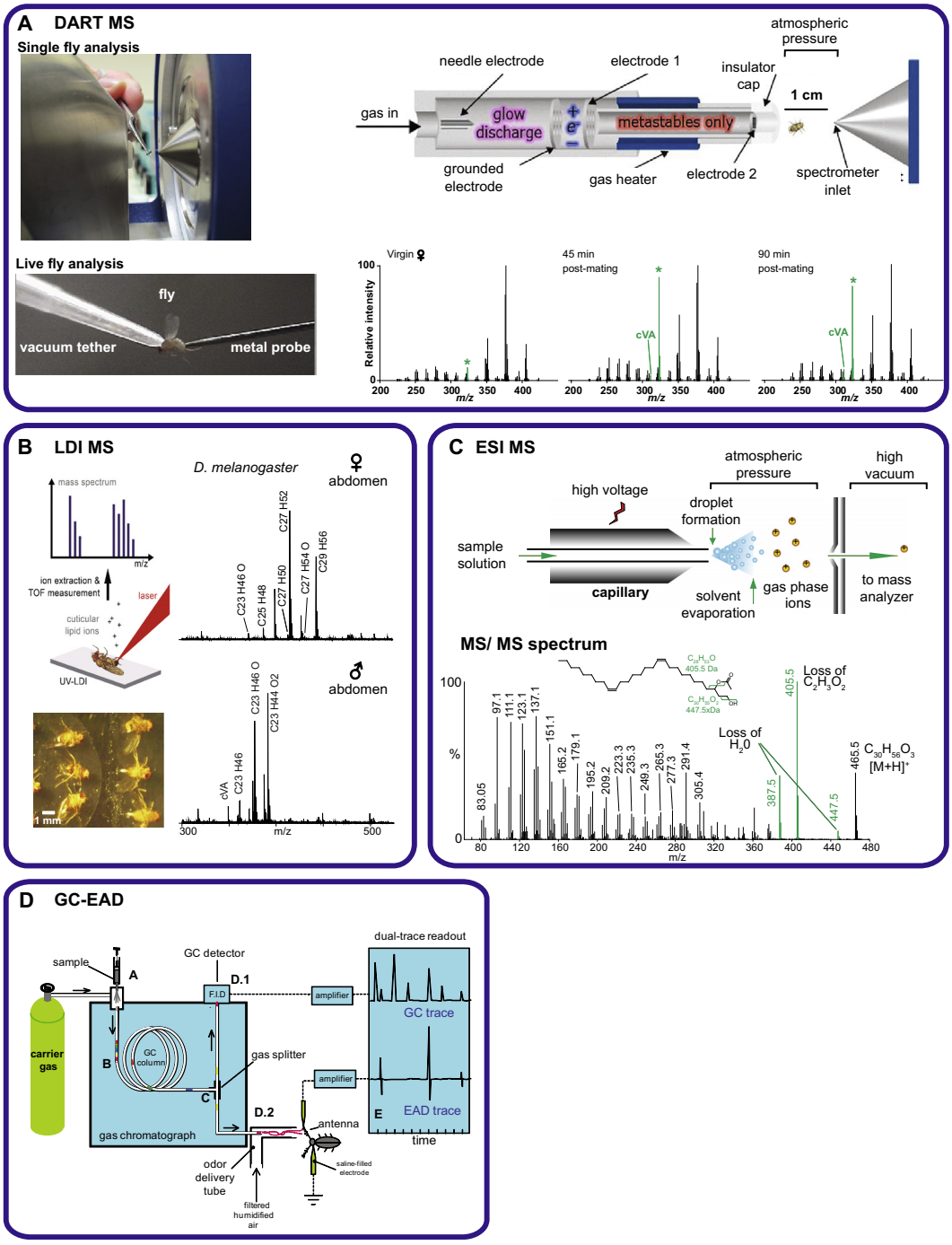

Fig. 3. Analytical methods for pheromone discovery and characterization. (A) DART MS ionizes small molecules directly from surfaces. An electric potential is applied to a gas (usually helium), generating a glow discharge containing metastable atoms. Metastable species desorb and ionize analytes directly from solid, liquid, or gas samples. The DART source schematic illustrates the ionization principle (reproduced from www.ionsense.com). For direct analysis of insect cuticles, single samples are placed directly in front of the source and the gas stream is heated to 250–300 °C. Live insect analysis is also possible by using a metal probe to sample the cuticular surface followed by analysis of the probe. Using this method, DART MS spectra revealed changes in cuticular profiles of the same fly before and after mating (green peaks; spectra adapted from [203]). (B) LDI MS probes the insect cuticular surface with a laser beam (diameter: ~100–200 μm), providing high spatial resolution profiles of single, intact insects, without the need for chemical matrix or sample extraction. Live *D. melanogaster* are shown mounted on the sample plate. Representative mass spectra from the cuticles of *Drosophila* abdomen reveal sex-specific profiles. The LDI image is reprinted with permission from Elsevier, ©2009 (see Acknowledgements). (C) For ESI MS analysis, sample solubilized in an organic solvent is loaded into a capillary. Application of high voltage to the liquid results in a fine mist of droplets at atmospheric pressure. As solvent evaporates and the droplets become smaller, analyte ions are liberated into the gas phase. Ions from a mixture can be selected by mass within the mass analyzer and subjected to fragmentation. The MS/MS spectrum shows collision-induced fragmentation of the *Drosophila* sex pheromone CH503 (intact molecular weight: 465.5 Da). Green peaks indicate loss of hydroxyl and acetyl functional groups. A series of signals in the lower molecular weight range differ by 14 mass units and are attributed to successive loss of methylene groups from the carbon backbone. (D) GC–EAD allows simultaneous measurement of antennal physiological activity and chemical identification by FID. Crude extract is separated on a GC column and the sample stream simultaneously shunted to a mass detector and to an insect electroantennogram preparation. Compounds that give positive electrical responses are possible chemical communication signals. Figure courtesy of B. Sullivan, U.S. Forest Service Southern Research Station.

DART MS allows detection of more polar and higher molecular weight molecules (including, e.g., triacylglycerides, long chain fatty alcohols, and sterols) that may be missed by GC–MS under standard conditions. Additionally, DART MS can be adapted for the analysis of pheromone profiles from live animals in parallel with behavior [203].

One major disadvantage of DART MS compared to GC–MS is that under standard conditions, saturated hydrocarbons are not detected. However, a method has been established for improved detection of these compounds in negative ion mode [204]. Another drawback is that absolute quantitation is complicated by two aspects. First, DART ionization can cause hydride extraction from saturated hydrocarbons, producing $[M-H]^+$ ions [205]. The mass (formally, mass to charge ratio, m/z) for the hydride-extracted hydrocarbons is indistinguishable from the $[M+H]^+$ signals of singly unsaturated hydrocarbons. Thus, the mass signal can be attributed to two different classes of hydrocarbons. Furthermore, signal intensity can vary depending on where the sample is placed in the source. Adding a standard to the sample surface or to the probe may help to provide an internal reference for relative quantification from solid objects. The use of a micromanipulator or standardized sample holder may also help with consistent sample placement.

5.3. Matrix-assisted laser desorption/ionization mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) allows analysis of molecules from a wide range of biomolecular classes. However, the method is most commonly applied to more polar compounds. A focused laser beam, typically with a UV wavelength of 337 or 355 nm and a diameter of 200 μm or less, is used to irradiate the liquid or solid sample. The sample is coated or co-crystallized with a chemical matrix which facilitates absorption of the laser energy and subsequent desorption and ionization. MALDI analysis is typically performed under vacuum conditions (either under high vacuum, $p < 10^{-6}$ mbar or intermediate pressure range, $p \sim 0.1$–2 mbar). One advantage of the method for lipid analysis is the improved capability for detecting more polar and higher molecular weight molecules. In addition, the ionization processes produce relatively few fragments and, in general, allow detection of intact molecular ions, thus facilitating the determination of elemental. These features were used to great effect when MALDI, in combination with a lithiated matrix, was applied to analyze cuticular extracts of termites, ants, cockroaches, and flesh flies [206]. Unexpectedly heavy apolar long-chain cuticular hydrocarbons, some containing more than 70 carbon atoms, were detected.

A variation of the method, laser desorption ionization (LDI) MS, omits the use of matrix. Insects or insect parts are placed directly into the instrument and analyzed with a focused laser beam under intermediate pressure conditions (1–2 mbar) in the source composition (Fig. 3B). Thermal processes are likely to contribute to the generation of ions and may be facilitated by endogenously-expressed chromophores in the cuticles which help to absorb the laser energy [207,208]. Ions are detected as metal-adduct ions, $[M+K]^+$ or $[M+Na]^+$. Alkenes, sugars, fatty acids, and higher molecular weight lipids such as triacylglycerides have been detected directly from intact insects [22,24,207]. Overall, MALDI and LDI MS are better suited for the analysis of non-volatile pheromones. As with DART MS, intact insects can be analyzed rapidly with minimal sample preparation. Under standard conditions, no visible damage is observed on the cuticles following laser interrogation.

One distinct advantage of LDI MS is the ability to provide controlled spatial profiling of specialized sites of pheromone production [24,209]. The lateral resolution, determined by the focal laser spot size, ranges from a few ten to a few hundred micrometers. This feature has been used to show site-specific changes in chemical profiles following behavioral interaction. For example, after *Drosophila* mating, male sex-pheromones were detected on female rear regions [22,85]. Similarly, male pheromones of the sepsid fly *Themira superba* were detected on the wings of females following transfer from males during mating [209]. Notably, the elevated pressure settings, which are an essential parameter for this method to work, are not currently possible with standard MALDI MS instruments and require customization of the ion source.

There are several drawbacks to using MALDI and LDI. First, as with DART MS, alkanes are not detected under standard conditions, except with the use of a matrix. In addition, since elemental composition determination is based on exact mass measurements alone, depending on the mass accuracy and resolution of the instrument, it may not be possible to distinguish between chemical species that are nearly isobaric with each other. Lastly, the analysis provides no information about stereochemistry. However, determination of double bond location and functional group position may be possible with derivatization prior to MS analysis, derivatization in gas phase during MS analysis, or by using tandem MS (see below).

The examples of DART and LDI MS described in this section have mostly involved the analysis of intact insects or crude extract. Minimizing sample preparation reduces the risk of sample loss, chemical modification (e.g., oxidation), and degradation. However, the chemical complexity of unfractionated samples hinders structural characterization of single molecules. Also, signals for less abundant compounds may be suppressed or be poorly resolved in the presence of more abundant molecules. To address the issue of chemical complexity, both DART and LDI MS can be paired with several off-line or on-line fractionation methods. For example, following separation of lipid extract by thin-layer chromatography (TLC) into broad chemical classes, extracts from the TLC plate or the plate itself can be analyzed with both MS methods [210,211]. Fractions resulting from liquid chromatography could similarly be prepared off-line prior to MS analysis.

5.4. Electrospray ionization mass spectrometry

Electrospray ionization (ESI) MS generates ions from liquid samples under atmospheric conditions. The sample, dissolved in solvent, is loaded into a fine glass or metal needle or capillary, which is placed in front of the MS inlet (Fig. 3C). Application of a high voltage leads to the formation of a fine mist of charged droplets. As the solvent evaporates, charge is retained by the analyte, generating ions which are then passed through the mass analyzer and to the detector (reviewed in [212]). ESI MS allows ionization of a broad range of biomolecules including apolar and polar lipids, peptides, proteins, and carbohydrates [213]. Furthermore, pairing liquid chromatography (LC) on-line with ESI MS can significantly enhance detection of low abundance molecules, increase analytical resolution, and, by using special chromatography columns, facilitate identification of stereoisomers [214]. For these reasons, ESI MS analysis serves as a powerful complement to GC–MS, particularly with respect to the analysis of non-volatile lipid pheromones. For example, a LC-ESI MS approach was used to identify putative nematode lipid pheromones from extract [215]. Despite these advantages and the availability of ESI MS in many core facilities, surprisingly few applications of ESI-MS for pheromone analysis are found in the literature.

5.5. On-line methods for structural elucidation

DART, LDI, and ESI can be coupled with a number of powerful on-line methods to improve structural elucidation. Tandem mass spectrometry (MS/MS) is a common feature of many commercial

MS instruments. Ions from a mixture are isolated in gas phase according to m/z by use of an ion selecting device (e.g., a quadrupole mass filter). The mass-selected ions are subjected to fragmentation by collision with an inert gas (e.g., argon). When applied to a broad array of neutral and polar lipid classes, this process allows some elucidation about functional group identity and position [216].

A recently developed on-line method named Ozone-induced dissociation (OzID) facilitates the determination of double bond position by exposing mass-selected ions to ozone vapor within the mass spectrometer. Ozone rapidly converts carbon-carbon double bonds to ozonides. Cleavage at the double bond site produces fragments ions with masses that are indicative of double bond position [217,218]. For example, OzID, was used to identify isobaric species and determining fatty acyl components found in *Drosophila* triacylglycerides pheromones [24].

Ion mobility spectrometry (IMS) is a third method of on-line chromatography that can be coupled with MS. Molecules in the gas phase are dragged by an electrical field through a drift tube containing a buffer gas. The transition time of the ions through the tube differs according to size, charge, and shape. IM is most effective for separating chemical classes of molecules (e.g., different phospholipid classes [219]). Since the ion mobility separation is de-coupled from the mass analysis, IMS can potentially be used to separate isobaric molecules if their collisional cross section (i.e., their shape) differs. For example, Dwivedi et al. successfully separated enantiomers of amino acids and sugars from racemic mixtures [220]. IMS MS has yet to be used for pheromone analysis.

5.6. Gas chromatography coupled to electroantennogram detection

One of the most useful tools for simultaneous chemical identification and assessment of functional activity is gas chromatography coupled to electroantennogram detection (GC–EAD). Chromatographic separation of a crude extract is divided into two different detectors: some of the effluent from the GC column is conveyed to a flame ionization detector while the other portion is blown over a biological sensor in the form of an insect (Fig. 3D). Electrodes inserted into the antennae or tarsi (legs) measure electrical activity from groups of sensory neurons. The method has been extremely useful for the identification of pheromones emitted from many different insect and arthropod species as well as volatile odors from plants that are attractive to insects [221–224]. For example, the alkyl aldehyde nonanal was identified as an attractive odor used by mosquitoes to find humans and birds [225]. One advantage of GC–EAD is that the method is sensitive to very low amounts (pg and lower) of ligand. This feature allows minor components of pheromone blends to be more readily identified. The EAD response is also quantitative: for each individual component, the EAD response is quantitatively scaled to the amount of the molecule detected. Differential physiological responses to different components of a pheromone blend can also be distinguished, providing information on which components are more likely to be functionally important. A more refined version of this system couples GC separation and detection to single sensillum recording. Electrical recordings are measured from single sensory cells rather than the whole antennae (which are comprised of many sensilla) allowing for functional mapping of individual neurons and their corresponding neural projections. This method has been used to spectacular effect to identify single classes of *Drosophila* olfactory receptor neurons that respond to ecologically-relevant odors [226,227].

5.7. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has long been considered the gold standard for absolute structural elucidation. However, several hundred micrograms to milligram amounts of pure material is needed for analysis, a requisite that can be challenging for low abundance molecules isolated from natural products. Recent developments in detection sensitivity and methods for spectral interpretation have allowed NMR to be used as a screening tool for identifying signaling molecules from complex mixtures (reviewed in [228]. By using a reduced-volume NMR probe, Dossey et al. were able to analyze defensive secretions from a single insect [229]. In addition, differential analyses of 2D NMR spectra allow spectra acquired from two different conditions to be compared. This strategy was used successfully to identify attractant pheromones from *Caenorhabditis elegans* by comparing active to inactive fractions [230]. One advantage of NMR compared to MS is that there is little bias in the types of analytes that are detected. Mass spectrometry ionization and sample preparation conditions can have considerable influence on the types of analytes that are detected. However, the greater analytical range provided by NMR also results in greater spectral complexity. Currently, the manual interpretation of NMR spectra is time-consuming and difficult. Improved computational methods that provide automated analysis will be necessary for effective analysis of complex biological mixtures.

6. Beyond behavior: applications to pest management and evolutionary biology

The impact of discoveries in chemical ecology has been far-reaching, providing direct applications to agricultural and conservation science as well as extending our understanding of how phenotypic traits diversify and how new species are formed.

6.1. Pest management

One major promise of chemical ecology is that the identification of signaling molecules and their function will allow us to better manage agricultural pests and disease vectors. Compared to the use of general pesticides which can be toxic to many animals, pheromone-based pest control is usually non-toxic and allows manipulation of behavior in a species specific-manner [231]. Three main strategies are used: mating disruption, mass trapping, and attract and kill. Mating disruption operates by permeating synthetic sex pheromones over a wide area, in this way interfering with chemical communication necessary for mate finding and female fertilization. Effective disruption depends on using data from field behavioral studies to optimize parameters such as the timing of chemical release, rate of release, chemical blend, and concentration. Mass trapping relies on attracting targets to a trap using a lure (e.g., attractants, aggregation signals, and oviposition signals) for one or both sexes. The attract and kill approach pairs an attractant with an insecticide thus killing insects by providing contact with a toxic bait. A lower concentration of pesticide is needed compared to an insecticide-alone approach. This strategy has led to the successful control of cotton boll weevils, houseflies, fruit flies, olive flies, and orchard moths and has helped to reduce the destruction of vineyards, orchards, and forests [6]. Traps that use a mixture of pheromone blends to attract more than one species of insects are also being developed [232,233].

One innovative approach for pheromone-mediated pest control uses genetic engineering to produce plants that are capable of emitting insect pheromones. Introduction of a terpene synthase gene into *Arabidopsis* resulted in production of (*E*)-β-farnenese, the aphid alarm pheromone [79]. Expression of this compound is intended to simultaneously repel aphids and attract the aphid parasitoid *Diaeretiella rapae*. Wheat plants bearing the terpene synthase gene are currently being field tested [234].

6.2. Pheromones as biomarkers of species and age

The cataloguing of insect chemical diversity can also be applied to conservation science and taxonomy. Correct species identification is crucial for surveys of ecological diversity and assessing the efficacy of pest control. Chemical profiling of cuticular lipids could provide a rapid, inexpensive way to identify species, especially in the case of cryptic species which are indistinguishable based on morphology alone. Notably, cuticular lipid profiles have proven to be useful for identification of a number of cryptic species of ants, butterflies, termites, and beetles [235–238]. Other large scale chemical surveys comparing hundreds of species of small ermine moths [239], bark beetles [240,241] and ants [242] have shown that pheromone blends exhibit distinct quantitative and qualitative differences between closely-related species.

In addition to species identification, lipid profiling can be helpful for age-grading, an important indicator of daily survival rates which is used to evaluate effective pest control methods. Several studies have shown that cuticular lipids vary with age and can thus be used to predict age [29,243,244]. While this approach has been validated for lab-raised mosquitoes, it remains to be tested on field mosquitoes [244]. The disadvantage of cuticular lipid profiling is that significant variation can be introduced due to environmental conditions (e.g., humidity, temperature), social conditions, and diet [245]. Moreover, accurate age-assessment could be difficult if the changes consist of subtle quantitative differences, requiring large samples sizes in order to establish statistical reliability. Nevertheless, cuticular lipid profiling offers a complementary approach to other molecular methods and morphological indicators used for age-grading and species determination.

6.3. Contribution of chemical ecology to evolutionary biology

One of the most fundamental and fascinating questions in biology is understanding how new species are formed. First hypothesized by Charles Darwin, the competition for mates is a driving force behind speciation [246,247]. One corollary of this theory is that female preferences for particular traits cause selection for males that express those traits in abundance [248–251]. Over successive generations, both the trait and the preference are stabilized. Since pheromones influence sex and species recognition in many systems, it stands to reason that a change in chemical signals could jumpstart speciation by significantly shifting mating preferences [252].

6.3.1. Evolution of chemical diversity in pheromone blends

Studies of how pheromone blends diverge across species and the underlying genetic mechanisms provide excellent opportunities for examining the formation of new species. In Lepidoptera, broad surveys of pheromone profiles have been performed and mapped to phylogeny [253–255]. Detailed transcriptomic data of pheromone glands are available for several species [256–259]. This depth of information has allowed differences in pheromone profiles between closely related species to be traced down to the level of single genes. For example, activation of a non-functional desaturase gene is thought to underlie the difference in double bond position found between two species of *Ostrinia*. Females of both *Ostrinia furnicalis* and *O. nubilalis* use tetradecenyl acetate as a sex pheromone. However, in *O. furnicalis*, the double bond is placed between C12 and C13 [260] whereas in *O. nubilalis*, the double bond is placed between C11 and C12 [261]. Within *O. nubilalis*, allelic variation in a fatty-acyl reductase *pg-FAR* gene underlies the distinct pheromone blends of the *E* and *Z* races (see Section 3.3). The differing ratios of the (*E*)-11- and (*Z*)-11-tetradecenyl acetate components are thought to be responsible for the strong reproductive isolation between the two races [171].

How does a shift in pheromone profile translate into a change in mating preference? Roelofs et al. hypothesized that attraction of a few rare males to the novel component would be sufficient. Offspring from such a pairing would likely inherit both the novel pheromone component as well as the behavioral preference to the component, giving rise to a new species [146]. However, recent findings indicate that new pheromone compounds can arise in populations without receivers having a preference for the compounds [262]. In the wasp *Nasonia vitripennis*, a cluster of three putative short chained reductases produces a novel male pheromone component, 4(*R*),5(*R*)-5-hydroxy-4-decanodile, which is not found in four closely related wasp species. Notably, neither females of the species nor those of a related wasp species respond to the pheromone. The results indicate that pheromone components can arise spontaneously and persist in the absence of a receiver.

6.3.2. Ecological influences on pheromone evolution

Environmental factors such as diet [263], humidity [116,264], and temperature [265] can also induce a change in pheromone profile. Work by Etges et al. has shown that in cactophilic *Drosophila arizonae* and *Drosophila mojavensis*, cuticular hydrocarbon profiles shift due to host plant diet and that this shift mediates reproductive isolation between closely related species [266,267]. In many Dipteran species, cuticular hydrocarbons play a role in providing a waxy layer on the cuticle to prevent desiccation as well as acting as pheromones [268]. The two different roles makes cuticular hydrocarbons potential "dual traits" which could explain the mechanisms underlying ecological speciation [269]. These examples indicate that small shifts in chemistry whether due to environment or spontaneous mutation can lead to large behavioral changes and eventually, speciation events.

7. Conclusions and future directions

Chemical ecology has come a long way in the past five decades or so since bombykol was first identified. Thousands of individuals are no longer required for the purposes of structural identification. Single insect analysis by NMR and mass spectrometry has become more commonplace and the amount of information that one can obtain from a single specimen is startling. In addition, the biochemistry and regulation of pheromone production are so clearly delineated in some systems that plants can be engineered to emit insect pheromones with the proper stereochemistry in order to control the behavior of pests (and their predators). Rapid progress has been enabled by developments in related fields of lipid biochemistry since many of the same challenges are faced – namely, structural elucidation, separation of chiral compounds, detection of low abundance molecules within a complex matrix, and enantioselective synthesis. Coupling MS with methods of gas-phase separation such as ion mobility mass spectrometry is one exciting future refinement that will help in the detection and separation of isobaric molecules [270]. Additionally, imaging mass spectrometry could be used to produce chemical maps of insect tissue sections and potentially be useful for mapping pheromone production glands and biosynthetic precursors residing in the glands [271,272]. Moreover, the combination of bioinformatics and next generation sequencing transcriptomic approaches opens the door for decoding pheromone biochemistry in many species of insects. It may be the case that at long last, analytical capabilities are finally starting to have a chance of keeping up with the chemical complexity found in nature. Ultimately however, pheromones are defined by their behavioral function. Chemical information has to be related back to behavior – a science for which an "–Omics" is, unfortunately, not yet available. As we began to unravel the meaning of these chemical messages used by insects for communication,

the study of these small lipid molecules will contribute to the management of our global food security as well as provide important insights in the field of evolutionary biology.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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