

REVIEW

Genetic basis of chemical communication in eusocial insects

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Social behavior is one of the most fascinating and complex behaviors in humans and animals. A fundamental process of social behavior is communication among individuals. It relies on the capability of the nervous system to sense, process, and interpret various signals (e.g., pheromones) and respond with appropriate decisions and actions. Eusocial insects, including ants, some bees, some wasps, and termites, display intriguing cooperative social behavior. Recent advances in genetic and genomic studies have revealed key genes that are involved in pheromone synthesis, chemosensory perception, and physiological and behavioral responses to varied pheromones. In this review, we highlight the genes and pathways that regulate queen pheromone-mediated social communication, discuss the evolutionary changes in genetic systems, and outline prospects of functional studies in sociobiology.

Complex systems require the interaction of their subunits, which is achieved through various ways of information transfer. In an organism, cells communicate through signals, including autocrine and paracrine signals for local communication and hormones for long-distance communication. Communication is also important when individuals organize themselves in groups and operate like cells in an organism. Many eusocial insects such as ants, some bees, some wasps, and termites are organized in this way and are thus commonly viewed as superorganisms: The individuals form a social unit (colony) with a strong division of labor (Seeley 1989; Hölldobler and Wilson 1990, 2009). They display complex cooperative behaviors with the following common type of organization: Inside the nest, the queen is engaged in egg-laying, while her helper workers carry out all other tasks such as brood care, nest maintenance, and colony defense. Outside the nest, different workers forage, alone or together, and bring food back to share with their nestmates and brood (Hölldobler and Wilson 1990; Ross and Matthews 1991; Seeley 1995). Efficient cooperation in eusocial insect superorgan-

isms is based primarily on chemosensation and, to a lesser extent, on visual and tactile communication.

Chemosensation is ubiquitous in animals, as they regularly interact with their chemical-rich environments. Some chemicals mediate animal behaviors, and animals consequently benefit from the evolution of a chemosensory neural system to sense chemical cues and to process their encoded information. Chemical cues are any chemical feature of the world that can be used to extract relevant information (see Box 1; Wyatt 2014). Such chemical cues can evolve into pheromones, the chemical signals emitted by one individual and perceived by another individual of the same species, and induce a behavioral or physiological response (Karlson and Lüscher 1959). In the most common scenario of the sender-precursor hypothesis of pheromone evolution (see Box 1), a chemical cue is produced by the sender, which is linked to its physiological condition but not yet established for communication. This chemical cue is subsequently sensed by an organism (same or different species) via activation of its chemosensory receptor neurons, leading to behavioral and physiological changes in the receiver. If the receiver benefits from the information about the condition of the sender through the perception of the cue, the receiver's sensory system is selected for improved cue discrimination and information extraction (Fig. 1). If the sender benefits from the receiver's response, the previous cue becomes a signal and is now selected to transfer information more efficiently to the receiver. This initiates a positive feedback loop with stronger and clearer signaling by the sender (signal ritualization) and more refined perception and response by the receiver (Fig. 1; e.g., Bradbury and Vehrencamp 2011). Alternatively, pheromones can evolve through receiver sensory bias (see Box 1; Stöckl and Steiger 2017), but we do not differentiate between these two evolutionary pathways at the mechanistic level of genetic regulation in this review.

Pheromones are widely used by eusocial insects to coordinate the organization of their colonies. In general,

[**Keywords:** chemosensory system; communication; genetic regulation; pheromone; signal evolution; social behavior]

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.346965>. 120.

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Box 1. Terminology**Positive selection**

Positive selection or directional selection shifts trait evolution in one direction. It can, for example, be demonstrated when the rate of nonsynonymous substitutions divided by the rate of synonymous substitutions in a set of homologous protein-coding genes is >1 ($d_N/d_S > 1$).

Queen pheromone

A queen pheromone is released by reproductive individuals in a eusocial insect colony (usually queens) that primarily inhibits reproduction in brood-care workers. It can have various other behavioral effects such as attracting the attention of workers. A more differentiated evolutionary perspective separates fertility signals that are learned and can

also be expressed by workers from queen pheromones that induce innate responses (Smith and Liebig 2017).

Signals versus cues

A signal is a trait that evolves in a sender to provide information to a receiver in a way that induces a change in the behavior of the receiver for the benefit of both parties (Abbott et al. 2010). A cue can also convey information, but it has not been shaped by natural selection for that function. In other words, the difference between a signal and a cue is the function of it: A signal has evolved exclusively for the purpose of communication. A cue is produced for something else but is incidentally used to extract information (Wyatt 2014).

Sender-precursor hypothesis

The sender-precursor hypothesis describes coevolution of a signal produced by the sender and a response in the receiver such that both the sender and receiver benefit from the signal. The signal originates from a cue that is associated with a condition of the subsequent sender.

Receiver sensory bias

A receiver evolves its sensory system in a context irrelevant to the sender, while natural selection favors the cues from the sender to trigger this pre-existing response in the receiver. This can lead to the exploitation of the receiver by the sender or to the evolution of a signal that benefits both parties.

animals use a large diversity of pheromones in many contexts. Major pheromones of eusocial insects include queen pheromones, sex pheromones, alarm pheromones, and trail pheromones with different roles (Hölldobler and Wilson 1990; Wyatt 2014; Leonhardt et al. 2016). Pheromone quality, quantity, and release rate may change in response to environmental context. Pheromone production may vary due to external factors, such as the absence of pheromones from competitors, as well as multiple internal factors, such as the sender's genetics, age, sex, hormonal state, or previous experience (Wyatt 2014). A better understanding of pheromone evolution requires an understanding of the mechanisms of communication at the physiological and molecular level.

In this article, we review our current understanding of the genes involved in signal evolution in the context of queen pheromones in eusocial insects. Queen pheromones play a central role in the organization of insect societies; e.g., by mediating reproduction. We divide the process of chemical communication into three parts: pheromone production in the sender, pheromone perception in the receiver, and pheromone-induced neural, behavioral, and physiological responses in the receiver (Fig. 1). We discuss recent progress in studying the genetic basis of communication.

Pheromone production

In insects, pheromone production and structure are highly variable. Pheromones are normally synthesized in a variety of glands located in different parts of the insect body, with ducts to the outside. For example, 149 exocrine glands have been identified across eusocial insects, although not all of them are involved in pheromone production (Billen and Šobotník 2015). In addition, cuticular hydrocarbon pheromones are synthesized in oenocytes

(a group of cells often located in the abdomen close to the cuticle) and secreted through the cuticle to the surface of the insect body (Lockey 1988). Because pheromones evolved from a wide range of chemical cues, the rich chemical diversity of insect pheromones, including those

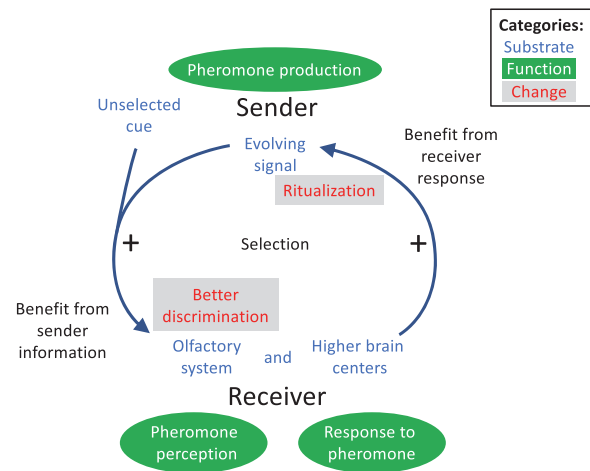


Figure 1. Sender-precursor model for the evolution of pheromones. An unselected cue that is secreted by a sender and is associated with a condition of the sender is sensed by a receiver through its olfactory system. If the receiver benefits from the information about the sender's condition, the receiver's olfactory system and higher brain centers are selected for better discrimination with associated changes in physiology and behavior. Conversely, if the sender benefits from the receiver's response, the cue is now under selection and becomes a chemical signal (pheromone) used for communication. This leads to a positive feedback loop with selection for a stronger and clearer signal in the sender (ritualization) and better discrimination by the receiver until costs of further modifications outweigh the benefits of signal ritualization and/or receiver adaptations.

of eusocial insects, is not surprising (Hölldobler and Wilson 1990; Vander Meer et al. 1998; Bordereau and Pasteels 2011). Any molecule, as long as it can be produced by senders and perceived by the chemosensory, primarily olfactory, system in receivers may potentially evolve into a pheromone.

A pheromone may also evolve to have multiple functions. This can be seen in the queen pheromones of eusocial insects. The main function of queen pheromones is the inhibition of worker reproduction in a colony by signaling the presence and fertility of the residing queen (Seeley 1985; Keller and Nonacs 1993). However, the pheromone can additionally help the queen, for example, allowing workers to recognize reproductive individuals (queen and king in termites), to induce workers to attend to her/them in a “royal court” (Slessor et al. 1988; Funaro et al. 2018), or induce subordinate behavior in nonreproductive workers (Smith et al. 2012b). Workers may also use queen pheromones to differentiate between queen-laid and worker-laid eggs in a colony (Endler et al. 2004; Oi et al. 2015) or use fertility signals to identify “cheaters”; i.e., workers that activate ovaries in the presence of the queen (Smith et al. 2009). Queen pheromones can also be involved in the suppression of the rearing of new queens and/or males (Vargo and Fletcher 1986; Winston et al. 1990; Oliveira et al. 2020) or suppression of reproductive competitors (Monnin et al. 2002; Smith et al. 2012a).

Although the main function of a pheromone might be the same across species, the chemicals used as pheromones can be quite different. The well-studied cases of queen pheromones in honeybees and in other species are good examples. The key insight into the role of queen pheromones in eusocial insects started with the identification of the first queen pheromone from the honeybee *Apis mellifera*, with its main compounds 9-oxo-2-decenoic acid (9-ODA) and 9-hydroxy-2-decenoic acid (9-HDA), which constitute the queen mandibular pheromone (QMP) synthesized in the mandibular gland in the honeybee that functions in inhibiting worker reproduction (Butler 1957; Winston and Slessor 1992; Hoover et al. 2003). QMP also acts as an attractant for workers, as well as a sex pheromone to attract males during mating flights (Plettner et al. 1996). However, the use of QMP is not conserved in other bee species. In the sweat bee *Lasioglossum malachurum*, for example, macrocyclic lactones act as a queen pheromone (Steitz and Ayasse 2020), while data from stingless bees and bumble bees suggest that cuticular hydrocarbon (CHC) profiles or CHC components function as queen pheromones (Nunes et al. 2014; Van Oystaeyen et al. 2014; but see, e.g., Amsalem et al. 2017; Melgarejo et al. 2018 for different results in bumble bees).

Cuticular hydrocarbons are ubiquitously produced in insects to prevent desiccation and infections (Howard and Blomquist 2005). In some groups of solitary insects, CHCs are used for mate recognition and as sex pheromones (see examples in Blomquist and Bagnerès 2010). Across eusocial insects, CHCs show queen-specific patterns that suggest their broad use as queen pheromones (Monnin 2006; Peeters and Liebig 2009; Liebig 2010; Van

Oystaeyen et al. 2014). Indeed, CHCs are known to function as queen pheromones in several ant and at least one wasp species (Endler et al. 2004; Smith et al. 2009, 2012b, 2016; Holman et al. 2010; Van Oystaeyen et al. 2014). In addition, a combination of CHCs with other compounds, such as tetrahydrofurans, are used as queen pheromone in *Odontomachus* ants (Smith et al. 2016), while the monocyclic diterpene neocembrene has a queen pheromone function in Pharaoh ants (Oliveira et al. 2020). In the subterranean termites *Reticulitermes flavipes* and *R. speratus*, the CHC heneicosane and a mixture of butylbutyrate and 2-methyl-1butanol are used as queen pheromone (Matsuura et al. 2010; Funaro et al. 2018). Although it has been suggested that the use of CHCs as queen pheromones in eusocial insects is conserved (Van Oystaeyen et al. 2014), the prevalence of CHCs as queen pheromones might be due to the omnipresence of CHCs and corresponding olfactory receptors in insects. This in turn might make the CHC queen pheromones in different eusocial insect lineages a case of parallel evolution (Smith and Liebig 2017; Bolnick et al. 2018), which does not, however, exclude the evolution of other chemicals as queen pheromones (Steitz and Ayasse 2020).

We next explore pheromone production in two examples: the queen mandibular pheromone in honeybees and CHCs in some other species, because in both cases their biosynthesis and function have been widely studied at the genetic level.

Queen pheromones and their biosynthesis in honeybees

QMP in honeybee queens mainly contains 9-ODA and 9-HDA, while workers synthesize 10-hydroxy-2-decenoic acid (10-HDA) (Plettner et al. 1996). Both castes have the biosynthetic machinery to produce all these compounds but differ in the selectivity in the production of one group of pheromones over another. The common precursor, the 18-carbon stearic acid (or stearyl-CoA), is synthesized by fatty acid synthase (FAS) (Fig. 2) and other enzymes, such as carboxylases, transacylases, and elongases (for review, see Wakil et al. 1983). The pathways then bifurcate by adding a hydroxyl group to different positions on the hydrocarbon chain: the penultimate carbon in queens versus the terminal carbon in workers, respectively. These steps are probably catalyzed by enzymes that belong to the cytochrome P450 (CYP) family (Plettner et al. 1998). This step is followed by β -oxidation to shorten the hydrocarbon from an 18- to a 10-carbon chain and completed by hydroxyl group oxidation to generate 9-HDA and 9-ODA in queens and 10-HDA in workers (Fig. 2).

Cytochrome P450 (CYP) enzymes, also called monooxygenases, catalyze reactions that add an oxygen atom to substrates, thereby hydroxylating the substrates. CYP proteins are involved in multiple biological processes, including drug and pesticide detoxification, fatty acid metabolism, and synthesis of important insect hormones, such as 20-hydroxyecdysone and juvenile hormone (JH) (Feyereisen 1999), which regulate insect growth, development, and reproduction. In the bifurcated pathways to generate queen pheromone 9-HDA/ODA and the worker-

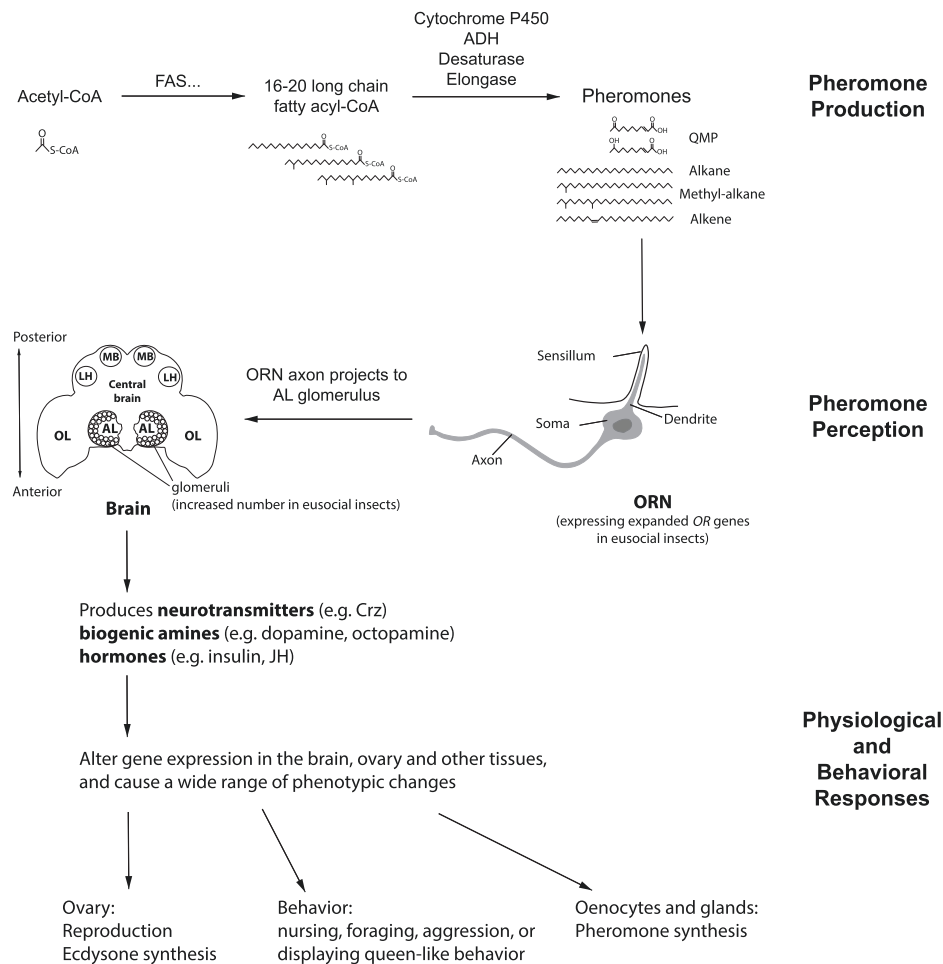


Figure 2. Pheromone production, perception, and its induced responses. (*Top panel*) The precursors of sender's QMP and CHC pheromones, normally 16- to 20-carbon fatty acyl-CoA (or fatty acid), are synthesized from acetyl-CoA, the multistep reactions catalyzed by fatty acid synthase (FAS) and other enzymes. QMP synthesis is catalyzed by enzymes such as cytochrome P450 mono-oxygenases and alcohol dehydrogenases (ADHs), while CHC synthesis is catalyzed by cytochrome P450, elongases, and desaturases. The most common forms of CHC pheromones are alkanes (saturated) and methyl-alkanes, and alkenes (unsaturated). (*Middle panel*) Pheromones are sensed primarily by the receiver's odorant receptors (ORs) expressed in peripheral neurons (ORNs), whose dendrites are located in the sensillum, and axons project to glomeruli in the antennal lobe (AL). The OR genes and number of glomeruli have been expanded in the evolution of hymenopteran insects, notably in those organized in societies. The signal is further processed in the mushroom bodies (MB), the lateral horn (LH), and the central brain. (OL) Optic lobe. (*Bottom panel*) The altered neuronal activity in the brain may cause the secretion of neurotransmitters, biogenic amines, and hormones, leading to systemic changes in physiology (e.g., reproduction and pheromone synthesis) and behavior in the receiver.

specific 10-HDA, it is likely that two groups of CYP proteins catalyze the hydroxylation reactions at different carbon positions on stearic acid (Plettner et al. 1996). There are ~50–100 CYP genes in most hymenopteran eusocial insect species (Nelson 2018). The functions of CYP proteins in pheromone synthesis are likely highly specific. Although it is not yet clear which CYPs are involved in QMP synthesis, some candidate genes have been identified among 17 CYP genes that are differentially expressed between queens and workers (Malka et al. 2014).

Differential expression of CYP genes may represent the first step in the evolution of QMP, because it creates the queen- versus worker-specific pathways that give rise to two distinct end products, linking to their differential fer-

tility status in the colony. Based on the theory of signal evolution, when a receiver responds to a cue against background noise, natural selection would be expected to reinforce the signal by fine-tuning the biosynthetic process (Fig. 1). Indeed, alcohol dehydrogenases (ADHs) that catalyze the last step only in the queen pheromone synthesis are up-regulated in the queen mandibular gland, thereby strengthening the signal produced by queens (Malka et al. 2014).

Cuticular hydrocarbons as pheromones

CHCs serve as queen pheromones in many eusocial species. In addition, CHCs are broadly used as cues for

recognition of colony members, which is based on heritable variation in CHC production between colonies (e.g., van Zweden and D'Ettorre 2010; Walsh et al. 2020). The widespread use of CHCs as queen pheromones or fertility signals in eusocial insects might be due to their ubiquitous presence on the insect cuticle, the ability of insects to perceive hydrocarbons, and possible links between hydrocarbon biosynthesis and a physiological condition. In *H. saltator*, for example, CHC profiles in workers are characterized by short chain hydrocarbons, while in reproductive workers the whole CHC profile is shifted to longer chain hydrocarbons (Liebig et al. 2000). When workers activate their ovaries, this shift is initiated and when ovaries become inactive again, their CHC profile reverts back (Liebig et al. 2000). Besides ovarian activity, task, age, mating status, and social stress are other factors that can lead to CHC variation (Sprenger and Menzel 2020). This sensitivity of the CHC profile to various intrinsic factors makes CHCs excellent precursors for a pheromone because the cue linked to a sender's physiological condition and a receiver's ability to recognize that cue are already present, which matches the core hypothesis of the sender-precursor model of pheromone evolution (Fig. 1). Thus, the mechanism of CHC biosynthesis is an important piece in understanding the evolution of CHCs as pheromones.

CHCs vary substantially, differing primarily in chain length, the position of double bonds and the position of methyl-groups in the carbon chain (Martin and Drijfhout 2009). CHC chain lengths are typically in the range of 25 to 39 carbons in eusocial insects, but lengths of 18–45 carbons have been observed. This range is limited by the increased volatility of shorter chains and the higher viscosity or even crystallinity of larger compounds, although carbon chain modifications also contribute to differences in both their volatility and viscosity (Gibbs 2002). Within and between species, a variety of different CHCs occur; e.g., different ant species display varied profiles of CHCs, including nonbranched, monomethyl-, dimethyl-, trimethyl-, and tetramethyl-alkanes, as well as monoenes, dienes (with two double bonds), trienes, and methylalkenes and methylated dienes. Among these hydrocarbons, dimethyl-alkanes and monomethyl-alkanes display the most dramatic structural variations (Martin and Drijfhout 2009), which makes them more distinct and thus more likely to be used as pheromones (Holman et al. 2010). This does not, however, preclude the use of other CHC structures as pheromones, such as straight-chain alkanes or alkenes in ants and termites (Fig. 2; Smith et al. 2009; Funaro et al. 2018). These compounds are less frequent in ant CHC profiles (Martin and Drijfhout 2009), which is an alternative explanation to structural complexity for their less frequent use as pheromones.

CHC biosynthesis normally starts with 16- to 20-carbon fatty-acyl-CoAs, such as stearyl-CoA, followed by elongation via fatty acyl-CoA elongases, formation of double bonds via acyl-CoA desaturases, and removal of CoA via reductases and cytochrome P450 in tandem with the coenzyme NADPH. In addition, the methyl groups in methyl-branched hydrocarbons arise from substitution

of malonyl-CoA with methylmalonyl-CoA during the initial assembly of the fatty acid chain (Howard and Blomquist 2005). Thus, most methyl branches are placed very early in the overall biosynthesis.

There are multiple elongases in the genome of eusocial insects: for example, 11 elongases in *Harpegnathos saltator* (Bonasio et al. 2010). Interestingly, recent phylogenetic analysis of desaturases of 15 insects, including social Hymenoptera demonstrated that insect desaturases represent an ancient gene family with eight subfamilies that differ strongly in their degree of conservation and the frequency of gene gain and loss. In three subfamilies, ants exhibit particularly large expansions of desaturase genes, which display species-, sex-, and caste-specific gene expression profiles (Helmkamp et al. 2015). These data suggest that some desaturases have undergone positive selection (see Box 1) during the evolution of ants.

A subfamily CYP4G genes encode P450 enzymes used by insects to produce cuticular hydrocarbons (Nelson 2018). The final step in the formation of the CHC is performed by a P450 enzyme that functions as an oxidative decarbonylase (Qiu et al. 2012). In this step, the precursor aldehyde is transformed into a hydrocarbon with the release of carbon dioxide. The essential role in the production of specific CHCs involved in insect communication has been demonstrated in a knockdown study in a termite. The knockdown of a P450 enzyme in queens of the termite *Cryptotermes secundus* led to significant changes in their royal CHC profile with subsequent lack of queen recognition by workers (Hoffmann et al. 2014).

The role of CHCs in communication and desiccation resistance demonstrates how important P450 enzymes are for insects. In fact, the active site sequences of the P450 subfamily CYP4G are highly conserved. Thus, it is surprising that the evolution of P450 genes is characterized by apparently random birth and death processes. This discrepancy might be explained by their two unrelated functions in desiccation resistance and communication: While CYP4G genes ensure production of CHCs for sufficient desiccation resistance, the specific CHC profile composition is not conserved and thus allows for random birth and death of CYP4G during evolution, thereby producing variation as a basis for their function in chemical communication (Feyereisen 2020).

Plasticity of pheromone synthesis

The production of queen pheromones varies with reproductive status and between castes, demonstrating its dynamic nature and genetic control. In the cape honeybee, *A. mellifera capensis*, for example, workers can functionally take the reproductive role in queenless colonies. When this happens, workers display queen-like physiology and behavior, suggesting a strong phenotypic plasticity in this species, including queen pheromone production. In fact, when workers replace the queen, they begin to synthesize QMP, in tandem with altered expression of P450 (CYP) genes (Malka et al. 2009; Wu et al. 2017), indicating the plasticity of enzyme production in response to an altered environment.

Plasticity in the production of CHC queen pheromones is probably widespread considering changes in the CHC profiles of reproductive individuals in ant, bee, and wasp societies (Peeters and Liebig 2009; Liebig 2010). Specifically, the level of queen- or reproductive-specific CHC expression is positively correlated with the level of ovarian activity in, for example, the ponerine ants *Dinoponera quadriceps* and *H. saltator* (Peeters et al. 1999; Liebig et al. 2000). In one of the most extreme cases, the queen CHC profiles in the ant *Camponotus floridanus* changes from 0% to 60% reproductive-specific CHCs, when queens increase their egg-laying rates during colony development from the foundation stage to colony sizes with more than 1000 workers (Endler et al. 2006). These compounds are exclusively in the shorter-chain part of the CHC profile. This example suggests strong changes in the regulation of genes associated with the production of the appropriate CHCs. In fact, the transition of workers to reproductive individuals after queen removal in the ant *H. saltator* is associated with a wide range of changes in their gene expression profile, including CYP genes, elongases, and desaturases (Bonasio et al. 2010). Concurrently, the CHC profile shifts from short-chain to long-chain hydrocarbons, with a strong increase in a queen-specific hydrocarbon, 13,23-dimethylheptatriacontane (13,23 dimethyl-C37), one of the likely queen pheromone compounds in *H. saltator* (Liebig et al. 2000). Why we find specific hydrocarbons associated with queen pheromones seems unclear. Although CHC profiles of ant workers from different species are associated with varying ecological conditions such as humidity or temperatures (Sprenger and Menzel 2020), reproductive queens in established ground nesting colonies are largely protected from desiccation, which thus should not constrain their CHC profiles and allow for large variations within the range of CHCs along the species-specific gene repertoire involved in CHC synthesis.

Summary of pheromone production

Genomic and transcriptomic evolutionary comparisons across species should also help with explaining the large variation in fecundity-related expression of CHC profiles in ants. For example, are genes involved in producing CHC queen pheromones more under stabilizing selection compared with genes involved in producing the species-specific CHC profile? Transcriptome analysis has been widely used to identify candidate genes involved in pheromone synthesis. To further understand the evolution of pheromones, specific enzymes need to be identified for each step in pheromone synthesis; for example, the CYP genes that determine the queen versus worker biosynthetic pathways in honeybees. In other words, functional analysis via genetic manipulations should follow the transcriptome analysis that identifies candidate enzymes. For example, CRISPR-mediated functional studies have been performed in eusocial insects (see “Conclusion and Perspective”), and these types of studies will facilitate the characterization of enzymes involved in regulating the pheromone biosynthesis, as well as the evolutionary analysis of these enzymes.

Pheromone perception in peripheral receptor neurons

When a sender-produced pheromone or cue reaches a receiver, it first stimulates activation of peripheral chemosensory neurons, which in turn induce behavioral and physiological responses. The chemosensory system is essential to discriminate among a large variety of chemicals in the environment. Chemosensory neurons in insects are localized in hair-like structures, called sensilla, on antennae and other appendages, with axons projecting to the brain (Fig. 2). They express odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), Pickpocket (Ppk), and Transient receptor potential (Trp) receptors (Joseph and Carlson 2015). The odorant receptor neurons (ORNs) that express ORs are specialized in detecting most volatile chemicals, including low volatility CHCs, while some volatiles, such as CO₂, can be sensed by GRs (Kwon et al. 2007). Besides receptor proteins, neuronal cells as well as glial cells surrounding neurons in the sensilla also express accessory proteins. For example, sensory neuron membrane protein (SNMP) likely acts as an essential cofactor of certain odorant receptors (Benton et al. 2007); odorant binding proteins (OBPs) and chemosensory proteins (CSPs) escort chemicals from the cuticular pore of the sensillum hair to the cell membrane of the chemosensory neurons (Leal 2013; McKenzie et al. 2014; Pelosi et al. 2014).

Chemosensory neurons in insects utilize a general decoding rule of “one neuron, one receptor.” ORNs normally only express one OR gene, and the axons of all ORNs expressing the same OR gene converge to the same glomerulus located in the antennal lobe (AL) in the brain (Fig. 2; Vosshall et al. 2000; Hansson and Stensmyr 2011; Grabe and Sachse 2018; Yan et al. 2020). However, exceptions have been found in *Drosophila* and mosquitoes. For example, some neurons express two or more OR genes, and some OR-expressing neurons also express IRs (Task et al. 2020; Younger et al. 2020; McLaughlin et al. 2021). In addition, different OR-expressing and IR-expressing neurons show coconvergence onto the same glomerulus in mosquitoes (Younger et al. 2020).

The insect OR gene family evolved from another family of chemosensory receptors, gustatory receptor (GR) genes (Brand et al. 2018; Robertson 2019). They encode seven-transmembrane domain proteins that display an inverted topology compared with G protein-coupled receptors, the corresponding chemosensory receptors in mammals. Insect ORs presumably act as ligand-gated ion channels, formed by heterotetramers, four-subunit protein complexes. Each heterotetramer contains two molecules of an obligate coreceptor called Orco, plus two molecules of a ligand-binding tuning OR (Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008; Butterwick et al. 2018). In contrast to *Orco* genes, which are highly conserved among insects, OR genes have undergone a high rate of birth and death. As a result, they show little conservation between orders, while within Hymenoptera, among wasps, bees, and ants, conservation has been found in some OR gene subfamilies, such as 9-exon ORs (see below; Zhou et al. 2012, 2015; McKenzie et al. 2016).

We next explore pheromone perception in more detail at the evolutionary and functional level of the chemosensory system.

Evolution of the chemosensory system in eusocial insects

When a receiver extracts information from a chemical cue in the first step of pheromone evolution, its chemosensory system already possesses olfactory receptors to sense these chemicals. Selection acts on the sender to make the pheromone more distinct, as well as on the receiver to recognize and respond to the pheromone more efficiently (Fig. 1). The latter includes the peripheral sensory system and subsequent sensory processing areas of the brain (Fig. 2).

Most insect species contain <100 *OR* genes and a similar number of glomeruli, the subunits of the antennal lobes. This number is strongly expanded in the flour beetle *Tribolium castaneum* and in Hymenoptera, including wasps, bees, and ants (for review, see Yan et al. 2020 and more bee species in Brand and Ramírez 2017). In ants and at least one social wasp, there are 300–500 *OR* genes, which cluster into 27 subfamilies, with the 9-exon subfamily being the largest, containing between one-third and one-half of the known *OR* genes (Zhou et al. 2012, 2015; McKenzie and Kronauer 2018; Legan et al. 2021). Given that *OR* genes are already expanded in parasitic solitary wasps, such as *Nasonia vitripennis* (225 *OR* genes) (Zhou et al. 2012), it is generally assumed that they started to expand as an adaptation to complex cues in hosts to assist in host recognition (Zhou et al. 2012; Yan et al. 2020). In contrast, the nonhymenopteran eusocial insects, termites in the order of Blattodea, do not show expansion of the *OR* gene family but rather an expansion of *IR* gene family (Terapon et al. 2014; Harrison et al. 2018), consistent with the termite chemosensory system having evolved independently for its role in eusocial organization.

The expansion of the *OR* family in the Hymenoptera is associated with positive selection (Roux et al. 2014; Zhou et al. 2015; Saad et al. 2018) in both solitary as well as social species, suggesting that it can be driven by factors associated with both eusocial organization and solitary life. The expansion of *OR* genes (or *IR* genes in termites) in the ancestors of eusocial insects may have been a pre-existing sensory sensitivity in receivers that helped enhance communication and discrimination abilities associated with colonial life. The combination of inputs from more narrowly tuned individual receptors enhances detection of slight differences among structurally similar chemicals or profiles of odorants (Wyatt 2014; Grabe and Sachse 2018; Yan et al. 2020), which is useful, for example, for the discrimination of complex CHC profiles of colony members versus outsiders. Due to the diversity of queen pheromones used in different eusocial species, the *OR*s that are used to sense queen pheromones must vary as well. The diverse gene gains or losses across eusocial insects match these expectations (Zhou et al. 2015). The *OR* diversity associated with the many different cues and pheromones can be achieved by tandem duplications (e.g., McKenzie and Kronauer 2018). Duplicated *OR*s in ants are associated with positive selection of amino acid positions at ligand binding sites of the re-

ceptor molecule that indicate neofunctionalization and a shift to the perception of novel odorants (Engsontia et al. 2015; Saad et al. 2018).

Function of odorant receptors and their role in neural development

At the level of peripheral neurons and receptors, some insect odorant receptors are very narrowly tuned, responding only to one or a limited range of ligands, whereas others are broadly tuned and act as “generalists,” responding to a variety of ligands (Grabe and Sachse 2018). During signal evolution, narrowly tuned receptors may have undergone strong positive selection for critical odors or signals, while broadly tuned receptors likely provide pre-existing sensitivity to a wide range to chemicals, the initial step of signal evolution that allows certain cues to induce a response in receivers. On the other hand, a pheromone or cue may activate only a single ORN type or a panel of ORNs to induce behavioral and physiological responses in receivers. In eusocial insects, specifically dedicated coding and combinatorial processing are both involved in mediating chemical responses (Su et al. 2009; Grabe and Sachse 2018).

The role of some *OR*s in social organization has already been elucidated. The 9-exon *OR*s, for example, seem to be essential for the establishment of the organization of colony structure and for communication between castes. Indeed, (1) the 9-exon subfamily is the largest among 27 *OR* subfamilies in hymenopteran eusocial insects, and (2) 9-exon *OR*s are mainly expressed in females (Zhou et al. 2012; McKenzie et al. 2016; Legan et al. 2021). The expansion of *OR* genes may allow an increase in the specificity and sensitivity of reception, leading to the hypothesis that 9-exon *OR*s are specialized in detecting queen pheromones and CHC cues. In fact, many *OR*s in the 9-exon family in the ant *H. saltator* are sensitive to CHCs (Pask et al. 2017), although other *OR*s outside of this group also respond to CHCs (Slone et al. 2017). This suggests a mechanism of combinatorial processing. For example, two *H. saltator* *OR*s (HsOR263, HsOR271) respond to 13,23-dimethyl-C37, a likely component of the *H. saltator* queen pheromone, which also indicates the specificity of certain *OR*s in pheromone response (Pask et al. 2017; Slone et al. 2017). Likewise, in honeybees, the *OR* AmOR11 specifically responds to 9-ODA, one of the main components of the queen pheromone QMP (Wanner et al. 2007).

It is commonly assumed that, unlike mammals, the development of most insect neurons is hardwired and does not depend on neuronal activity (Yan et al. 2020). For instance, knockout of *Orco* in *Drosophila* does not affect the ORN development (Larsson et al. 2004) but results in postdevelopment defects in axons and glomeruli (Chiang et al. 2009). However, recent evidence in other insect species, such as ants, suggests that development of the chemosensory system is dependent on the expression of *Orco* genes (Trible et al. 2017; Yan et al. 2017; Maguire et al. 2020). In the ants *H. saltator* and *Ooceraea biroi*, loss-of-function mutations in *Orco* strongly reduce the number of ORNs and glomeruli (Trible et al. 2017; Yan

et al. 2017), indicating its function in regulating neural development. The underlying mechanism is not clear yet. One explanation is that neuronal survival in ants is activity-dependent; alternatively, lack of *Orco* may lead to misfolding of OR proteins and subsequent cell death. Interestingly, *Orco* and *OR* genes are expressed early in neural development in ants as well as in mosquitoes and are required for the correct development of glomeruli in *O. biroi* (Maguire et al. 2020; Ryba et al. 2020). In contrast, *Drosophila Orco* is only expressed at late stages of ORN development and after maturation of glomeruli (Larsson et al. 2004). Furthermore, overexpression of one *OR* gene in mosquito neurons suppresses the expression of endogenous ORs, a phenomenon reminiscent of mice, but not in *Drosophila* (Maguire et al. 2020).

Summary of pheromone perception

As shown above, recent studies have challenged our previous understanding of the development of ORNs and indicated the complexity of the insect chemosensory system. More insects, including eusocial species, need to be explored. Further data from different insects will help us determine whether, despite the differences in odorant receptor structure in insects and mammals, the development of their ORNs may have undergone convergent evolution and shares larger similarities than previously thought.

Physiological and behavioral responses to pheromones

In insects, pheromones or chemical cues usually activate a unique combination of projection neurons (PNs), either directly or mediated by local interneurons in AL glomeruli. These PNs project to the mushroom bodies (MB) and lateral horn (LH) where the signal is integrated (Fig. 2; Su et al. 2009). The processing of integrated signals leads to subsequent physiological and behavioral responses via two main pathways: (1) activated neurosecretory cells secrete neurotransmitters and hormones to induce local and global changes in internal physiological conditions, and (2) axon projections along the ventral nerve cord (VNC) activate muscle cells to produce behaviors (Gilbert 2012). Eusocial insects largely share with solitary insects the essential genes that regulate neural development and activity, while certain genes have evolved to form a social gene toolkit in which genes have been co-opted to regulate social communication and social behavior (Amdam et al. 2006; Robinson et al. 2008).

While brain structures are highly conserved among insects, specific neural circuits display striking variations between species, sexes, age, castes, and varied environmental conditions (Hammer and Menzel 1995), which potentially leads to different responses to queen pheromones. QMP has variable effects on workers of different ages and task group. This pheromone controls social hierarchy and division of labor in the colony and also regulates a wide range of social behavior in honeybee workers. First, QMP restricts aggressive behavior, such as stinging, in

workers (Kolmes and Njehu 1990). Second, QMP regulates behavior in nurse workers by suppressing behaviors related to rearing new queens, which maintains colony stability (Winston et al. 1990). Third, QMP regulates behavior in foragers through its effect on JH levels. Foraging behavior is positively correlated with JH levels in eusocial insects (Pankiw et al. 1998), and QMP delays the transition from nursing to foraging via the down-regulation of JH, *krüppel homolog 1* (a JH-responsive gene), and cyclic guanosine monophosphate (cGMP) (Pankiw et al. 1998; Grozinger et al. 2003; Fussnecker et al. 2011). QMP also induced age-dependent attraction in workers. Young nurses are normally attracted by QMP, while old foragers are not. The attractive response to QMP is closely associated with lower levels of dopamine and dopamine receptors (Beggs et al. 2007; Vergoz et al. 2009), which highlights the importance of pathways downstream from pheromone receptors for differential responses to pheromones (Fig. 2).

In contrast to pheromone receptors and pheromone production, which are subject to rapid evolution and strong positive selection, pathways downstream from the pheromone receptors are largely conserved. For example, bees and ants diverged 150 million years ago and produce distinct queen pheromone molecules, but they share queen pheromone-responsive genes and pathways, including olfactory perception, neurotransmission, lipid metabolism, and transport, as well as reproduction (Holman et al. 2019). Five major royal jelly proteins (MRJPs) show increased expression in workers that are not exposed to queen pheromones, consistent with the need to rear new queens when the previous old queen dies. While the role of MRJPs in ants is not clear, one MRJP ranked the second queen pheromone-responsive gene in the ant *Lasius niger*, also showing up-regulation when not exposed to its queen pheromone (Holman et al. 2019).

Further studies in social wasps, ants, and termites have identified a large number of queen pheromone-responsive genes and pathways, notably the insulin pathway, G protein-coupled receptors (GPCRs), neurotransmitters, and vitellogenin (Libbrecht et al. 2013; Berens et al. 2015; Gospocic et al. 2017; Calkins et al. 2019; Haroon et al. 2020). Functional studies have been performed to reveal the role of key genes (e.g., *Corazonin* [*Crz*], which encodes a neuropeptide) in regulating behavior and reproduction: The queen pheromone may lead to the increased secretion of *Crz* that promotes worker foraging behavior. It also suppresses worker reproduction via down-regulation of vitellogenin, a profertility protein, in the fat body (Gospocic et al. 2017).

Genes that control transcription and cell identity also display caste-specific expression and pheromone response. These genes are known to regulate epigenetic processes in eusocial insects (Bonasio 2012; Yan et al. 2014, 2015; Allis et al. 2015; Opachaloemphan et al. 2018). Epigenetic processes that are responsive to queen pheromone include DNA methylation in honeybees (Holman et al. 2016) and ants (e.g., Bonasio et al. 2012; for review, see Yan et al. 2015), as well as histone modifications. For example, histone lysine 27 acetylation (H3K27ac) regulates caste determination, neural

development, and olfaction-mediated learning and memory in the carpenter ant *Camponotus floridanus*. Manipulation of H3K27 acetylation can drive major workers to forage (Simola et al. 2016). Furthermore, a key regulator, neuronal corepressor for element-1-silencing transcription factor (CoREST) appears to mediate the effects of histone deacetylation and further regulates juvenile hormone esterase and epoxide hydrolase, two enzymes that degrade juvenile hormone, thereby controlling ant foraging behavior (Glastad et al. 2020).

One of the downstream effects of pheromone exposure (or lack of it) is alteration of pheromone production in receivers. Variable levels of hormones are associated with differential production of CHCs and pheromones in insects. In *Drosophila*, the loss of ovaries and its secreted ecdysone lead to the reduction of the synthesis of long-chain hydrocarbons (Baron et al. 2018), while reduced insulin signaling decreases the synthesis of sex pheromone and decreases female sexual attractiveness to males (Kuo et al. 2012). Although the mechanisms are not clear, caste-specific hormone profiles may also regulate pheromone production in eusocial insects: Workers that are released from the inhibiting effects of a queen pheromone may show an increase in insulin and ecdysone synthesis and a decrease in JH synthesis, which subsequently induce the production of queen pheromones and other reproductive-specific compounds (Fig. 2). For example, the cape honeybee *A. mellifera capensis* develops the ability to reproduce in the absence of the host queen pheromone QMP, which is accompanied by changes in pheromone production (Mumoki et al. 2018). During the transition to reproductive status, the alcohol dehydrogenase (ADH) that converts 9-HDA to 9-ODA (QMP) is dramatically up-regulated in the mandibular gland (five times higher in queenless workers compared with queenright workers). Likewise, as described above, the transition from nonreproductive to reproductive status as a consequence of the lack of queen pheromones is associated with alterations of CHC profiles and the production of queen pheromones in transitioning ants in *H. saltator* and *D. quadricaps* (Peeters et al. 1999; Liebig et al. 2000), among other species (Peeters and Liebig 2009; Liebig 2010). The queen-like pheromone profile established in some workers plays an important role in regulating other workers' behavior and suppressing their reproductive potential.

Summary of responses to pheromones

In social insects, pheromones coordinate cooperation in their societies by inducing a wide range of physiological and behavioral responses. Although large efforts have been made, we know little about how these responses are regulated. For example, how do signals from peripheral neurons integrate in the central brain and how does the central brain regulate the secretion of hormones in eusocial insects? Analysis of neuronal circuits and functions in the brain will benefit from advanced techniques, such as single-cell transcriptome analysis that has been used for eusocial insect research (Sheng et al. 2020), as well as development of genetic tools, as described below.

Conclusion and perspective

Recent progresses in genomics, genetics, and epigenetics of eusocial insects have greatly enhanced our understanding of the genetic basis of social communication. Genomic studies, notably transcriptomic analyses, have identified a large number of developmental stage- and caste-specific genes at the three key steps of communication: pheromone synthesis, pheromone perception, and the resulting physiological and behavioral changes. However, the precise functions of these candidate genes are most often not yet clear. Full functional analysis will require further development of genetic tools for eusocial insects. These efforts will be enhanced by the fact that some eusocial insect species produce a large proportion of reproductively active individuals, which will facilitate the establishment of mutant or transgenic lines (Yan et al. 2014). So far, mutant and/or transgenic lines have been developed in the honeybee *A. mellifera* and the ant species, *H. saltator*, *O. biroi*, and *S. invicta* (Schulte et al. 2014; Kohno et al. 2016; Tribble et al. 2017; Yan et al. 2017; Kohno and Kubo 2018; Hu et al. 2019; Roth et al. 2019; Chiu et al. 2020). These genetic manipulations, and more to follow, will pave the way toward a better understanding of the role of genes in controlling social communication, neural development, and pheromone-induced physiology and behavior in eusocial insects.

Competing interest statement

The authors declare no competing interests.

Acknowledgments

We thank Claude Desplan, Jocelyn Millar, Danny Reinberg, and Adrian Smith for their comments and suggestions. H.Y. acknowledges support from the National Science Foundation I/UCRC, the Center for Arthropod Management Technologies under grant number IIP1821914, and by industry partners. J.L. acknowledges funding from the National Science Foundation (grant no. 2027237). This review benefited from a wealth of research articles, but due to length limitations, some relevant publications could not be cited.

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Genes Dev. 2021, **35**:

Access the most recent version at doi:[10.1101/gad.346965.120](https://doi.org/10.1101/gad.346965.120)

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