Deciphering the Rosetta Stone of Insect Chemical Communication

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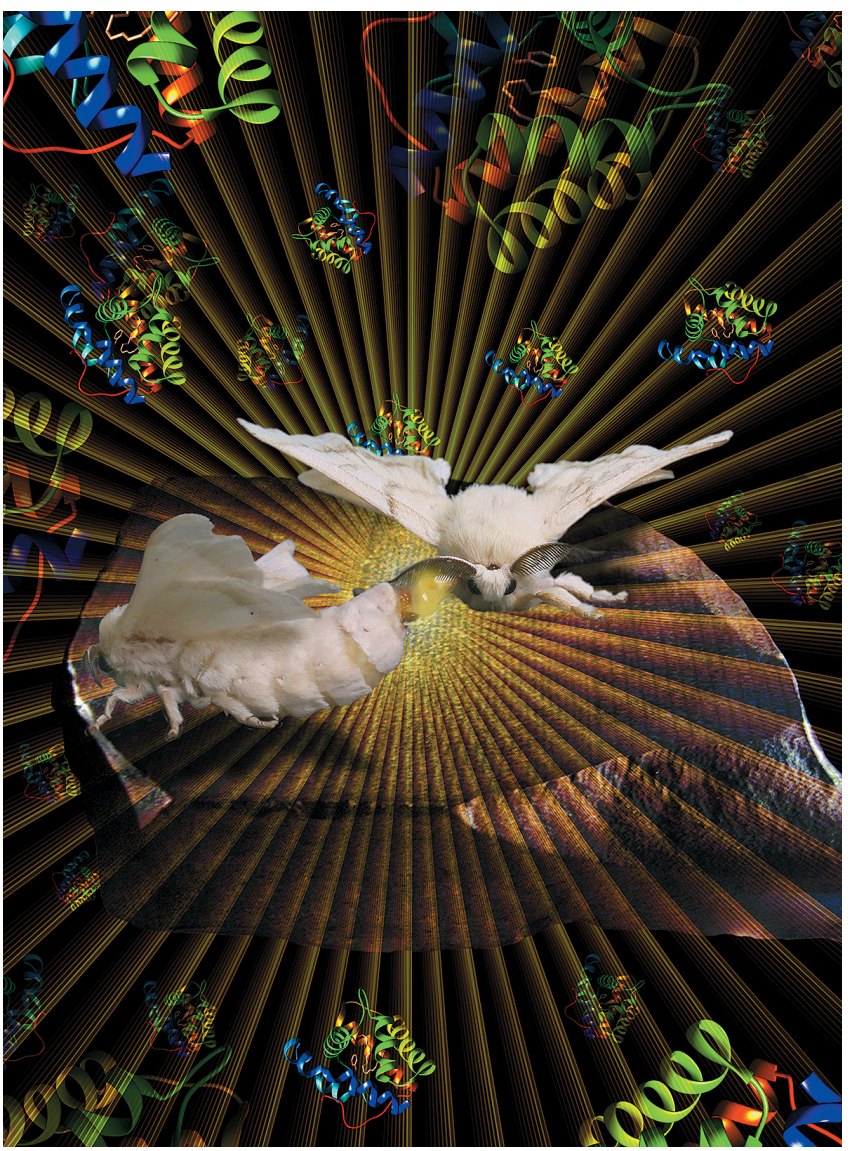

Imagine a world with an abundance of pesticide-free produce, no more flies and yellow jackets at picnics, and—more importantly—no diseases transmitted by mosquitoes and other arthropods. Welcome to Utopia! In the real world, however, chemical ecologists are pushing the envelope towards these ambitious goals. They are working diligently to decipher the "Rosetta Stone" of insect chemical communication to reduce our dependence on conventional agricultural chemicals and to mitigate mosquito bites and consequent transmission of vector-borne diseases. These may seem like utopian goals, but we must never underestimate the potential of scientific knowledge to grow exponentially and beyond our imaginations. The proverb "shoot for the moon; even if you miss, you will land among the stars" is exactly what chemical ecologists are doing. They rely on molecules, large or small, for their moonshot.

The Discovery of Insect Sex Pheromones

In the nineteenth century, French entomologist Jean-Henri Fabre and American entomologist Joseph Albert Lintner independently observed that female moths remotely advertise to conspecific males their readiness to copulate. After winning the Nobel Prize for chemistry in 1939 for

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the identification of sex hormones, German chemist Adolph Butenandt studied chemical communication in the silkworm moth, *Bombyx mori* L. Curiosity and the prospect of practical applications drove him to pursue this research. In the late 1950s, after 20 years of extracting female abdominal pheromone glands and about a century after Fabre's observations, Butenandt identified the chemical compound mediating male–female communication in the silkworm moth, (10*E*,12*Z*)-hexadecadien-1-ol (common name, bombykol) (Butenandt et al. 1959). Butenandt was indeed the founder of the field of molecular basis of insect olfaction. The term pheromone ("carriers of excitement" in Greek) was then coined to refer to chemicals produced by an individual and recognized by conspecifics to induce a defined behavioral response, and later the more generic term semiochemicals was created for chemicals that carry a message. At the time of the bombykol discovery, identifying pheromones and semiochemicals from 7,000 species of insects would have been considered an overambitious goal. After all, it took two decades of research to identify just the first sex pheromone. Nevertheless, this is what chemical ecologists have already accomplished in about six decades.

Simple and Complex Sex Pheromones. Because the majority of compounds thus far identified are alcohols, aldehydes, and acetates of long unsaturated hydrocarbon chains, an initial misconception surfaced that sex pheromone chemistry is "trivial." Some compounds are indeed very simple. Other pheromones (and semiochemicals involved in insect defense) are very complex, and the major challenge of elucidating their structures is the minute amount produced by insects, sometimes below the detection limit of most analytical instruments. Cockroaches are among the most sophisticated "natural product chemists." Full characterization and synthesis of the periplanones proved so challenging that it attracted the brightest and the best in the field, including Koji Nakanishi (Adams et al. 1979). The intriguing chemistry of male-produced pheromones from butterflies sparked the interest of Jerrold Meinwald, who discovered the first pyrrolizidine alkaloids as a sex pheromone (Meinwald et al. 1966). Beetles, too, utilize complex chemicals as pheromones. Even when the chemistry is not that complex, the signal can be sophisticated. Females of the Japanese beetle, *Popillia japonica* Newman, produce (5*R*)-5-[(*Z*)-dec-1-enyl]oxolan-2-one (japonilure) as a sex pheromone (Tumlinson et al. 1977) with high enantiomeric purity, and males' sophisticated olfactory system can detect even minute amounts of the antipode as a behavioral antagonist—a deterrent to males. In short, the pheromone is an attractant and its mirror image is a deterrent. For another species native to Japan, the Osaka beetle, *Anomala osakana* Sawada, these stereoisomers have just the reverse role; that is, the chemistry and stereochemistry of the sex pheromone from the Japanese beetle is the same as the behavioral antagonist for the Osaka beetle (Leal 1996), and vice-versa.

Pheromones in IPM. Today, chemical ecologists and entomologists throughout the world are using these semiochemicals to minimize the use of conventional agricultural chemicals. They deploy traps baited with synthetic pheromones to monitor populations of agricultural pests and are lowering the use of pesticides, intervening only when they are really needed; i.e., when population levels are detrimental to crop production. Alternatively, they lure insects with their own scents, or other attractants, to areas treated with toxic chemicals, instead of indiscriminatingly spraying fields populated with both pests and beneficial insects. The most sophisticated use of insects' communication channels is a process called mating disruption. Researchers permeate the field with minimal quantities of synthetic sex pheromones—large amounts compared to what insects produce, but a drop in the ocean compared to conventional pesticides—to mislead males and disrupt female–male communication. These environmentally friendly approaches to reduce insect pest populations may still be a little more costly than applications of conventional pesticides, but are definitely more affordable when the societal cost of environmental contamination is included in the equation. Look at it this way: in today's society, a shopper has to pay for plastic bags in supermarkets or at least be given a choice between plastic and paper. The most environmentally conscious tend to avoid plastic bags and/or recycle them. In much the same way, chemical ecologists want to deliver more environmentally safe alternatives for pest control and medical entomology.

Signal Production. The chemical signal itself is one theme in a trilogy of insect chemical communication; the others are the biosynthetic pathways involved in making these species-specific pheromones (pheromone production) and the intricate biochemical machinery for high fidelity and acute reception of pheromones (pheromone reception). Neither Butenandt nor his contemporaries could have envisioned the influence of the bombykol discovery on new fields of pheromone research, particularly pheromone production and reception. Starting late in the last century, legendary chemical ecologist Wendell L. Roelofs (Bjostad and Roelofs 1983), his collaborators, and many other research groups deciphered an important facet of pheromone biosynthesis in moths, exploring the enzymes involved. With a unique Δ11-desaturase, in combination with chain-shortening reactions, reductases, and oxidases, moths produce combinations of unsaturated acetates, aldehydes, and alcohols that form an unlimited range of potential blends of species-specific sex pheromones. Although they may share a few or even all constituents, the species-specific signature of a moth's sex pheromone blend is dictated by the ratio of the constituents. Ashok Raina's discovery (Raina and Klun 1984) of a peptide named pheromone biosynthesis activating neuropeptide (PBAN) paved the way to a better understanding of how a moth's central nervous system controls the biosynthetic pathway and, consequently, pheromone release. Our knowledge of the biosynthetic component of insect's chemical communication continues to grow, but a short-sighted expectation that research has to have an immediate application led to significant reduction in funding opportunities in this endeavor.

The discovery of bombykol opened new research avenues as scientists were empowered with a simple,

quantifiable tool—the pheromone—to investigate insect chemical communication physiologically, behaviorally, and neurologically. Iconic German physiologist Dietrich Schneider, a contemporary of Adolf Butenandt, developed devices to record electrical signals from insect antennae (the electroantennogram, EAG) (Schneider 1969) as well as from individual sensilla in antennae (a single sensillum recording, SSR) when the antennal preparations were exposed to pheromones. Later researchers made miniature EAG devices and Swiss scientist Heinrich Arn combined EAG with a gas chromatograph (GC-EAD) to make a stable, real-time biodetector (Heinrich et al. 1975). Instead of the 500,000 glands needed by Butenandt to identify bombykol, today one could, in principle, identify a sex pheromone with a single gland from a female moth and one antenna from a conspecific male. These techniques, all based on Schneider's invention, led to the already discussed explosion in the identification of new sex pheromones and other semiochemicals.

New Research Avenues. Research by Schneider and his graduate student, Karl-Ernst Kaissling, demonstrated for the first time the inordinate selectivity (fidelity) and unmatched sensitivity of the insect's olfactory system. Together with Juergen Boeckh, Veit Lacher, Ernst Priesner, and Gerhard Kasang, Kaissling demonstrated early on that minimal structural changes to pheromone molecules render them inactive, whereas a single molecule of a natural sex pheromone was estimated to be sufficient to activate an olfactory receptor neuron (ORN) in male antennae (Kaissling and Priesner 1970). In the 70s, John Kennedy developed a wind tunnel to measure pheromone searching behavior of flying insects and to accumulate data supporting his theory of anemotaxis—oriented movement in response to wind direction (Kennedy and Marsh 1974). In the early 80s, John Hildebrand discovered a structure in the brain of the male moth, the macroglomerular complex (Hildebrand and Matsumoto 1981), dedicated to the reception and processing of pheromone signals, including integration of behavioral antagonists and pheromones. As we now know, the entire olfactory process encompasses the reception of pheromones and other semiochemicals by specialized organs (antennae and maxillary palps) in the periphery; processing of signals in the antennal lobe, including perception of pheromones and behavioral antagonists in the macroglomerular complex; integration of odor in the protocerebrum with other sensory modalities; and ultimate translation into behavior. All these processes are part of pheromone perception, but I will focus on peripheral reception of odorants.

Pheromone Reception

Reception of pheromones starts with a "violation" of an organic chemistry textbook rule of solubility: like dissolves like. Pheromones and other semiochemicals are hydrophobic compounds and they must solubilize in aqueous solutions to reach their detectors, the odorant receptors (ORs) housed in insect antennae (and maxillary palps in some species). ORs are embedded in the dendritic membrane of olfactory receptor neurons (ORNs) inside odorant-detecting sensilla, which are surrounded by an aqueous solution, the sensillar lymph (Leal 2013). This compartmentalization is essential for OR protection from harmful chemicals in the environment, as well as to add to the selectivity of the olfactory system. Additionally, it supports a filtering mechanism: only chemicals that can be ferried through the sensillar lymph are able to reach ORs. Thus, the selective pressure on OR genes for evolving specific receptors is less stringent than if all chemicals were to indiscriminately reach ORs. Odorant-binding proteins (OBPs; also named pheromone-binding proteins (PBPs) in the specific case of pheromone reception), first discovered by Richard Vogt in Lynn Riddiford's laboratory (Vogt and Riddiford 1981), are accomplices in breaching the organic chemists' rule. OBPs solubilize and transport pheromones or semiochemicals, ferrying them from the port of entry of the antennal sensilla (the pore tubules), through the sensillar lymph, and delivering them to ORs at the end of the journey. In moths, and likely also in mosquitoes, these small (ca. 15 KDa) globular proteins perform these tasks in a spectacular way. To unravel OBP functions, I have been fortunate to collaborate with world-class structural biologists, including Harvard Professor Jon Clardy and Nobel laureate Kurt Wüthrich, among others. In the sunset of the last century, our laboratory serendipitously discovered that the silkworm moth PBP, BmorPBP (renamed BmorPBP1 when multiple PBPs were discovered later), undergoes a pH-dependent conformational change (Wojtasek and Leal 1999). Although their structures were still terra incognita, we postulated that there were two forms of PBPs that are important for pheromone binding and release. We prepared pure samples of recombinant, labelled BmorPBP1, which we shipped to Wüthrich's laboratory for NMR analysis. Although our sample was pure as indicated by multiple chromatographic techniques, native and SDS gel electrophoresis as well as liquid chromatography coupled with electrospray ionization mass spectrometry NMR analysis in Wüthrich's laboratory suggested two species—a possible "impurity." We suspected that the protein sample had undergone degradation during shipment to Switzerland. Surprisingly, the sample returned from Switzerland was still pure, by our standards. A thorough analysis of BmorPBP1 by circular dichroism (CD) and fluorescence showed that the protein was also very stable. It could be heated to near the boiling point and retained the CD spectrum when returned to room temperature: a seemingly "rock-solid" protein. Hubert Wojtasek, then a postdoctoral scholar in my lab, discovered that this remarkably stable protein was very sensitive to pH changes. As indicated by near-UV CD, the tertiary structure of the protein changed dramatically when the pH was reduced, with a clear transition between pH 6 and pH 5, but the secondary structure (as suggested by far-UV CD) remained nearly unchanged. These observations substantiated our hypothesis of the two forms of BmorPBP1 (Wojtasek and Leal 1999).

Structural Biology in Entomology. After attempts that consumed several months, the Clardy group, then at Cornell University, was able to crystallize BmorPBP1, but as the structure had no suitable models, the diffraction data could not be analyzed by molecular replacement with

a related protein. In my lab, Larisa Nikonova prepared a sample of selenomethionyl BmorPBP1 that allowed Clardy's graduate student, Benjamin Sandler, to solve the phase problem and determine the structure after years of work. *Voila!* The structure of BmorPBP1 (Sandler et al. 2000) was a surprise to all of us: six α-helices, four of them converging to form a large flask-shaped cavity that encapsulated bombykol. Thus, BmorPBP1 solubilizes bombykol by holding the pheromone (mostly with van der Waals interactions) in a hydrophobic pocket in the core of the protein. Twenty-one glutamate or aspartate residues and 14 lysine or arginine residues on the outer surface of the protein make BmorPBP1 as well as BmorPBP1-bombykol highly water-soluble. To crystalize BmorPBP1, Clardy's lab needed to use concentrations at least twice what one would have expected for a protein of its size. Almost simultaneously, the Wüthrich lab elucidated the structure of BmorPBP1 at low pH (4.5) (Horst et al. 2001). The NMR structure provided unambiguous evidence for a pH-dependent conformational change. At low pH, the C-terminus forms a seventh α-helix, which replaced bombykol in the binding site. Thus, the two forms of BmorPBP1 we hypothesized earlier on the basis of CD and fluorescence data are indeed conformations of the protein; i.e., $BmorPBP1^{A}$ (the acid form) and $BmorPBP1^{B}$ (the basic form).

Their 3D structures differed markedly, particularly with respect to the C-terminus, which is at the surface of $BmorPBP1^{B}$ and located in the binding pocket of $BmorPBP1^{A}$. This is physiologically relevant, given that the surface of dendrites where the receptors are located is negatively charged (as observed earlier by Thomas Keil [Keil 1984] in Kaissling's laboratory) and thus, in the vicinity of the membrane, the pH is reduced due to accumulation of protons. With stopped flow fluorescence analysis, we have demonstrated that $BmorPBP1^{B}$ is rapidly converted into $BmorPBP1^{A}$ (half lifetime, ≈9 ms) (Leal et al. 2005) when the pH is lowered, as found close to the surface of dendrites. Thus, the pH-mediated conformational change is within the timescale of pheromone reception (on average 500 ms).

Recently, NMR studies demonstrated that in solution at the pH of the sensillar lymph pH (6.5), unliganded BmorPBP1 exists predominantly in the conformation $BmorPBP1^{A}$, but the equilibrium is shifted toward the B conformation in the presence of ligands that effectively compete with the C-terminal helix for the binding site (Damberger et al.). pH titration of BmorPBP1 showed that the B → A transition midpoint was at pH 7.3 for the apo-protein, 5.4 for BmorPBP1-bombykol, and 5.8 when the protein was bound to hexadecane-1-ol or hexadecanal. The pH at the surface of the dendrites (or pore tubules) is lower than the bulk pH (pH of the sensillar lymph), increasing with the distance from negatively-charged surfaces. At the lower pH in the environs of the pore tubules, the stability of the A-conformation of BmorPBP1 is enhanced and weak binding ligands are unable to displace the C-terminal helix, whereas the affinity of bombykol is sufficient to displace the C-terminal α-helix from the binding pocket. Pheromone is then taken up into $BmorPBP1^{B}$-bombykol, and diffuses towards the region of higher pH. BmorPBP therefore neatly performs the trick of filtering against weaker ligands and yet enhancing the stability of the complex during its journey towards the receptors. Bombykol is thus made "soluble" and (in contrast to weaker binding ligands) it is protected from aggressive enzymes in the sensillar lymph. When reaching dendritic surfaces, the complex undergoes a conformational change, with the C-terminus occupying the binding pocket and ejecting bombykol to activate ORs. Ligands with higher affinity than bombykol would exhibit a lower midpoint in the pH transition and the C-terminal helix would be unable to compete for the binding cavity. Therefore, such a ligand would remain trapped and unable to access the receptors. Although high affinity ligand might be released to a certain extent by mass action, the off rate or ligand release rate is very slow without the help of the C-terminal dodecapeptide. Indeed, the BmorPBP1 conformational change expedites release of pheromone by 10,000-fold (Leal et al. 2005). Slow release kinetics would make the strongly binding ligand ineffective for olfaction. On the other hand, a ligand with lower affinity than bombykol exhibits a conformational transition at a higher pH midpoint, and therefore both uptake at the pore tubules and release to the receptor would occur farther from the pore or the receptor, respectively, making weaker binding ligands more vulnerable to degradation. In summary, many compounds may bind to BmorPBP1, but some will remain trapped, while others will be dropped out of reach of receptors. Bombykol and perhaps just a few other closely related compounds are released at "the right time and the right place." The second layer of filtering is then achieved by selectivity of the receptors (see below).

Signal Termination. Stray molecules in the sensillar lymph, be they ligands prematurely dropped by an OBP or pheromones released from their receptors, are prone to degradation by odorant-degrading enzymes (ODEs) (Kaissling 2013), as postulated by Gerhard Kasang and later discovered by Richard Vogt (Vogt and Riddiford 1981). After I had isolated to purity and obtained internal amino acid sequences, Yuko Ishida in my lab cloned and expressed for the first time pheromone-degrading enzymes (PDEs) from moths (Ishida and Leal 2005) and beetles (Ishida and Leal 2008). The kinetic properties of both recombinant and native PDEs at the pH of the sensillar lymph were consistent with rapid inactivation of pheromones. Although these findings support the notion that pheromones are enzymatically inactivated, there is not enough evidence to support a generic mechanism of enzymatic inactivation of pheromones. Some of the antennae-specific enzymes are cytosolic, so the ligands would have to be transported from the sensillar lymph through the membrane to reach these enzymes. This would be a slow process compared to the time scale of olfaction, unless there is an as yet unknown molecular mechanism to expedite transport through the membrane. It is worth mentioning that sensory neuron membrane proteins are embedded in the membrane in the vicinity of receptors, and their relatives in vertebrate animals internalize multiple ligands, including fatty acids, but

there is no evidence that they internalize pheromones. An alternative hypothesis to enzymatic degradation yet to be rigorously tested is that pheromones are trapped and inactivated by OBPs.

Parapheromones. One of the moonshot goals in chemical ecology and olfaction is to devise non-natural compounds that interfere with odorant reception in insects of agricultural and medical importance. Some of these compounds are referred to as parapheromones, as they mimic naturally occurring (sex) pheromones; others are receptor antagonists. In order to be effective, both types of compounds must pass through the sensillar lymph and reach the well-protected ORs. Thus, research on the molecular basis of binding, transport, and release of odorants by OBPs is essential to accomplish these goals. For these non-natural compounds to be effective, leading compounds must be optimized so that they retain their function(s) on receptor(s), but are properly transported through the aqueous barrier (the sensillar lymph) and delivered to ORs in vivo.

Odorant Receptors. Not until the turn of the century did John Carlson's and other laboratories discovered ORs. Ironically, Carlson's prospecting of the first *OR* genes in the genome of the fruit fly, *Drosophila melanogaster*, was based mostly on a bioinformatics approach designed to fish out G-protein-coupled receptor (GPCR)-like olfactory genes (Clyne et al. 1999). We now know that insect ORs are not GPCRs; they are seven transmembrane ion channels, with "inverse" topology (Benton et al. 2006) as compared to GPCRs; i.e., the N and C termini are intra- and extra-cellular, respectively. The discovery of the first insect *OR* genes thus underscores the importance of a working hypothesis; the hypothesis is a driver, important for the advancement of science. Shortly thereafter, in Leslie Vosshall's laboratory at Rockefeller, chemical ecologist Matthias Larsson discovered an obligatory co-receptor, initially named Or83b (Larsson et al. 2004). Now, orthologs of the noncanonical OR83b have been identified in moths, mosquitoes, and many other insect species. Because they all apparently perform the same function, OR83b and its orthologs were renamed odorant receptor co-receptor (Orco). Orco is a seven-transmembrane protein with the same topology as insect ORs. The ground-breaking discoveries of ORs and Orco formed the cornerstones of the spectacular explosion in our understanding of the molecular basis of odorant reception. Together, OR and Orco form ligand-gated nonselective cation channels (OR-Orco complexes) with odorants activating only the canonical unit, so the OR is thus sometimes referred to as the receptor "binding unit." As insect genomes have been sequenced, putative ORs from multiple insect species (including but not limited to moths, mosquitoes, honey bees, and ants) have been identified at a pace faster than anyone could have imagined even a decade ago. The entire repertoire of *OR* genes in the fruit fly has been elegantly deorphanized and mapped to ORNs and sensilla type as well as ORN projections in the antennal lobe. A functional assay to interrogate receptors vis-à-vis odorants, the "empty neuron" system, played a significant part is this endeavor. This system has also been used to painstakingly deorphanize ORs from the malaria mosquito, *Anopheles gambiae* (Carey et al. 2010). Additionally, heterologous expression of ORs, particularly in the *Xenopus* oocyte recording system, helped deorphanize moth, mosquito, and honey bee ORs.

Despite these and other new approaches, deorphanization is still the rate-limiting step in full characterization of insect ORs. With the advent of next-generation sequencing, the number of putative OR genes will grow exponentially. The massive genomic data that will be generated in the next 5-10 years are certain to be 100- or perhaps 1,000-fold larger than the data generated in the last 50+ years after the discovery of bombykol. To catalyze this fast-growing field, we must have state-of-the-art, high-throughput approaches to de-orphanize ORs, much faster and more user-friendly than the automated *Xenopus* oocyte recording system. After all, it is now possible to investigate the effect of any environmental or physiological condition on the olfactory system by studying the entire repertoire of genes affected. We can blood-feed mosquitoes, let them starve, expose them to repellents, and infect them with pathogens. We can expose moths over extended periods to sex pheromones, inflict honey bees with environmental contaminants, and then investigate these effects on all olfactory genes. Sequencing of *D. melanogaster* genome was a large-scale project that, among many benefits, led to the discovery of OR genes. Very soon, sequencing the genome of a new species will be a master's or even an undergraduate thesis project. The future is bright, but handling the data will be challenging. It was just about a decade ago that our laboratory isolated and identified the first olfactory protein from a mosquito species—the odorant binding protein CquiOBP1—by extracting proteins from antennae and legs, running gel electrophoresis, sequencing bands, and cloning (Ishida et al. 2002). We have just investigated the entire repertoire of olfactory genes in the same species by transcriptome (RNA-Seq analysis). The difference is that we have now identified hundreds of genes, including the complete set of OBPs and 36 novel OR genes (Leal et al. 2013). However, functional analysis is still the rate-limiting step.

New Challenges

Solving 3D structures of odorant (or pheromone) receptors in complex with the activating signal molecules is the Holy Grail of insect olfaction, but we shall see surprises in a not-too-distant future. For now, it has been established that (1) the binding pocket is located on the extracellular halves of its transmembrane (TM) domains (Guo and Kim 2010); (2) a residue located at the predicted interface between TM-3 and extracellular loop (ECL) 2 plays a role in activation in *Drosophila* (Nichols and Luetje 2010); (3) membrane-embedded binding pockets might be covered by ECL-2 in ORs from moths and mosquitoes (Xu and Leal 2013); and (4) TM-3 is part of the binding pocket in a moth OR and one of its residues is essential for pheromone specificity (Leary et al. 2012). Experiments in the oocyte interrogation system showed that the bombykol receptor from the silkworm moth, BmorOR1, is more sensitive to bombykol, but responds

to the related aldehyde, bombykal, with about one order of magnitude higher threshold. Interestingly, the bombykol ORN does not respond to bombykal, except when it is challenged at >10,000-fold higher doses. The discrepancies between the responses recorded from the intact neuronal system and the "naked" receptor thus support the notion that BmorPBP1 plays an essential role in augmenting selectivity. By contrast, BmorPBP1 seems to indiscriminately bind to stereoisomers of bombykol (Hooper et al. 2009), but the receptor is very selective. BmorOR1•BmorOrco-expressing oocytes responded to the natural isomer (10*E*,12*Z*)-bombykol, but not to the other three isomers (Xu et al. 2012a). These findings suggest that PBPs and ORs work in a two-step filtering system. It is, therefore, important to study OR and OBP structures when devising novel, non-natural compounds aimed at interfering with the insect's olfactory system in vivo. One may argue that it may be far-fetched to devise parapheromones given the "lock-and-key" selectivity of the system. However, the system is not "bulletproof," and it is worth exploring its loopholes. A few decades ago, entomologists discovered by a trial-and-error approach that formates two carbons shorter than the natural ligand can be used to replace unstable aldehyde pheromones. A few decades later, we demonstrated that these analogs trick both pheromone-binding proteins and odorant receptors involved in the reception of aldehydes (Xu et al. 2012b). Thus, a more educated approach based on structures of PBPs and ORs is likely to succeed in the design of next-generation semiochemicals.

Intriguing Insect Repellents

One of the most remarkable non-natural compounds identified in insect olfaction is the insect repellent DEET (*N,N*-diethyl-3-methylbenzamide). It works to variable degree of efficacy against all arthropods tested. Repellents have been used for hundreds of years, but modern repellents started to be developed at USDA laboratories by trial-and-error testing of thousands of candidate compounds. DEET was discovered in 1953, but even today it remains an enigma in insect chemical ecology. More recently, other good repellents have been developed and are now commercially available, including 3-(*N*-acetyl-*N*-butyl) aminopropanoic acid ethyl ester (IR3535), 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester (picaridin, icaridin), and *p*-menthane-3,8-diol (PMD). Interestingly, PMD was missed in the USDA screening in the '50s, but isolated from a Chinese plant in the '90s and later registered for commercial use. A myriad of other insect repellents have been discovered, but DEET remains the gold standard. Most of the new products do not reach the market or are not competitive with DEET for one or more of the following reasons: DEET is cheap, effective for 6-8 h, and side effects and toxicity are so minimal that it has been deemed safer for human use, including children and pregnant women. However, DEET may dissolve plastic and synthetic fabrics, causing damage to the frames of eyeglasses and watch bands, for example. DEET (used in combination with permethtrin) has the potential to reduce malaria incidence, but (though cheap for some) it is not affordable to most people living in malaria-afflicted regions where methods of control are sorely needed. Therefore, chemical ecologists are deeply interested in understanding its mode of action so as to lay the foundation for the development of "safer" and cheaper insect repellents.

Various properties of DEET have been reported, but some seem to be rather pharmacological than physiological properties. Experiments in vitro demonstrated that DEET inhibits acetylcholinesterase, but this is unlikely to be related to its mode of action. It has been reported to block receptors and jam the olfactory system, but the most convincing evidence in the literature is that DEET acts directly on both the olfactory and gustatory system. In short, DEET repels from a distance as an olfactory signal (odorant) (Syed and Leal 2008) and reduces biting as a gustatory signal (tastant) (Lee et al. 2010). The mechanism by which DEET is detected as an odorant is still controversial, however. It has been unambiguously demonstrated that mutants of the yellow fever mosquito, *Aedes aegypti*, engineered to interfere with Orco expression were not repelled by DEET (DeGennaro et al. 2013). Since the *orco* mutant did not express Orco protein, which is an obligatory co-receptor, it was convincingly concluded that DEET is detected by OR(s). On the other hand, it has been suggested that DEET is detected by an entirely different group of olfactory proteins, the ionotropic receptors (which do not require Orco), particularly IR40a (Kain et al. 2013). These two seemingly dichotomous research findings need to be reconciled. Importantly, the evidence that DEET is detected by IR40a in the fruit fly is solid, but so far there is no supporting evidence that the ortholog receptor in *A. aegypti* is in fact a DEET receptor. The *Drosophila*-based findings have been extended to all insect species, including mosquitoes, on the basis of the existence of DmelIR40a orthologs in all insect species investigated to date, including the three medically most relevant mosquito species, *Aedes aegypti, Anophles gambiae*, and *Culex quinquefasciatus*. Although DEET is a generic insect repellent, the assumption that all species utilize the same type of receptor for DEET detection may not be true. DEET is a non-natural chemical signal, so the selective pressure for its reception may have been different in different species. The silkworm moth and, intriguingly enough, the fruit fly have receptors for bombykol, but they share no more than 17 percent identity. More specific to the case at hand, AgamOR40 in larvae of the malaria mosquito responds to DEET, but its ortholog in *C. quinquefasciatus*, CquiOR91 (formerly CquiOR40), does not. Thus, DEET acts differently on ortholog ORs from two different mosquito species, so there could be differences between the fruit fly and the yellow fever mosquito in DEET detection.

Another pertinent, 60-year-old question is: why does DEET work? Is it mimicking some natural compound(s)? Is this a plant-derived honest signal? It is worth mentioning that mosquitoes are vegetarian blood-feeders. They do rely on flowers as sources of energy for flight. Thus, when mosquitoes detect DEET and stay away, are they detecting "useful" information? Perhaps answering those questions is as important as understanding DEET's mode of action. If there are chemicals for which DEET works an "analog,"

the structure of the natural product should be a more effective lead compound to design next-generation repellents.

The Future is Bright

The field of chemical ecology is broader than portrayed in this article and has an enormous potential to contribute to a more environmentally conscious planet. Chemical ecologists will continue to explore earth and sea to find lead compounds for the development of novel drugs. They will continue to build upon the foundation of Butenandt and other pioneers to unravel the intricacies of insect chemical communication. Agricultural fields will be made greener with parasitoids being systematically recruited with semiochemicals to reduce pest populations. Databases (genomes) will allow us to predict which pheromone is utilized by a new species or was utilized by an extinct species. In the near future (perhaps not soon after this article is printed, but not too far from now) the 3D structures of insect ORs will be solved. We will get an in-depth understanding of the molecular basis of insect olfaction and will be able to use that foundation to design green chemicals for controlling populations of agricultural pests and insects of medical importance. We will develop better ways to keep mosquitoes at bay, reduce transmission, and save human lives.

Postscript

Recently, it has been unambiguously demonstrated that in the southern house mosquito, *Culex quinquefasciatus*, DEET is detected by an odorant receptor, CquiOR136, which is also sensitive to methyl jasmonate. These findings suggest that DEET might work by mimicking defensive compound(s) from plants. (See Xu et al., Mosquito odorant receptor for DEET and methyl jasmonate. Proceedings of the National Academy of Sciences of the United States of America, *doi: 10.1073/pnas.1417244111*)

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References Cited

Adams, M., A. Adams, K. Nakanishi, W. C. Still, E. V. Arnold, J. Clardy, and C. J. Persoons. 1979. Sex pheromone of the American cockroach: absolute configuration of periplanone-B. J. Am. Chem. Soc. 101: 2495-2498.

Benton, R., S. Sachse, S. W. Michnick, and L. B. Vosshall. 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. PLoS Biol 4: e20.

Bjostad, L. B., and W. L. Roelofs. 1983. Sex pheromone biosynthesis in *Trichoplusia ni*: key steps involve delta-11 desaturation and chain-shortening. Science 220: 1387-1389.

Butenandt, A., R. Beckmann, D. Stamm, and E. Hecker. 1959. Uber den sexual lockstoff des seidenspinners *Bombyx mori* - Reindarstellung und konstitution. Zeitschrift Fur Naturforschung Part B-Chemie Biochemie Biophysik Biologie Und Verwandten Gebiete 14: 283-284.

Carey, A. F., G. Wang, C. Y. Su, L. J. Zwiebel, and J. R. Carlson. 2010. Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464: 66-71.

Clyne, P. J., C. G. Warr, M. R. Freeman, D. Lessing, J. Kim, and J. R. Carlson. 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. Neuron 22: 327-338.

Damberger, F. F., E. Michel, Y. Ishida, W. S. Leal, and K. Wüthrich. 2013. Pheromone discrimination by a pH-tuned polymorphism of the *Bombyx mori* pheromone-binding protein. Proc. Natl. Acad. Sci. U.S.A.: in press.

DeGennaro, M., C. S. McBride, L. Seeholzer, T. Nakagawa, E. J. Dennis, C. Goldman, N. Jasinskiene, A. A. James, and L. B. Vosshall. 2013. Orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature 498: 487-91.

Guo, S., and J. Kim. 2010. Dissecting the molecular mechanism of *Drosophila* odorant receptors through activity modeling and comparative analysis. Proteins 78: 381-99.

Heinrich , A., E. Stadler, and S. Rauscher. 1975. The electroantennographic detector: A selective and sensitive tool in gas chromatographic analysis of insect pheromones. Z. Naturforsch. C 30: 722-725.

Hildebrand, J. G., and S. G. Matsumoto. 1981. Olfactory mechanisms in the moth *Manduca sexta*: Response characteristics and morphology of central neurons in the antennal lobes. Proceedings of the Royal Society B 213: 249-277.

Hooper, A. M., S. Dufour, X. He, A. Muck, J. J. Zhou, R. Almeida, L. M. Field, A. Svatos, and J. A. Pickett. 2009. High-throughput ESI-MS analysis of binding between the *Bombyx mori* pheromone-binding protein BmorPBP1, its pheromone components and some analogues. Chem. Commun. (Camb): 5725-7.

Horst, R., F. Damberger, P. Luginbuhl, P. Guntert, G. Peng, L. Nikonova, W. S. Leal, and K. Wuthrich. 2001. NMR structure reveals intramolecular regulation mechanism for pheromone binding and release. Proc. Natl. Acad. Sci. U.S.A. 98: 14374-14379.

Ishida, Y., and W. S. Leal. 2005. Rapid inactivation of a moth pheromone. Proc. Natl. Acad. Sci. U.S.A. 102: 14075-9.

Ishida, Y., and W. S. Leal. 2008. Chiral discrimination of the Japanese beetle sex pheromone and a behavioral antagonist by a pheromone-degrading enzyme. Proc. Natl. Acad. Sci. U.S.A. 105: 9076-80.

Ishida, Y., A. J. Cornel, and W. S. Leal. 2002. Identification and cloning of a female antenna-specific odorant-binding protein in the mosquito *Culex quinquefasciatus*. J. Chem. Ecol. 28(4): 867-71.

Kain, P., S. M. Boyle, S. K. Tharadra, T. Guda, C. Pham, A. Dahanukar, and A. Ray. 2013. Odour receptors and neurons for DEET and new insect repellents. Nature 502(7472):507-12.

Kaissling, K.-E. 2013. Kinetics of olfactory responses might largely depend on the odorant–receptor interaction and the odorant deactivation postulated for flux detectors. J. Comp. Physiol. DOI: 10.1007/s00359-013-0812-z

Kaissling, K.-E., and E. Priesner. 1970. Die Riechschwelle des Seidenspinners. Naturwissenschaften 57: 23-28.

Keil, T. A. 1984. Surface coats of pore tubules and olfactory sensory dendrites of a silkmoth revealed by cationic markers. Tissue Cell 16: 705-17.

Kennedy, J. S., and D. Marsh. 1974. Pheromone-regulated anemotaxis in flying moths. Science 184: 999-1001.

Larsson, M. C., A. I. Domingos, W. D. Jones, M. E. Chiappe, H. Amrein, and L. B. Vosshall. 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43: 703-14.

Leal, W. S. 1996. Chemical communication in scarab beetles: Reciprocal behavioral agonist-antagonist activities of chiral pheromones. Proc. Natl. Acad. Sci. U.S.A. 93: 12112-12115.

Leal, W. S. 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu. Rev. Entomol. 58: 373-91.

Leal, W. S., Y.-M. Choo, P. Xu, C. S. B. d. Silva, and C. Ueira-Vieira. 2013. Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. Proc. Natl. Acad. Sci. U.S.A.: in press.

Leal, W. S., A. M. Chen, Y. Ishida, V. P. Chiang, M. L. Erickson, T. I. Morgan, and J. M. Tsuruda. 2005. Kinetics and molecular properties of pheromone binding and release. Proc. Natl. Acad. Sci. U.S.A. 102: 5386-91.

Leary, G. P., J. E. Allen, P. L. Bunger, J. B. Luginbill, C. E. Linn, Jr., I. E. Macallister, M. P. Kavanaugh, and K. W. Wanner. 2012. Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. Proc. Natl. Acad. Sci. U.S.A. 109: 14081-6.

Lee, Y., S. H. Kim, and C. Montell. 2010. Avoiding DEET through insect gustatory receptors. Neuron 67: 555-61.

Meinwald, J., Y. C. Meinwald, J. W. Wheeler, T. Eisner, and L. P. Brower. 1966. Major components in the exocrine secretion of a male butterfly (Lycorea). Science 151: 583-585.

Nichols, A. S., and C. W. Luetje. 2010. Transmembrane segment 3 of *Drosophila melanogaster* odorant receptor subunit 85b contributes to ligand-receptor interactions. J. Biol. Chem. 285: 11854-62.

Raina, A. K., and J. A. Klun. 1984. Brain factor control of sex pheromone production in the female corn earworm moth. Science 225: 531-533.

Sandler, B. H., L. Nikonova, W. S. Leal, and J. Clardy. 2000. Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. Chem. Biol. 7: 143-151.

Schneider, D. 1969. Insect olfaction: deciphering system for chemical messages. Science 163: 1031-1037.

Syed, Z., and W. S. Leal. 2008. Mosquitoes smell and avoid the insect repellent DEET. Proc. Natl. Acad. Sci. U.S.A. 105: 13598-603.

Tumlinson, J. H., M. G. Klein, R. E. Doolittle, T. L. Ladd, and A. T. Proveaux. 1977. Identification of the female Japanese beetle sex pheromone: inhibition of male response by an enantiomer. Science 197: 789-92.

Vogt, R. G., and L. M. Riddiford. 1981. Pheromone binding and inactivation by moth antennae. Nature 293: 161-163.

Wojtasek, H., and W. S. Leal. 1999. Conformational change in the pheromone-binding protein form *Bombyx mori* induced by pH and by interaction with membrane. The Journal of Biological Chemistry 274: 30950-30956.

Xu, P., and W. S. Leal. 2013. Probing insect odorant receptors with their cognate ligands: insights into structural features. Biochem. Biophys. Res. Commun. 435: 477-82.

Xu, P., A. M. Hooper, J. A. Pickett, and W. S. Leal. 2012a. Specificity determinants of the silkworm moth sex pheromone. PLoS One 7: e44190.

Xu, P., S. F. Garczynski, E. Atungulu, Z. Syed, Y. M. Choo, D. M. Vidal, C. H. Zitelli, and W. S. Leal. 2012b. Moth sex pheromone receptors and deceitful parapheromones. PLoS One 7: e41653.

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