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1 **Progress in the use of genetic methods to study insect behavior outside *Drosophila***

2

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12

13 Abstract

14 In the span of a decade we have seen a rapid progress in the application of genetic tools and genome editing
15 approaches in “non-model” insects. It is now possible to target sensory receptor genes and neurons, explore
16 their functional roles and manipulate behavioral responses in these insects. In this review, we focus on the latest
17 examples from Diptera, Lepidoptera and Hymenoptera of how applications of genetic tools advanced our
18 understanding of diverse behavioral phenomena. We further discuss genetic methods that could be applied to
19 study insect behavior in the future.

20 Introduction

21 Insects are the most numerous and diverse animal taxa on the planet. They have evolved different adaptations
22 in sensory function and neural circuitry towards performing basic behavioral tasks such as finding mates, food
23 and oviposition sites [1]. The availability of advanced genetic tools in *Drosophila melanogaster* has allowed us
24 to perform sophisticated genetic experiments to investigate everything from gene expression to brain and
25 behavior. The vinegar fly is a wonderful model for investigating odor- and light-directed locomotion and
26 courtship, but offers little insight into pollination, phyto- or haemotophagy and eusociality. From the perspective
27 of meeting the global challenges of the 21st century *D. melanogaster* fails as it is neither a crop pest nor a
28 disease vector and we are only beginning to understand its natural behaviors [2]. Paradoxically, only limited
29 genetic tools are employed for insects with better studied behavior in their ecological context [3]. The
30 application of genetic techniques in non-drosophilids is often directed by pioneering *D. melanogaster* studies
31 that have uncovered phenotypes for candidate homologous gene targets. However, while a genetic tool can
32 often be successfully ported between species (e.g. fluorescent markers [4], gene editing [5,6] or transgene
33 binary expression systems [7–9]), the behavioral outcome of targeting homologous genes is less predictable
34 and needs to be studied on species-by-species basis [e.g. 10–13].

35 A number of recent studies have employed gene silencing or editing in Diptera, Lepidoptera, Hymenoptera,
36 Orthoptera, Hemiptera, Coleoptera and Blattodea (**Table 1, Box 1**) in order to understand their behaviors. The
37 majority of these studies focused on olfaction, reflecting its importance for insect fitness and survival, and the
38 multi-sensory nature of many natural behaviors. These advances herald exciting times in studying the genetic
39 basis of insect behaviors, with increased focus on the organism itself and reduced focus on its use as a genetic
40 model. Here we review the latest progress in the use of genetic tools in behavioral studies, taking a closer look
41 at insect-plant interaction, social behaviors, human host-seeking and oviposition. We also highlight studies that
42 can potentially help decipher the neuronal basis of behavior.

Box 1. Overview of genetic methods

RNA-interference (RNAi)

The technique of suppressing gene transcription through the application of RNA-interference (RNAi) was first described in 1998 in the nematode *Caenorhabditis elegans* [14]. Since its discovery, this technique has been widely used in insects [15–20] to silence genes of interest by exogenous or endogenous delivery of double-stranded RNA (dsRNA) or small interfering RNA (siRNA). In target cells, dsRNA is cleaved to siRNA by the Dicer enzyme and is incorporated into the RNA-induced silencing complex (RISC) to direct degradation of complementary endogenous mRNA of the targeted gene. The flexibility of this technique is partially attributed to the fact that only the sequence of the gene, and not that of its chromosomal location or regulatory untranslated regions, is required to design dsRNAs. However, the technique is susceptible to variable or no results depending on insect species, gene, tissue, and method of delivery [16,21,22]. Yet, the ability to reduce (knock-down) and not completely ablate (knock-out) the function of a targeted gene at different stages of development permits the analysis of early regulators of sensory behaviors that are also essential for overall survival. Multiple methods of delivery, such as injections at any developmental stage, feeding, transgenic expression and soaking (**Fig. 1**), enable manipulation of insect behaviors in laboratory and the field for research or pest control purposes. Interestingly, pre-blastoderm embryo injections of mRNA has been successfully applied as a forward genetics approach to upregulate gene expression to study sex determination in mosquitoes [23].

Directed mutagenesis by ZFNs, TALENs and CRISPR/Cas9

Reverse genetics is central to associating genes with a biological function. Classic methods of altering genomic DNA using X-rays to induce chromosomal breakage *in situ* revolutionized our ability to associate a particular genomic locus with a behavior [24]. Later, genome sequencing helped map these loci to specific genes and, most recently, gene function was explored through targeted mutagenesis. Targeting a particular gene of interest became feasible for non-model organisms with the help of Zinc-finger nucleases (ZFNs) [25,26] and Transcription activator-like effector nucleases (TALENs) [27]. However, costs and time associated with engineering these proteins prevented a quick adoption of these methods. The discovery of CRISPR/Cas9 system, the part of the bacterial adaptive immune system [5,6,28], permits a fairly quick and inexpensive mechanism for the targeted modification of DNA with the ability to generate deletions from a single base pair to hundreds of kilobase pairs [29]. It is currently the fastest and most effective method of genome editing in diverse organisms from bacteria to human [30]. The application of the technique requires a source of Cas9 protein and the custom-designed guide-RNAs (sgRNAs) that are complimentary to the gene of interest. The sgRNAs bind the Cas9 and deliver it to the desired location in the genome. Cas9 induces a double-stranded break that is naturally repaired either through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanism. The latter mechanism allows researchers to design specific DNA homology templates surrounding the repair site, adding elements such as transgenes to be incorporated into the target site. In most cases, components of the CRISPR/Cas9 system (Cas9, sgRNAs and a DNA homology template) are injected into a pre-blastoderm embryo. To overcome high costs and workload, and embryo lethality associated with injection, new methods of delivery directly into a gravid female are now being developed [31,32].

Since its introduction as a gene editing tool in 2012, CRISPR/Cas9 system has advanced research in many insect species, including flies [33–38], sandflies [39], mosquitoes [40–43], moths [13,44–47], butterflies [48,49], crickets [50], locusts [51], planthoppers [52], honeybees [53,54], wasps [55], ants [11,12], beetles [56], aphids [57] and psyllid bugs [31].

Transposon Mutagenesis

The workhorse of *Drosophila melanogaster* genetics is the P-element [58]. The P-element is a sequence of nucleotides recognized by a transposase found in wild *Drosophila* and applicable for insertion based mutagenesis in lab strains of *D. melanogaster*. These transposable elements allow researchers to insert genes and gene reporters into the germ line of the vinegar fly driving research in reverse and forward genetics. However, the p-element is narrowly applicable to other insect species. The piggyBac transposable element was discovered in the cabbage looper moth, *Trichoplusia ni* [59,60] and has been applied broadly to generate random insertions of transgenes in non-model insects [61].

Insect-plant interaction

Insects and plants have co-evolved for approximately 400 million years and many insects rely on the sensory perception of plant cues to elicit quick and adaptive behaviors [62]. Plants are also not passive in these interactions, exemplified by the diversity of flower colors or plant odors driven by the selectivity of their pollinators or voraciousness of their pest.

The crepuscular hawkmoth *Manduca sexta* uses both visual and olfactory cues to locate its host plant, the Western Jimsonweed, *Datura wrightii* [3,13], which produces a relatively large, white upright trumpet flower with a strong odor bouquet. Mediating the detection of this floral bouquet are a subset of diversely evolved insect chemosensory receptors, one group of which is encoded by the odorant receptor (OR) genes. The ORs form ligand-gated cation channels with a highly-conserved insect co-receptor ORCO [10,63], and determine the channel's odorant-binding specificity. The ORCO gene is thus necessary for proper function of most olfactory sensory neurons in an insect. Mutating ORCO provides a means of shutting down a large portion of the olfactory system and evaluating its importance in behaviors in insects (**Table 1**). Recently, CRISPR/Cas9 was used to generate an ORCO knock-out (KO) in *M. sexta* [13]. Wind tunnel experiments on ORCO mutants demonstrated that while the nectar-filled and fragrant flower provides a strong visual cue, ORCO-dependent olfaction is needed to complete the sensory behavior of hovering, unfurling the proboscis, and feeding [13]. Interestingly, ORCO-independent sensory processes, such as vision, perception of humidity, and CO₂ do not compensate for the innate behavior involved in seeking out the *Datura* flower (**Fig 2A**). This study also investigated the role of ORCO in hawkmoth plant-seeking for oviposition. The hawkmoth caterpillar is an herbivore and *Datura* is a preferred food source, often to the detriment of the plant. A gravid female hawkmoth evaluates a suitable host plant via olfactory cues from plant leaves [64], and this host-seeking behavior is significantly disrupted in ORCO mutants. However, a number of gravid ORCO-mutant *M. sexta* were still able to locate their host [13], implying that other ORCO-independent olfactory cues may direct this host plant seeking behavior. Thus, the hierarchy of sensory cues and the mode of their integration may vary in multi-sensory behaviors and can only be understood by testing a reverse genetic phenotype in a semi-natural environment. Further implementation of genetic methods, e.g. live imaging of neuronal responses as done in mosquitoes [65,66], could help us understand the gene-specific representation of sensory cues in an insect's brain.

Contact chemoreception mediated by gustatory receptors (GRs) is important for oviposition in many insects, especially in Lepidoptera [67]. Female swallowtail butterflies *Papilio xuthus* evaluate the suitability of a substrate while drumming their front legs against the leaves of their Rutacea (citrus) plant host. Synephrine, a citrus plant alkaloid, induces a physiological response in the female tarsi [68]. Gustatory receptor *PxutGR01* was found to be expressed only in females and respond to synephrine when heterologously expressed in an insect cell line [67]. An injection of dsRNA in the pupae downregulated the *PxutGR01* transcript and physiological response to synephrine was reduced in the tarsi of adults. While there was no change in the drumming activity, the oviposition behavior in response to synephrine was reduced in the knock-down individuals, demonstrating that this GR is responsible for the evaluation of synephrine. Laying eggs on the right plant is important since caterpillars need to overcome the plant's defense mechanisms and feed the moment they hatch, and not all leaves provide adequate nutrients to support growth and development. The peripheral sensory system mediating this choice has been studied further in the monophagous silkworm, *Bombyx mori*. This moth is cultivated for its silk cocoons and has been a Lepidopteran model for the development of genetic tools [69]. Additionally, many behaviorally abnormal strains of *B. mori* have been cultivated and their genetic loci characterized. The silkworm feeds exclusively on the leaves of the common mulberry plant and a specific cultivated strain was found to have an abnormal food preference. A putative bitter sensing gustatory receptor *BmorGr66* was identified within the mapped genetic loci of the abnormal strain [70]. The application of CRISPR/Cas9 to mutate the *BmorGr66* led to the silkworm accepting foods like fruits and grains in addition to mulberry leaves [70]. Electrophysiological analyses of the mutants did not reveal any general sweet or bitter contact chemoreception deficit; the ligand for mulberry leaf preference remains to be identified. CRISPR/Cas9 mutation of ORCO in *B.mori* silkworms also determined that OR-related olfaction was important for feeding behaviors; the ORCO-mutants had trouble localizing the mulberry leaves in a test arena [71]. These studies highlight the importance of single sensory receptors for complex phenomena like foraging preference. Identification of genes like *BmorGr66* may further instruct genetic pest control strategies.

146 Social interactions

147

148 Eusocial insects live in complex societies and interact with each other in fascinating ways to maintain social
149 integrity [72,73]. However, understanding the genetic basis of these sensory behaviors has been hindered by
150 the lack of genetic tools, which are particularly difficult to establish when only very few female individuals
151 reproduce sexually, and generation times often span many months. Moreover, these females often have to be
152 isolated to start new colonies. Genetic crossing and outcrossing routines are thus difficult to achieve in eusocial
153 insects in a laboratory setting.

154 Pheromones play a crucial role in regulating social behaviors in eusocial Hymenoptera like ant communities.
155 Antennal olfactory neurons respond to conspecific cuticular hydrocarbons in *Camponotus floridanus* ants [74],
156 and ORCO-dependent receptors of the ponerine ant *Harpegnathos saltator* respond to its cuticular
157 hydrocarbons and pheromones when ectopically expressed in *Drosophila* [75]. Recent studies have taken the
158 next step in genetic characterization of the role of olfaction for intraspecific communications of two species of
159 ants, using CRISPR/Cas9 for the first time in eusocial insects to mutate the ORCO gene [11,12]. Workers of
160 *Harpegnathos saltator* present a unique advantage that facilitates a genetic modification – all workers of this
161 species normally mate, and can take over the queen's place after the queen dies or is removed from the colony
162 [11]. The unmated workers may thus lay haploid eggs that develop into males, or the workers may be allowed
163 to mate and lay diploid eggs that produce females. Another ant species, the clonal raider ant *Ooceraea biroi*
164 was selected for these experiments because it reproduces asexually via parthenogenesis, thus overcoming the
165 obstacles related to difficult genetic crosses [12]. In addition, these ants are blind, which simplifies analysis of
166 their behaviors in response to multisensory cues.

167 ORCO mutant individuals of two species showed deficiencies in olfactory response to pheromones and other
168 volatiles, and abnormal social behaviors (**Fig 2B**). For instance, the ORCO-mutant *O. biroi* could not detect and
169 follow the pheromone trail to their nest, spending a significant amount of time wandering. Additionally, a
170 permanent Sharpie marker line drawn on a surface often deters wild type ants from approaching the line,
171 however, the ORCO-mutants were not repelled and often crossed these lines [12]. Both studies also reported a
172 surprising defect in olfactory neurodevelopment. The ORCO-mutants exhibited a dramatic reduction of olfactory
173 receptor neurons in the antennae [12] and the number of glomeruli in their antennal lobe. Interestingly, ORCO
174 mutation leads to no visible changes in the brain of *Drosophila* [10], and only minor reduction in the relative
175 volume of pheromone-specific glomeruli in *M. sexta* [13]. These results reveal that olfactory neurodevelopment
176 in the ant is largely dependent on the presence of functional ORCO, and raise intriguing questions about the
177 role of ORCO and other olfactory genes in neurodevelopment of other insects.

178 Within the ant colony necrophoric behavior, the removal of dead individuals from the nest by workers, is an
179 important innate behavior that is triggered by olfactory cues from dead individuals [76]. A study on the red
180 fire ant *Solenopsis invicta* showed that a chemosensory protein gene *Si-CSP1*, which is highly expressed in the
181 antenna of workers, is involved in detecting volatile oleic and linoleic acids from dead nestmates and in
182 regulating the necrophoric behavior of *S. invicta* workers. The behavior was suppressed by RNAi through
183 feeding with siRNA mixed into sugar water [77], demonstrating that siRNA feeding is a feasible method of
184 genetic intervention in red fire ants, and could even be a means of population control.

185 Attempts are currently underway to introduce genetic tools into another eusocial insect, the honeybee.
186 CRISPR/Cas9-mediated genetic editing of the *major royal jelly protein 1 (MRJP1)* gene and a mushroom-body-
187 specific protein *mKast* were successful (as verified by genotyping) in honeybees but did not affect the normal
188 development of drones [53,54]. These studies pave way for generating genome-edited honeybee workers for
189 investigating their neurodevelopment, innate and learnt behaviors. This work is especially exciting given the
190 economic importance of honeybees as pollinators and the long history of learning and memory studies on
191 honeybees.

192

193 Human host-seeking

194

195 Insects are vectors of malaria, Zika, Dengue, yellow fever, Chagas and other lethal diseases. Female mosquitoes
196 (**Fig 2C**) and triatomine bugs [78] target their human and animal hosts in order to obtain a blood meal and
197 develop their eggs. Zinc-finger nucleases (ZFNs) were used to mutagenize the ORCO [25] and CO₂ co-receptor

198 GR3 [26] of *Aedes aegypti* mosquitoes. The mutations impaired the mosquitoes' ability to detect components of
199 human body odor and CO₂, but still left them able to find humans. Mosquitoes with a CRISPR/Cas9-generated
200 mutation for ionotropic olfactory co-receptor, *IR8a*, were impaired in their ability to respond to acidic odorants
201 that are components of human sweat [79]. This mutation also significantly reduced attraction of female
202 mosquitoes to a human arm. The attraction was reduced further, but not abolished, in mosquitoes carrying two
203 mutations, *IR8a+ORCO* or *IR8a+GR3* indicating that other cues outside chemosensation mediate attraction to
204 humans. One of these cues is human body heat. For example, mutation in *Aedes TRPA1* gene affects the
205 mosquitoes' preference for human body temperature (40 °C) and avoidance of warmer objects (50-55 °C)
206 [80]. These studies have highlighted the multisensory and additive nature of sensory cues mosquitoes employ in
207 finding humans (summarized in [81]), drawing certain parallels with plant host-seeking in hawkmoths.

208 Interestingly, female *Aedes aegypti* mosquitoes discontinue host-seeking for four days after a blood meal, and
209 resume after the eggs have been laid. A recent study has discovered that human neuro-peptide Y (NPY) Y2
210 receptor agonists efficiently target the *Aedes* NPY-like receptor 7 (NPYLR7), suppressing mosquito attraction to
211 humans [82]. NPY antagonists had the opposite effect, leading to increased host-seeking. Mosquitoes that
212 carried a CRISPR/Cas9-induced mutation in NPYLR7 resumed host-seeking only one day after the blood meal,
213 in contrast to four days in wild-type mosquitoes. A drug screen, conducted on wild-type and NPYLR7-mutant
214 mosquitoes, identified six NPYLR7-specific agonists that suppress mosquito attraction to humans. These findings
215 suggest an exciting new pathway for behavioral analysis of mosquitoes and the potential for vector disease
216 control by deploying mosquito drug feeders.

217 The neuronal circuits that underlie mosquito host-seeking are currently unknown. Thus the next step is to
218 investigate how multimodal sensory stimuli and systemic signals are processed in the mosquito brain. First steps
219 in this direction have been taken in *Anopheles gambiae* [83,84] and *Aedes aegypti* [85] by employing the
220 fluorescent calcium indicator GCaMP to image live neuronal responses from the peripheral organs and the brain
221 of mosquitoes. The same approach has been taken to study *Aedes* oviposition choices [65,66].

222

223 Oviposition

224

225 *Drosophila* neuroscience heavily relies on transgenic lines, and in particular on three orthogonal binary
226 expression systems (reviewed in [86]). Reporter transgenes are especially useful for labelling neurons,
227 monitoring or manipulating their functional responses. Generating transgenic lines in other insects has, until
228 recently, been hampered by the need to identify and clone out the native enhancer and promoter region for
229 the gene of interest (although see [83,85,87,88]). The advance of CRISPR/Cas9 has removed this requirement,
230 and now allows us to introduce a transgene, with a T2A or a similar linker, immediately into or after the coding
231 sequence of a gene [89]. By using live Ca²⁺ imaging of genetically encoded activity indicators, we can now
232 investigate the neuronal basis of the observed behavioral phenotypes. This method has been elegantly used to
233 study oviposition choices in *Aedes aegypti* [65,66] (Fig 2D). Gravid mosquito females lay their eggs in or near
234 water sources, because their larvae and pupae are aquatic. Female *Aedes*, mutant for the *pickpocket* cation
235 channel subunit gene *ppk30*, lay fewer eggs and fail to avoid water with high salinity that is harmful for their
236 larvae [66]. Live Ca²⁺ imaging of ventral nerve cord that is innervated by *ppk30* expressing neurons from the
237 mosquito legs, has shown that these neurons responds both to water and to NaCl, implying that there must be a
238 parallel neuronal pathway that prevents oviposition in salty water in wild-type *Aedes*. Live Ca²⁺ imaging from
239 the *Aedes* antennal lobe has been used to observe sparse neuronal responses to geosmin, an oviposition
240 attractant [65]. The preference for geosmin has been abolished in ORCO mutant mosquitoes, indicating that, as
241 in *Drosophila*, geosmin binds an ORCO-dependent receptor. *Drosophila*, however, find geosmin repulsive [90]
242 and avoid it in oviposition and other assays.

243

244 In summary, these latest studies have shed light on general principles that guide insect behavior. Not surprisingly,
245 complex behaviors such as host-seeking and oviposition in moth and mosquitoes are controlled by multisensory
246 cues. The relative importance of these cues is different for different behaviors, and depends on the internal
247 state of the animal (fed, hungry, host-seeking, etc). Mutations of the highly conserved ORCO gene in different
248 species lead to strikingly different developmental and behavioral consequences, highlighting the necessity of

249 an era of comparative genetic studies. These are also instrumental for the development of pest and disease
250 vector control strategies.

251

252 Outlook

253 CRISPR/Cas9 provides a unique opportunity to use gene editing to study the molecular and neuronal basis of
254 insect behavior, ranging from sensory perception to memory formation and retrieval [50,91]. Either mutating a
255 gene of interest or simultaneously introducing transgenes into precisely defined locations with CRISPR/Cas9
256 would permit the functional re-programming of neurons. The successful use of transgenes to monitor neuronal
257 responses in mosquitoes will be undoubtedly followed by similar studies in other non-model insect organisms.
258 Work on *Drosophila* has developed multiple methods for activating or silencing neurons by ectopic expression
259 of sensory receptors or ion channels [86,92]. These techniques are now being adapted to other insects [e.g. 93],
260 promising us greater understanding of the neural basis of insect behaviors.

261 Gene knockouts deliver a unique opportunity for observing the comparative evolution of gene function. For
262 example, the ORCO gene knock out has been generated in 8 species (**Table 1**). ORCO is a highly conserved
263 gene with putative chaperone function and forms functional co-receptors with the highly diverse ORs. OR gene
264 numbers range from 10 to > 300 across species and are tuned to diverse natural ligands [1,94]. The Orco KO
265 has consistently demonstrated disrupted neurophysiological responses to a range of odorants and pheromones.
266 However, insect OR-mediated behaviors distinctively integrate with other sensory modalities (**Fig 2**). For
267 instance, copulation behaviors continue to occur in ORCO KO *D. melanogaster*, presumably through the flexible
268 multi-sensory nature of their mating cues. On the other hand, the strict OR-mediated perception of pheromones
269 is critical for copulation behaviors in some Lepidoptera [13,47]. A more striking example involves the role of
270 ORCO in neurodevelopment, where the loss of ORCO leads to dramatic reduction and loss of olfactory glomeruli
271 and olfactory sensory projections from the antennae, indicating a developmental role for ORCO via an unknown
272 mechanism in ants [11,12]. The application of genetic techniques to other genes and their respective homologues
273 will no doubt advance our understanding of many novel biological phenomena based on expanding
274 comparative observations.

275 However, care should be taken in understanding certain unforeseen effects of current gene editing techniques.
276 CRISPR-Cas9 may introduce unintended mutations beyond the targeted genes and *in silico* methods of off-
277 target detection are often unverified in current non-model organisms. These off-target effects may provide
278 misleading information for behavioral phenotypes or disrupt other factors involved in fitness or fecundity.
279 Especially when a genetic rescue lines are not feasible, techniques in testing off-target effects *in vivo* should be
280 considered. Research is quickly advancing in the development of rapid and accurate techniques applying
281 methods in next generation sequencing to identify sites that go through the natural cellular nucleotide repair
282 mechanism after CRISPR application, providing a reliable and un-biased method of off-target detection in any
283 organism [95,96].

284 Advances in genetic techniques in other insect species will also have practical implications in pest management
285 of major crop and disease vectors. For example, new methods of RNAi delivery now allow applications of it in
286 field conditions for crop protection [17,19]. Additionally, a combination of CRISPR/Cas9 and RNAi can lead to
287 the generation of more insects susceptible to RNAi transcript downregulation. Releases of sterile [97] or
288 bacteria-carrying [98] mosquitoes have been adopted as methods to limit mosquito population. Gene-drive
289 technology now allows us to propagate various genetic modifications and transgenes throughout an insect
290 population [41]. These modifications do not need to eliminate an insect population, but may also rely on
291 manipulating insect behavior, e.g. to divert them from economically important crops or from ourselves. Ultimately
292 the application of these techniques and the observations gained from different insects may provide the
293 conceptual framework to better address these challenges.

294

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300 limitations or our ignorance.

301 **Figures**

302 **Figure 1. Ways to deliver RNAi in insects.**

303 **A. Soaking.** Immersion in dsRNA solution against a detoxification enzyme gene has been successfully used in
304 adult fleas [99]. The fleas were incubated at 4C, which excludes active ingestion of dsRNAs.

305 **B. Feeding.** RNAi feeding has been applied in e.g. larval mosquitoes [100–103] and *Tribolium* [104], triatomine
306 bug nymphs [105], caterpillars [106], ants [76] and aphids [104,107]. dsRNA may be mixed directly into food
307 [76,104], or presented in the form of nanoparticles [100,101] that slow down the degradation of dsRNA.
308 Bacteria [102] and yeast [103] have been genetically engineered to produce siRNA. Finally, plants may be
309 genetically modified to produce siRNA or sprayed with dsRNA against insect genes [107].

310 **C. Injections.** Injection of dsRNA or siRNA is the most common laboratory delivery method. Injections may be
311 given at any of an insect's life stages (e.g. embryos [23,108], larvae [109–111], pupae [91,112–114], adults
312 [115,116]). While labor-intensive, this method normally provides the highest efficiency of gene silencing, with
313 the caveat that giving the injection may impair an animal's survival.

314 **D. Transgenes.** Transgenic expression of dsRNA is most commonly used in *Drosophila*, where thousands of UAS-
315 RNAi lines have been established, and may now be used by simple genetic crosses with a driver line of interest.
316 The same transgenic approach is feasible in other insects, but the need to create a stable transgenic line has so
317 far prevented its implementation.

318

319 **Figure 2. Insect behaviors, studied with genetic tools.**

320 **A. Insect-plant interactions.** Flowers provide visual and olfactory cues (odor bouquet, relative humidity and
321 CO₂), while the leaves of the plant also provide olfactory and gustatory cues for adult female butterflies and
322 moths, and their caterpillars. ORCO-mutant *Manduca sexta* moths are impaired in their foraging behaviors [13]
323 (left). Caterpillars choose their food based on its taste. *Bombyx mori* caterpillars with mutated GR66 receptor
324 expanded their food preference from mulberry leaves to fruit and grains [70] (right).

325 **B. Social behaviors.** Social behaviors of ants heavily rely on olfactory perception of pheromones. Recent studies
326 have shown that ORCO-mutant ants are seriously impaired in their social interactions, indicating that ORCO-
327 dependent olfactory receptor neurons are necessary for pheromone perception [11,12].

328 **C. Human host-seeking.** Female mosquitoes, as moths, integrate multiple sensory cues to find their human host.
329 Mutations in ORCO [25], GR3 [26] and IR8a [79] receptors that detect human body odors, CO₂ and acidic
330 components of human sweat respectively, have significantly reduced the ability of *Aedes aegypti* to find humans.

331 **D. Oviposition.** Female mosquitoes lay their eggs in or near water, and their larvae and pupae develop in
332 water. Thus, oviposition sites need to be carefully selected by the females. Two recent studies have found that
333 *Aedes aegypti* mosquitoes prefer to lay their eggs in geosmin-scented water [65], and tend to avoid salty water
334 [66]. Neurons that respond to salt and water were found in the mosquitoes' legs, and geosmin-sensing neurons
335 – in the antennae.

336

337 **References**

338 * of special interest

339 ** of outstanding interest

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Table 1. Applications of genetic methods to study behavior (non-exhaustive list)

Order	Species	Method	Target	Phenotype	Reference
Diptera	<i>Aedes aegypti</i>	chitosan-siRNA nanoparticle feeding	<i>SEMA1a</i>	Impaired larval light avoidance due to improper targeting of photoreceptor neurons	[100]
	<i>Aedes aegypti</i>	chitosan-siRNA nanoparticle feeding	<i>SEMA1a</i>	Impaired larval yeast attraction due to improper targeting of olfactory neurons	[101]
	<i>Aedes aegypti</i>	CRISPR/Cas9; RNAi injection	<i>DOP1</i>	Impaired olfactory learning	[91]
	<i>Aedes aegypti</i>	ZFN	<i>ORCO</i>	Loss of OR-mediated olfaction. Disrupted host localization	[25]
	<i>Aedes aegypti</i>	ZFN	<i>GR3</i>	Impaired CO ₂ detection and host localization	[26]
	<i>Aedes aegypti</i>	ZFN	<i>TRPA1</i>	Impaired avoidance of high temperatures	[80]
	<i>Aedes aegypti</i>	CRISPR/Cas9	<i>PPK301</i> (also 304, 216, 306)	Impaired oviposition decisions in response to salty water	[66]
	<i>Aedes aegypti</i>	CRISPR/Cas9	<i>NPYLR7</i>	Abnormal host-seeking after a recent blood meal	[82]
	<i>Aedes aegypti</i>	CRISPR/Cas9	<i>IR8a</i>	Impaired detection of lactic acid and host localization	[79]
	<i>Aedes aegypti</i>	GCaMP imaging	<i>ORCO, Ubi-GCamp6s</i>	Olfactory responses to geosmin observed <i>in vivo</i> . Demonstration that geosmin is oviposition attractant.	[65]
	<i>Aedes aegypti</i>	GCaMP imaging	<i>Ubi-GCamp6s</i>	In vivo recordings from antennal and optic lobes, evidence of visual - olfactory integration.	[117]
	<i>Anopheles gambiae</i>	RNAi injection	<i>OR7, OR40, IR76b</i>	Impaired larval olfactory behavior	[109]
	<i>Anopheles gambiae</i>	RNAi injection	<i>TRPA1</i>	Impaired larval thermotaxis	[110]
	<i>Culex quinquefasciatus</i>	RNAi injection	<i>OR37, OR99</i>	Impaired oviposition preference for 4-ethylphenol	[113]
Lepidoptera	<i>Spodoptera littoralis</i>	CRISPR/Cas9	<i>ORCO</i>	Disrupted antennal function towards plant host and pheromone volatiles. Disrupted mating.	[47]
	<i>Spodoptera exigua</i>	RNAi injection	<i>Se-uv, Se-bl, Se-lw</i>	Phototaxis towards green light	[118]
	<i>Manduca sexta</i>	CRISPR/Cas9	<i>ORCO</i>	Disrupted plant host localization and foraging behaviors. Disrupted mating	[13]
	<i>Ostrinia furnacalis</i>	TALEN	<i>ORCO</i>	Ablated pheromone response	[119]
	<i>Bombyx mori</i>	CRISPR/Cas9	<i>GR66</i>	Feeding assay used to determine a gustatory receptor involved in the deterring generalist feeding behavior	[70]
	<i>Bombyx mori</i>	CRISPR/Cas9	<i>ORCO</i>	Pheromone detection	[71]
	<i>Papilio xuthus</i>	RNAi	<i>PxutGr01</i>	Tarsal contact chemosensation of plant host compounds	[68]

	<i>Helicoverpa armigera</i>	RNAi injection	Sex peptide receptor	Oviposition and ovary development	[120]
	<i>Danaus plexipus</i>	ZFN, TALENs, CRISPR/Cas9	CRY2, CLK	Group eclosion behavior	[49, 21]
	<i>Heliconius melpomene</i> , <i>Heliconius cydno</i>	QTL analysis		Mating preference	[122]
Hymenoptera	<i>Ooceraea biroi</i>	CRISPR/Cas9	ORCO	Deficiencies in social behavior and fitness. Disrupted antennal lobe development	[12]
	<i>Harpegnathos saltator</i>	CRISPR/Cas9	ORCO	Deficiencies in social behavior and fitness. Disrupted antennal lobe development	[11]
	<i>Solenopsis invicta</i>	RNAi feeding	Si-CSP1	Chemosensory protein, involved in necrophoric behavior	[76]
	<i>Nasonia vitripennis</i>	Genetic crosses, RNAi, hybrids, QTL analysis	NV10127-29	Production and perception of male sex pheromone components	[123]
	<i>Nasonia vitripennis</i>	Hybrids, genotyping		Egg-laying preference	[124]
Orthoptera	<i>Locusta migratoria</i>	CRISPR/Cas9	ORCO	Olfactory response to conspecifics	[51]
	<i>Locusta migratoria</i>	RNAi injection	CSP3, TO1	Olfactory response to conspecifics	[125]
	<i>Gryllus bimaculatus</i>	CRISPR/Cas9	DOP1	Appetitive and aversive olfactory learning	[50]
	<i>Gryllus bimaculatus</i>	RNAi injection	OA1, DOP1, DOP2	Appetitive and aversive learning	[126]
Coleoptera	<i>Tribolium castaneum</i>	RNAi injection	TRP channels	Motor behaviors based on anatomical defects of hind leg folding; tonic immobilization	[127]
	<i>Tribolium castaneum</i>	RNAi injection	TcT6H	Mobility	[128]
	<i>Tribolium castaneum</i>	RNAi injection	TRPA1	Thermotaxis	[129]
	<i>Tenebrio molitor</i>	RNAi injection	ORCO	Impaired mate recognition	[130]
Hemiptera	<i>Rhodnius prolixus</i>	RNAi injection	ORCO	Impaired host localization, ecdysis, survival, oviposition rate and blood ingestion	[78]
	<i>Nilaparvata lugens</i>	RNAi injection	CSP8	Decreased olfactory attraction	[131]
	<i>Laodelphax striatellus</i>	RNAi feeding	ORCO	Olfactory host-seeking	[132]
Blattodea	<i>Periplaneta americana</i> , <i>Blattella germanica</i>	RNAi injection	CRY1, CRY2, TIMELESS	Responses to magnetic field	[133]
	<i>Periplaneta americana</i>	RNAi injection	Opsins Trp Channels	Electrophysiological characterization of phototransduction	[134]