

Review

A molecular toolkit for superorganisms

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Social insects, such as ants, bees, wasps, and termites, draw biologists' attention due to their distinctive lifestyles. As experimental systems, they provide unique opportunities to study organismal differentiation, division of labor, longevity, and the evolution of development. Ants are particularly attractive because several ant species can be propagated in the laboratory. However, the same lifestyle that makes social insects interesting also hampers the use of molecular genetic techniques. Here, we summarize the efforts of the ant research community to surmount these hurdles and obtain novel mechanistic insight into the biology of social insects. We review current approaches and propose novel ones involving genomics, transcriptomics, chromatin and DNA methylation profiling, RNA interference (RNAi), and genome editing in ants and discuss future experimental strategies.

Molecular biology in ants

Most studies that investigate the mechanistic detail of how cells and organisms function are performed in model species, such as yeast, *Drosophila*, and mice. Research on these easy-to-breed species laid the foundation of modern biology. As studies in model organisms rely on decades of knowledge acquired by thousands of laboratories and on vast community resources, performing similar research in non-model organisms may seem a daunting task. However, recent technological advances have provided new and improved experimental tools that can be applied to a great variety of species. Investigations in non-model organisms bring value because gene functions evolve rapidly, and studying the same process in different organisms always has potential to provide novel mechanistic insight [1]. More importantly, some species have interesting traits not present in model organisms.

Social insects, such as ants, bees, wasps, and termites, receive considerable attention due to their unique lifestyles. In some insect societies, the roles played by different individuals are so segregated and specialized that their colonies are sometimes called 'superorganisms', drawing an analogy between individuals in the colony and organs in the body. Sociality in most species relies on the existence of castes, or groups of individuals with distinct roles in the colony. In many cases, members of different castes are genetically identical, yet they display different behaviors (e.g., soldiers hunt and nurses care for the brood), physiology (e.g., queens lay eggs while other castes rarely do), and lifespan (queens live considerably longer than workers) [2]. These biological properties raise many important questions. What signaling mechanisms translate environmental cues and social context into molecular cues that specify the caste in developing larvae? How broadly do the mechanisms of cellular differentiation and fate maintenance that have been discovered in cell cultures apply to whole organisms? How are the brains organized to drive altruistic behaviors in nonreproducing members of the colony? Which mechanisms allow queens to live longer than workers while reproduction is linked to decreased lifespan in most other animals? How did sociality evolve and what selective pressure acts on the underlying genes? Ants represent a particularly attractive model to address these questions owing to their diversity and the fact that some species can be propagated in the laboratory indefinitely [3–6]. Here, we summarize the tools

Highlights

While many social insects remain genetically intractable, several ant species possess features which enable their propagation in the laboratory and allow mechanistic, molecular genetic research on caste-specific behaviors and physiology, developmental plasticity, and aging.

In ants and other non-model organisms, gene expression studies have become the major source of candidate genes. The recent boom in single-cell transcriptomics complemented by chromatin-based techniques further increase the power of this approach to discover regulators of various biological processes.

Historically, experimental validation of the candidate genes in ants has been done by RNAi injections. Recently, CRISPR-Cas9 mutagenesis was performed. Next, transgenic technology will allow precise genetic manipulation and enable the use of other approaches, such as imaging of neural activity.

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of molecular biology which have been applied to ants and suggest novel alternatives and potentially useful techniques for future research. This review is intended to serve as a primer for anyone who is interested in applying molecular techniques to ants and non-model species of insects in general.

Genome assembly and annotation

The application of most molecular biological techniques requires knowing the sequence either of the gene of interest or of the entire genome. Therefore, a genome assembly is one of the first resources that needs to be generated for a new non-model species (Figure 1). Conveniently, ants tend to have small-to-intermediate genome sizes: the genomes assembled to date by the Global Ant Genomics Alliance (GAGA) range from 191 Mb to 599 Mb [7]. This means that sequencing is relatively inexpensive, and genome assembly suffers from fewer computational challenges than the assembly of the large and repeat-rich genomes of some other insects [8]. Additionally, most ant species have haploid males, and sequencing a single male helps avoid assembly problems caused by heterozygosity and haplotypes [9]. There are several approaches to *de novo* genome assembly. First, ‘classical’ short-read sequencing can generate an assembly that will be highly fragmented but will nevertheless recover the majority of genes [10,11]. However, many applications require better contiguity. It can be achieved by utilizing long reads (which yield longer contigs), linked reads or proximity ligation methods (which improve scaffolding), or a combination thereof (Table 1). A high-quality genome assembly can be constructed using Oxford Nanopore or PacBio long reads alone, as exemplified by a mosquito genome assembled using DNA from a single individual [12]. A linked-read technology, best known as the 10X Genomics’ discontinued Chromium Genome platform, can also produce contiguous, haplotype-resolved genome assemblies [13–15]. Newer, low-cost alternatives, include **single-tube long fragment reads (stLFR) and haplotagging** (see Glossary). In both cases, scaffold links can be further enhanced by proximity ligation methods such as **high-throughput chromosome conformation capture (Hi-C) and Chicago** [16,17]. Ant genome assemblies at near-chromosome resolution have been constructed by combining long reads with either stLFR or Hi-C, although using the newest PacBio long read technology (HiFi reads) alone has also yielded highly contiguous assemblies [7]. Finally, there are several ongoing massive sequencing projects that continuously generate new genome assemblies of arthropods (e.g., i5k) or ants specifically (e.g., GAGA) [7,18]. These sequencing efforts have the potential to identify the genomic basis of evolutionary innovations in ants and provide vital genomic resources for molecular biological studies in previously unexplored species. Thus, an ever-increasing number of ant genomes are being publicly released and generating a genome assembly on demand is also reasonably straightforward. The optimal benefit–cost ratio is achieved by assembling PacBio Hi-Fi or Oxford Nanopore long reads.

The most common gene annotation strategies rely on empirical evidence of gene structure provided by gene expression data, such as RNA-seq. The caveat of transcriptomics is that only a fraction of genes are detected at a given life stage in a given tissue. Therefore, best annotation accuracy is achieved when RNA-seq is performed on different developmental stages, sexes, castes, and tissues, and the data are combined [19,20]. Additionally, certain applications require accurate annotation of specific transcript features, such as alternative isoforms or untranslated regions. Long-read RNA-seq using PacBio or Oxford Nanopore, which captures whole-length transcripts, may address this and dramatically improve genome-wide analyses [21,22]. In many cases, genes can be annotated in a semi-automated fashion using the Eukaryotic Genome Annotation Pipeline. It is a comprehensive annotation workflow developed by NCBI which can be run on any publicly deposited genome assembly, as long as it satisfies all the quality requirements [23].

Glossary

Assay for transposase-accessible chromatin (ATAC-seq): chromatin is exposed to transposomes, which insert sequencing barcodes into accessible DNA more frequently than into compacted DNA. More sequences are derived from accessible regions.

Bisulfite sequencing: DNA is treated with bisulfite ions, which convert cytosines, but not 5-methylcytosines, to uracils. Treated DNA is sequenced and mapped to the genome to infer the location of 5-methylcytosines.

CBP: cyclic adenosine monophosphate response element-binding protein binding protein. A transcriptional coactivator and a histone acetyltransferase.

ChIP-seq: chromatin immunoprecipitation. Chromatin is fragmented and incubated with an antibody against the protein of interest. The antibody-chromatin complexes are pulled down, DNA is eluted and sequenced to determine where the protein was bound.

Clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9): technology used to change DNA sequence. Cas9 binds guide RNAs that direct the protein to a complementary DNA target, which Cas9 then cleaves.

CoREST: corepressor for element-1-silencing transcription factor. A transcriptional repressor.

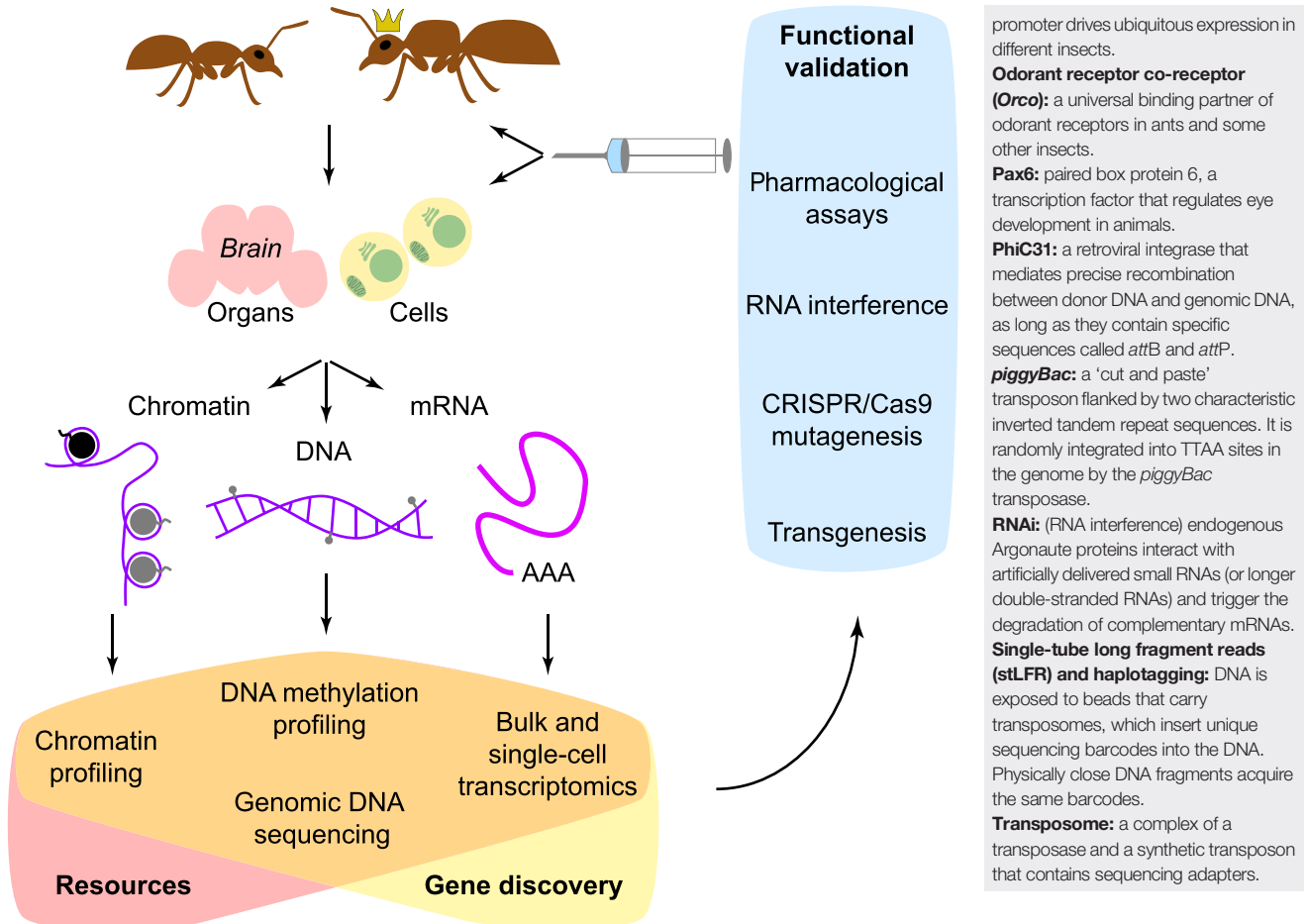
Epidermal growth factor receptor (Egfr): a member of a signaling pathway that controls growth and development.

H3K27ac: acetylated lysine-27 of the histone H3. A histone post-translational modification.

High-throughput chromosome conformation capture (Hi-C) and Chicago: native (Hi-C) or *in vitro* reconstituted (Chicago) chromatin is crosslinked, and the DNA within is fragmented, ligated, and sequenced. Physically interacting DNA fragments are recovered together.

Homology-directed repair (HDR): double-strand breaks produced by Cas9 can be repaired using homologous DNA as a template. DNA containing a custom insert flanked by sequences homologous to the ends of the double-strand break is usually co-injected with the ribonucleoprotein.

Immediate-early gene 1 (ie1): of lepidopteran nucleopolyhedroviruses. Its



Trends in Genetics

Figure 1. Experimental approaches in ant biology. See Table 1 for a summary of specific experimental techniques, their advantages, and disadvantages. Abbreviation: CRISPR/Cas9, clustered regularly interspaced short palindromic repeats-associated protein 9.

Traditional and single-cell transcriptomics

In the absence of the ability to perform genetic screens, gene expression studies have become the main source of gene candidates for the regulators of various biological phenomena in non-model organisms [24]. Transcriptomics has some drawbacks, such as difficulty in prioritizing large lists of differentially expressed genes and limited ability to detect functionally important genes that are constitutively expressed [25]. Nevertheless, it has become an invaluable tool due to its accessibility and moderate cost (Table 1). Many pioneering studies are restricted to description of gene expression profiles in different individuals or conditions, implicating specific signaling pathways in the biological process of interest [3,26–28]. Some studies are further complemented by other exploratory methods to corroborate the proposed mechanism. For example, transcriptomic profiling of the antennae, combined with molecular evolution studies, linked the emergence of complex social behaviors with the evolution of odorant receptors and odorant binding proteins [29–32]. Finally, some studies take it a step further and experimentally validate the gene candidates identified. Identification of an insulin-like peptide, encoded by the only gene consistently differentially expressed between reproductive and nonreproductive individuals across seven ant species, led to an experiment where insulin

Table 1. Summary of the techniques discussed

Class of techniques	Technique	Published examples in ants	Advantages	Disadvantages ^a	Refs
De novo genome sequencing	Illumina short reads	YES	The least expensive	Fragmented assembly, poor recovery of low-complexity regions	[11]
	PacBio HiFi or Oxford Nanopore long reads	YES	Contiguous assembly	More expensive than short reads, high molecular weight DNA required, high error rate (Oxford Nanopore)	[7]
	stLFR or haplotagging linked reads	YES (stLFR)	Highly contiguous assembly when used in combination with long reads, less expensive than proximity-based ligation	High molecular weight DNA required	[7]
	Hi-C or Chicago proximity-based ligation	YES (Hi-C)	Highly contiguous assembly when used in combination with long reads	The most expensive, high molecular weight DNA required	[7]
Transcriptomics	Bulk RNA-seq	YES	Inexpensive, deep, analysis strategies well-established	Cell-specific signals are diluted or lost	[3,26–34]
	Single-cell RNA-seq	YES	Single-cell resolution, high statistical power to identify genes with correlated expression	Expensive, high-throughput methods (10X) result in shallow sequencing, low-throughput methods (SMART-Seq) profile low numbers of cells	[38]
Chromatin profiling: accessibility	Bulk ATAC-seq	YES	Less expensive, deep	Cell-specific signals are diluted or lost, high number of reads are derived from mitochondrial DNA	[46]
	Single-cell ATAC-seq	NO	Single-cell resolution, can be combined with RNA-seq	Expensive, shallow, high number of reads are derived from mitochondrial DNA	–
Chromatin profiling: localization	ChIP-seq	YES	Golden standard, analysis strategies well-established, straightforward normalization controls	High input amount of chromatin and high sequencing depth required, the coarsest resolution, nuclei isolation required in most protocols, limited number of commercially available antibodies suitable for non-model species, custom antibodies may be expensive	[44,47,48]
	CUT&RUN	NO	Low input amount of chromatin and moderate sequencing depth required, compatible with both nuclei and cells, the highest base-pair resolution	Limited normalization strategies, protocol longer than in CUT&Tag, limited number of commercially available antibodies suitable for non-model species, custom antibodies may be expensive	–
	CUT&Tag	NO	Low input amount of chromatin and low sequencing depth required, compatible with single-cell approaches, the fastest protocol	High number of reads are derived from mitochondrial DNA, nuclei isolation preferred, limited normalization strategies, resolution coarser than in CUT&RUN, limited number of commercially available antibodies suitable for non-model species, custom antibodies may be expensive	–

Table 1. (continued)

Class of techniques	Technique	Published examples in ants	Advantages	Disadvantages ^a	Refs
DNA methylation	Bisulfite sequencing	YES	Single-nucleotide resolution	C-T conversion reduces sequence complexity which may affect alignment, nucleotide polymorphisms may be missed or misidentified as methylation events	[57–62]
Localization	Immunohistochemistry	YES	Direct and quantitative way to determine protein localization at the subcellular level	Limited number of commercially available antibodies suitable for non-model species, custom antibodies may be expensive, raising antibodies to proteins that contain hydrophobic domains may be challenging, substantial protocol optimization is required in some cases	[33,38,64,70,76,77]
	'Traditional' RNA <i>in situ</i> hybridization	YES	Less expensive than hybridization chain reaction, suitable for shorter (<1 kb) mRNAs	Limited or no information about the subcellular localization of the encoded protein, less sensitive than hybridization chain reaction	[25,64]
	<i>In situ</i> hybridization chain reaction (HCR)	YES	Highly sensitive, quantitative	Limited or no information about the subcellular localization of the encoded protein, more expensive than 'traditional' <i>in situ</i> hybridization, designing a sufficient number of probes for shorter (<1 kb) mRNAs may be impossible	[100]
Pharmacological assays	Pharmacological assays	YES	No knowledge about gene sequence is required	Limited number of commercially available compounds, specificity may be difficult to verify, little control over tissue distribution when injecting or feeding	[34,47,48,64–66]
RNA interference	Injection of double-stranded or Dicer-substrate siRNAs	YES	Fast, no genetic tools are required	Highly invasive, efficiency is frequently low, limited control over tissue distribution	[4,48,49,64,70–72]
	Expression of double-stranded RNAs from an integrated transgene	NO	Possibility to control the strength, timing and tissue specificity of expression	Genetic tools need to be developed, time-consuming in sexual species with long generation time	–
Genome editing	Stable mutant lines generated using CRISPR/Cas9	YES	Heritable, all cells carry the same allele	Time-consuming in sexual species with long generation time, not suitable for lethal mutations and those that cause sterility or affect mating behavior, no tissue specificity	[76,77]
	Mosaic mutants generated using CRISPR/Cas9	YES	Fast, suitable for species that cannot be mated in the laboratory, suitable for lethal mutations and those that cause sterility or affect mating behavior	Heterogeneity within tissues may partially mask the mutant phenotype, not heritable unless the germline has been mutagenized	[76,77,79]

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Table 1. (continued)

Class of techniques	Technique	Published examples in ants	Advantages	Disadvantages ^a	Refs
	Transgenesis via CRISPR/Cas9-based homology-directed repair	NO	Integration in predefined location	Time-consuming in sexual species with long generation time, integration efficiency of large inserts may be low	–
	Transposon-mediated transgenesis	NO	Less limited by insert size than homology-directed repair	Time-consuming in sexual species with long generation time, (semi-)random integration may cause transgene silencing if inserted into heterochromatin	–

^aAbbreviation: SMART-seq, switching mechanism at 5' end of RNA template sequencing.

injected into nonreproductive individuals of the clonal raider ant *Ooceraea biroi* activated their ovaries [33]. Transcriptomic analyses of several tissues in the jumping ant *Harpegnathos saltator* implicated the neuropeptide corazonin, as well as vitellogenin, ecdysone, and juvenile hormone into the regulation of caste-specific traits. Their injection into the ants, combined in one case with RNAi, demonstrated their effects on behavior and physiology [4,34]. Thus, gene expression studies enable discovery of candidate regulators of important biological processes, which can be further experimentally validated.

Recently, transcriptomic approaches have gained an additional dimension thanks to the boom in single-cell mRNA sequencing (Table 1). It provides an unprecedented opportunity to analyze highly cell-heterogeneous tissues and organs, such as the brain, whereby gene expression changes that are masked in bulk transcriptomics can be mapped to specific cell types. Additionally, single-cell sequencing enables cell type composition analysis and inference of cell type-specific gene regulatory networks [35–37]. As these techniques are relatively new, only one study in ants has been published to date. Single-cell RNA-seq of non-visual brains in the workers and gamergates (pseudoqueens) of the jumping ant *H. saltator* revealed a higher proportion of ensheathing glia, which have neuroprotective properties, in the gamergate brains. Consistent with the shorter lifespan of workers, the proportion of this glial type declines with age, a phenomenon that appears to be conserved in *Drosophila* [38]. Given the rapid improvement in cost and performance of single-cell techniques, we expect more such studies in ants in the near future. Importantly, single-cell RNA-seq can be combined with chromatin accessibility profiling [assay for transposase-accessible chromatin (ATAC-seq), see later], enabling a comprehensive description of the cell regulatory landscape [39]. Several important limitations remain, such as the lack of cell-type specific markers in non-model species and the loss of spatial information during single-cell library preparation. Nevertheless, techniques to transfer cell type identities from *Drosophila* cell atlases, as well as methods that incorporate spatial information, have been developed and are being continuously improved [40–42].

Chromatin profiling

Caste-specific gene expression is associated with distinct chromatin states [43,44]. Therefore, the description of the latter has potential to identify genes differentially regulated between castes and the mechanism by which their differential expression is maintained. Chromatin profiling establishes the pattern of accessibility, describes the distribution of histone post-translational modifications, and determines the binding of transcription factors and other DNA-interacting proteins (Table 1). The most commonly used technique to profile accessible chromatin is ATAC-seq [45]. Recently published data for the pharaoh ant *Monomorium pharaonis* showed

that the brains of different castes and sexes have distinct ATAC-seq profiles, whereby chromatin accessibility mirrors the expression level of nearby genes [46]. Future studies in ants will take advantage of the modest requirements for the amount of input chromatin, which allows ATAC-seq to be performed at a single-cell resolution [39]. **ChIP-seq** maps histone modifications and DNA-binding proteins. ChIP-seq for the histone H3 and its several modifications, as well as for RNA polymerase II and the acetyltransferase **CBP**, was performed in different castes and sexes of the carpenter ant *Camponotus floridanus*. These chromatin maps enabled identification of regions differentially marked between the castes, whereby gene-proximal **H3K27ac** displayed the strongest correlation with gene expression, and its levels strongly discriminated between castes and sexes, especially in the regions bound by CBP [44]. This study paved the way for experimental manipulation of CBP-dependent histone acetylation, which alters the behavior of major workers [47]. Most importantly, it also led to the identification of a more downstream effector **CoREST**, which is a direct repressor of juvenile hormone-degrading genes (and thus an indirect activator of juvenile hormone signaling) in minor workers [48]. The significance of this finding is that it described in detail the regulation of juvenile hormone signaling, a pathway that appears to have been the main evolutionary target during the emergence of sociality [49,50].

Nevertheless, ChIP-seq suffers from several drawbacks. ChIP-seq typically requires a high amount of input chromatin, which limits its use in non-model organisms, especially those species that cannot be propagated in the laboratory. Additionally, it requires high sequencing depth, and the assay may need considerable optimization for each sample and antibody. By contrast, recently developed techniques called cleavage under targets and release using nuclease (CUT&RUN) and cleavage under targets and tagmentation (CUT&Tag) may overcome these limitations. They utilize nuclease- or **transposome**-tethered antibodies that are allowed to diffuse into immobilized cells or nuclei. DNA immediately adjacent to the antibody-recognized protein is cleaved (CUT&RUN) or directly tagged by sequencing adapters (CUT&Tag) [51,52]. CUT&RUN and CUT&Tag are more straightforward than ChIP-seq, as they provide better resolution, require lower sequencing depth, and a considerably lower amount of input material. Importantly, the CUT&Tag protocol is also compatible with single-cell approaches [53]. Therefore, these two techniques will be useful for chromatin profiling in ants in the future (Table 1).

DNA methylation profiling

DNA methylation, in particular 5-methylcytosine (5mC) methylation, has a well-known role in transcriptional silencing in mammals. Therefore, it attracted considerable attention among researchers studying social insects as it could potentially serve as an additional source of genes underlying caste-specific phenotypes [43,54]. However, the real picture appears to be more nuanced in ants and bees [55,56]. **Bisulfite sequencing** was used to contrast the levels of DNA methylation between reproductive and nonreproductive individuals in the carpenter ant *C. floridanus*, the jumping ant *H. saltator*, the clonal raider ant *O. biroi*, and the South American ant *Dinoponera quadricaps* (Table 1). Somewhat surprisingly, it identified few or no regions differentially methylated between reproductives and nonreproductives, and instead highlighted the association of DNA methylation with highly and constitutively expressed genes, as well as with constitutively included exons [57–59]. Conversely, pharmacological manipulation of DNA methylation changed the size distribution of *C. floridanus* workers. The methylation level of a specific locus that controls growth, **epidermal growth factor receptor (Egfr)**, negatively correlates with its expression level, suggesting that transcriptional silencing via DNA methylation is a plausible, albeit not universal, mechanism to regulate caste-specific gene expression [60]. A further potential function of DNA methylation was revealed by a study in the fire ant *Solenopsis invicta*, which showed a far greater difference in the pattern of DNA methylation between haploid and diploid males than between any diploid sexes or castes [61]. This intriguing finding suggests that DNA methylation may be involved in the regulation of gene dosage in

haploid males, although the requirement for such regulation remains hypothetical. Finally, a study in the species complex of the harvester ants *Pogonomyrmex barbatus/rugosus* found higher levels of DNA methylation in parental species than in their hybrids, possibly owing to allele-specific methylation [62]. Together, this shows considerable differences in the potential role of DNA methylation between ants and mammals. Surveying for genes differentially methylated between castes appears to have limited potential to uncover new genes regulating caste-specific phenotypes, and the results of such studies must be interpreted with caution due to the poorly understood role of DNA methylation in ants.

Pharmacological assays

Manipulating the activity of signaling pathways and other biochemical targets via treatment with chemicals is a classical experimental technique which still finds its use today (Table 1). For example, applying methoprene as a juvenile hormone analog is a well-established approach to activate juvenile hormone signaling [63]. Recently, methoprene was applied to the developing individuals of *Pheidole* to explore the mechanism and evolution of the worker subcaste decision [64,65]. Another example is a study in the jumping ant *H. saltator* where transcriptomics performed during the worker-to-pseudoqueen transition in adults implicated juvenile hormone and ecdysone signaling in the regulation of caste-specific physiology and gene expression. Injections of either methoprene or 20-hydroxyecdysone into the body and into the food helped to functionally validate this hypothesis [34]. In addition to hormones, chemical treatment is commonly used to manipulate histone acetylation machinery. The chromatin profiling of the carpenter ant *C. floridanus* described previously and a recent study in *Temnothorax rugatulus*, successfully used Trichostatin A and valproic acid to inhibit histone deacetylases, and C646 to inhibit histone acetylases [47,48,66]. Thus, pharmacological treatment is a straightforward way to manipulate various biochemical targets. As a disadvantage, administration of chemicals by injection, feeding, or topical application, provides limited control over their efficiency and distribution among target tissues. In the future, performing *ex vivo* experiments on organ explants or primary cell cultures may be a way to bring pharmacological assays into a more controlled biochemical setting, especially in the context of hormonal treatments which often induce a complex tissue crosstalk [67,68]. Separating different tissues will allow disentangling direct and indirect effects of the chemical, while treating isolated cells or tissues *in vitro* may provide a better control over drug delivery.

RNAi

RNAi has been one of the most accessible molecular techniques for functional studies in non-model organisms [69]. Injections of double-stranded RNAs (dsRNAs) have been performed into embryos, larvae, pupae, and adults of various ant species, to test the function of genes with known roles in other organisms and to validate candidates identified through transcriptomics [4,47,48,64,70–72]. For instance, injection of dsRNAs against a homolog of *vestigial*, which controls wing development in *Drosophila*, into soldier-destined larvae of *Pheidole hyatti* leads to the reduction of their rudimentary wing discs. As a consequence, head-to-body size ratio changes, too, providing functional evidence for the role of rudimentary wing discs in allometric scaling of worker subcastes [64]. Furthermore, injection or feeding invasive ant species with dsRNAs enables exploration of possible target genes for pest management [73,74]. Altogether, RNAi is a powerful tool that has been applied to a plethora of ant species at all developmental stages. Still, the tissue specificity is difficult to achieve, and RNAi is mostly performed in ants by injection, which is highly invasive. It also shows variable efficiency, although there are cases when this may be advantageous. For example, variable or low efficiency of RNAi may uncover a range of intermediate phenotypes between castes, lending insight into caste differentiation. In an attempt to overcome some of these limitations, the delivery technologies are being continuously improved. For example, adding liposomes or nanocarriers may stabilize

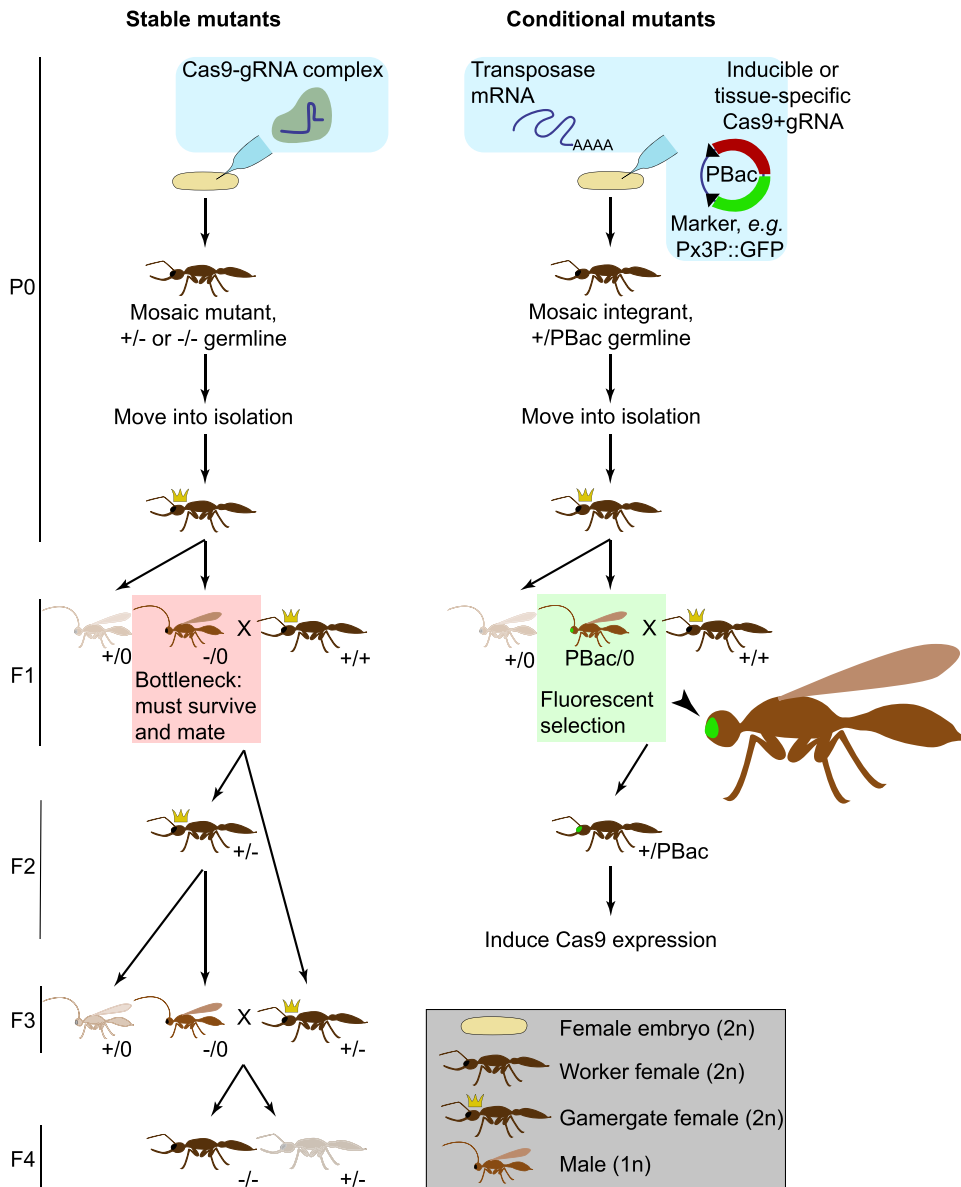
dsRNAs and enhance their uptake by cells [69]. Nevertheless, a real breakthrough will only come once transgenic technology is established (Table 1). Expression of dsRNAs from an integrated transgene will be more consistent across cells and tissues than delivery by injection or feeding. Using tissue-specific promoters will allow targeting specific tissues, and adoption of binary expression systems (e.g., Gal4-UAS) will provide a way to fine-tune the timing and level of dsRNA expression [75].

Genetic knockouts and knock-ins

Recently, the set of functional tools to study non-model organisms has been enriched by the advent of **clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9)** genome editing. The simplest application of CRISPR-based techniques only requires knowing the sequence of the target site and a way to deliver guide RNA (gRNA) and Cas9 to the tissue. Therefore, CRISPR-Cas9 genome editing has been used for functional genetic studies in a myriad of organisms. By contrast, in social insects, it is inherently limited by the difficulties or inability to set up genetic crosses in the laboratory. Yet, two studies were able to obtain stable mutant lines generated using CRISPR-Cas9 in the jumping ant *H. saltator* and the clonal raider ant *O. biroi*. Both studies targeted the gene **odorant receptor co-receptor (Orco)**. The mutants have a defective sense of smell and exhibit aberrant social behaviors that are mediated by pheromones. Unexpectedly, they also lack the majority of olfactory sensory neurons in the antennae, and the antennal lobes of the brain are strongly reduced. This suggests that the survival of olfactory sensory neurons during development depends on their activity, similar to what is observed in mammals and opposite to the 'hardwired' development in *Drosophila*, which yet again highlights the importance of performing functional studies in non-model species [76–78]. Recently, a proof-of-principle CRISPR-Cas9 experiment, which targeted the odorant-binding protein *Gp9* and epidermal growth factor, was also performed in the fire ant *S. invicta*. Although the study only examined the directly injected generation zero animals, mutations were observed at high frequency, demonstrating that efficient somatic genome editing can be achieved in species that cannot be reared in the laboratory [79].

Nevertheless, CRISPR-Cas9 experiments in ants remain a challenging endeavor (Table 1). First, gRNA and Cas9 injections into embryos create mosaic mutants. Thus, obtaining stable mutant lines requires controlled genetic crosses, but most embryos develop into nonreproductive workers which cannot be mated. To overcome this, the two *Orco* studies described previously were performed in species that have rather unique life cycles. *H. saltator* workers can be experimentally induced to become reproducing queen substitutes, called 'gamergates' (Figure 2), and *O. biroi* colonies consist of females, all of which reproduce parthenogenetically. Second, generation of mutants in sexual species takes a long time (although in asexual ones, such as *O. biroi*, homozygous mutants are obtained in the first generation). Homozygous *Orco* mutants in *H. saltator* could only be obtained in the fourth generation, which took a year and a half. Third, nurses in the colony tend to destroy injected embryos and reject the larvae that develop from them. Possible solutions include letting embryos hatch on agar plates in isolation from nurses and mixing experimental larvae with unmanipulated larvae [76,77,80]. Finally, future studies will likely need to focus on a small number of very important genes, some of which may be essential for survival, mating, or reproduction, making it difficult to obtain stable mutant lines or to analyze mutant phenotypes that manifest late in development (Figure 2).

A simple solution is to study mosaic mutants obtained as a result of somatic mutagenesis in injected embryos instead of establishing stable lines. The frequency of somatic mutations was relatively high in the previous experiments, which confirms the feasibility of this approach [77,79]. Generating mosaics does not require the ability to mate animals in the laboratory, making



Trends in Genetics

Figure 2. Comparison of experimental strategies to obtain stable and conditional mutants. This figure uses *Harpegnathos saltator* as an example but this is also applicable to other species. On the left, a previously used strategy to obtain *Orco* mutants (see [80] for method details). Cas9 protein and gRNA are combined *in vitro* and then injected into embryos. The embryos are allowed to hatch on agar plates and the larvae are placed into colonies with nursing workers (not shown). All animals develop into workers. Isolation of adult workers induces transition into an egg laying queen substitute ('gamergate'). Eggs laid by unfertilized females develop into males. Genotyping is done by Sanger sequencing. In males, DNA for genotyping is extracted from a clipped wing. Females are sacrificed and genotyped after a cross. P0 and F1–F4 are parental and offspring generations, respectively. 'X' stands for 'crossed to'. In genotype notations, '+' is a wild-type allele, '-' is a mutant allele, and '0' underscores that males are haploid. On the right, a proposed strategy to generate transgenic animals for conditional mutagenesis or other applications. Instead of Cas9-gRNA complexes, a mixture of mRNA that codes for a transposase (in this example, the *piggyBac* transposase) and a plasmid that contains the construct to be inserted are injected instead. Black arrowheads on the *piggyBac* plasmid in the upper right corner symbolize inverted terminal repeat sequences recognized by the transposase. 'PBac' in the genotype notations is an integrant allele. Green eye color symbolizes the expression of P_{x3P}::GFP as an example of a selection marker. Abbreviations: Cas9, associated protein 9; gRNA, guide RNA; *Orco*, odorant receptor co-receptor; *PBac*, *piggyBac*.

it the only viable approach in most ant species, and it permits studying homozygous lethal mutations [81,82]. Furthermore, mosaic mutant analysis may be combined with single-cell transcriptomics akin to pooled CRISPR screens in cell cultures [83]. Another strategy is to develop a technique to generate conditional mutants in those ants that can be propagated in the laboratory. While it may remain limited to species with unusual life cycles (see earlier), and experiments in true queens may be impossible, conditional mutagenesis will nevertheless be a big step in the development of a molecular toolkit for ants. A transgenic construct that carries Cas9 and gRNAs driven by a tissue-specific or inducible promoter along with a fluorescent selection marker, can be integrated into the genome using one of the transposon-based transgenic techniques widely used in other insects, such as *piggyBac* (Figure 2) [84–89]. Creation of such transgenes will provide a way to control genome editing in time and space and will enable studying essential genes. Importantly, transgenes are also expressed in heterozygous state, and skipping the crosses necessary to obtain homozygous lines may considerably speed up the experiments (Table 1).

To develop conditional mutagenesis, it will be first necessary to optimize the transgenesis strategy. *piggyBac* and its hyperactive version, as well as other transposons, such as *Hermes* and mariner-class transposons, have been successfully used in various insects, but the efficiency of each transposon may differ from species to species [84,85,87–90]. Thus, several strategies may need to be tried in ants simultaneously. Importantly, (semi-)random insertion of transposons into the genome means that multiple transgenic lines need to be constructed each time to compensate for possible mis-expression due to position effects [87,91]. As an alternative, CRISPR-Cas9-mediated **homology-directed repair (HDR)** may enable transgene integration [92,93]. Still, insertion of large fragments is inefficient in many species, limiting the use of HDR to targeted substitutions and insertions of small epitope tags. In such a case, HDR may at least be used to create ant lines suitable for **PhiC31**-assisted integration [91,93]. PhiC31 landing sites can be knocked-in using HDR into regions that are known or expected to be euchromatic. Ideally, such a landing site would be located in the intron of a haploinsufficient gene that produces a distinct morphological phenotype when mutated. Thus, successful integration would lead to a visible phenotype that can be used to screen for integrants.

Next, it is important to generate a construct that can be used both to test the transgenesis efficiency and as a selectable marker in subsequent transgenesis experiments. Fluorescent proteins driven by 3xP3, three binding sites for the eye selector transcription factor **Pax6**, have been shown to work in diverse insects, as well as crustaceans [85,86,94]. Thus, 3xP3 constructs may allow the selection of transformants by screening for fluorescence in the eye. Should the dark pigmentation of the eye conceal the fluorescent signal, the **immediate-early gene 1 (ie1)** promoter can be used instead, as it drives broad expression in the body throughout development [95,96]. In the best case, the only optimization step required will be to test several fluorescent proteins, which should additionally be codon-optimized for the focal species [97]. The final step will be to select upstream regulatory regions for Cas9 and gRNA expression [89]. Identification of tissue-specific promoters will make use of tissue and cell type-specific RNA-seq data that are already available and will greatly benefit from additional ATAC-seq data [5,19,34,38]. As for inducible expression, heat-shock and Tetracycline-inducible promoters work universally in insects, albeit with varying efficiency [93,98]. In summary, establishment of transgenic tools is the next step in the development of genetic techniques in ants. In addition to conditional CRISPR-Cas9 mutagenesis, the same genetic reagents, and the same experimental strategy will be used to perform RNAi, to create expression reporters, and to apply more sophisticated techniques, such as Calcium imaging in the nervous system.

Concluding remarks

Both classical and novel approaches described previously (genome assembly and annotation, bulk and single-cell transcriptomics, chromatin and DNA methylation profiling, pharmacological assay, RNAi, and genome editing) combined with the ability to rear ants in the laboratory, represent a formidable toolkit to tackle the unique biology of social insects (see [Outstanding questions](#) for examples of specific experimental questions for the future). Still, the application of molecular biological tools is inherently limited by the long life cycles and social lifestyles, prompting a somewhat different approach to molecular biology in ants and other non-model species in comparison to 'traditional' model organisms. As mutant or RNAi screens are virtually impossible, we predict that transcriptomics will remain the main source of candidate genes for functional studies. The explosion of single-cell techniques with their unprecedented resolution and novel statistical approaches to the inference of gene regulatory relationships has already powered the discovery of important biological regulators in *Drosophila* and has potential to do the same in ants [99]. When complemented by chromatin-based techniques, transcriptomics uncovers additional biological regulators providing a more comprehensive picture of the biological process in question. In the future, *in vivo* studies may be complemented by *ex vivo* experiments on isolated organs or cells and by *in vitro* experiments on cultured cells. These techniques may enhance the efficiency of functional manipulations and facilitate the experimental readout. Finally, establishment of transgenic techniques is the necessary next step in the development of functional tools which will enable more efficient and fine-tuned RNAi, conditional knockouts, expression reporters, and calcium imaging of neuronal activity.

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Declaration of interests

No interests are declared.

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Outstanding questions

Similar to mammals, the survival of developing olfactory sensory neurons in ants appears to depend on their activity. Is this mode of development linked to sociality or is it also present in solitary hymenopterans? Would conditional *Orco* knockout in adults (only affecting smell and not the development of the antennae or the brain) cause the same behavioral phenotypes as the early knockout?

The neuropeptide corazonin induces hunting behavior in workers. How is this achieved? Which areas of the brain are targeted by neurons that produce corazonin and how does the activity of these regions differ between foragers and nurses?

Studies in various social insects documented differences between castes in the volume of the entire brain, or specific regions. Volumetric changes appear to even occur in adulthood (e.g., during the transition from workers to pseudoqueens). Does this simply reflect pruning or growth of processes during synaptic remodeling, or do apoptosis and neurogenesis also take place? Which cell types are affected? How is this linked to caste-specific behaviors?

How do hormonal and other cues in larvae activate the mechanisms that regulate the establishment of different chromatin states in different castes?

In social insects, queens or their equivalents exhibit elevated expression of insulin-like peptides and produce many progeny while being long-lived. This seemingly contradicts what is known in relation to other animals, in which reproduction and insulin signaling correlates with shorter lifespan. How does insulin-like signaling selectively regulate reproduction without affecting physiological processes associated with aging? What is the contribution of different downstream branches of the insulin-like pathway(s)?

What are the targets of insulin-like peptides, juvenile hormone, and ecdysone in different tissues? How do tissues talk to each other?

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