Genetics and Evolution of Social Behavior in Insects

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Abstract

The study of insect social behavior has offered tremendous insight into the molecular mechanisms mediating behavioral and phenotypic plasticity. Genomic applications to the study of eusocial insect species, in particular, have led to several hypotheses for the processes underlying the molecular evolution of behavior. Advances in understanding the genetic control of social organization have also been made, suggesting an important role for supergenes in the evolution of divergent behavioral phenotypes. Intensive study of social phenotypes across species has revealed that behavior and caste are controlled by an interaction between genetic and environmentally mediated effects and, further, that gene expression and regulation mediate plastic responses to environmental signals. However, several key methodological flaws that are hindering progress in the study of insect social behavior remain. After reviewing the current state of knowledge, we outline ongoing challenges in experimental design that remain to be overcome in order to advance the field.

Keywords
genomics, gene expression, genetic toolkits, eusociality, division of labor, supergenes
INTRODUCTION

Social interactions among conspecifics span a rich diversity of behaviors, ranging from aggressive contests to courtship, parental care, and group living. Such interactions create interesting evolutionary dynamics as selection is induced by other members of the same species (108). Social behavior is also unique relative to other traits as it can be highly plastic within the lifetime of an individual and labile across evolutionary time (19). Thus, the study of social behavior within and between species is well suited to deepen our understanding of the proximate and ultimate mechanisms of phenotypic evolution (51). With relatively simple neural circuitry, insects are ideal models with which to study social behavior because they engage in a wealth of both simple and complex social interactions (35, 147). For example, Nicrophorus burying beetles engage in elaborate direct biparental care (41). The paper wasp Polistes fuscatus can discriminate among individuals on the basis of facial features (142). Drosophila melanogaster females engage in mate choice copying, a form of social learning (102). Finally, social caterpillars communicate to synchronously display defensive group postures (34). These examples, among many others, demonstrate the occurrence of complex social decision-making and communication systems in insects.

Insects are also ideal models for the study of social behavior because they span the extremes of social structure, from solitary species where individuals seldom interact, to massive societies where groups of individuals perform specific tasks. Between these two extremes, other species live in family groups, form nonkin aggregations, and compete in dominance hierarchies. The best-studied social structure along this continuum is that of eusocial species, which are characterized by the reproductive division of labor, cooperative brood care, and overlapping generations. The most striking features of eusocial species are (a) reproductive division of labor between the queen and worker castes and (b) behavioral division of labor within workers, such as groups that specialize in foraging or brood care. The origins of eusociality have been said to represent one of the major transitions in the evolution of life (153). Importantly, eusociality has independently evolved multiple times [once in ants, once in spheciform wasps, once or twice in vespid wasps, four times in bees, and once in termites (159)], making it a tractable system for studying the mechanisms underlying transitions between social structures and sociality in general. Depending on the stringency of the defining criteria, eusociality in insects has also evolved in aphids (149), thrips (36), and beetles (70). Interestingly, it appears that reversals from eusocial societies back to solitary nesting have also occurred many times (37, 164).

MOLECULAR EVOLUTION OF SOCIAL BEHAVIOR

Evolution of the molecular processes underlying social behavior can follow several different scenarios. When behavioral phenotypes are shared, the underlying mechanisms can be conserved, convergent, or different (51, 160, 166), leading to several general hypotheses about the multiple origins of social behavior in insects (159). Unfortunately, there has been some confusion about these hypotheses, which are not always well defined and are not mutually exclusive.

Genetic Toolkits Versus Novel Genes

Derived from evolutionary developmental biology, the genetic toolkit hypothesis predicts that conserved genes and/or gene networks are repeatedly co-opted in the evolution of behavioral phenotypes (160). Not surprisingly, direct support for the genetic toolkit hypothesis is mixed,
particularly given that the theory does not predict a specific extent of gene conservation (129, 159, 172). In response to this mixed support, it has been suggested that the evolution of novel social phenotypes instead involves rapidly evolving, taxonomically restricted genes with no significant homology to other known sequences (61, 152, 173). Such genes are free from the effects of pleiotropy and are important in trait evolution (72).

Determining the relative contribution of conserved and novel genes in the evolution of social behavior requires a more careful application of genomic approaches. Crucially, the importance of tissue specificity, both spatially and temporally, is often overlooked. Behavior is regulated by many different tissues and cell types, controlled by different and interacting molecular circuits, and each is likely to follow a different evolutionary trajectory depending on its function. For example, in specialized tissues such as glands and antennae, positively selected genes of high expression are often taxonomically restricted and/or located in the periphery of gene networks (58). This is not the case for neural tissue, where changes in gene regulation are more likely to underlie evolutionary change—in support of the toolkit hypothesis (48, 58). Studying specific circuits and molecular pathways in specified tissues, while applying a systems biology approach, offers the most promise for synthesizing these disparate findings.

Feeding-Related Pathways

Modifications to feeding- and nutrient-related molecular pathways have had important roles in the evolution of social behavior (43). One of the first of these pathways to be identified involved the gene foraging (for), which encodes a cyclic guanosine monophosphate–dependent protein kinase (PKG). In D. melanogaster, natural allelic variation in this gene affects larval and adult foraging behaviors, resulting in rover and sitter phenotypes (124). This gene also plays a role in regulating behavioral maturation in social insects, although, interestingly, the direction of effect differs between species. In honeybees, brain gene expression and for activity is increased in the transition from in-hive nest activities to foraging (13, 14), whereas in the ants Pogonomyrmex barbatus and Pheidole pallidula workers that forage have lower levels of for (57, 93).

Insulin/insulin-like growth factor signaling (IIS) also plays an important role in social behavior, linking changes in physiology to shifts in behavior (8). In Apis mellifera workers, this pathway is causally involved in the transition from nursing to foraging (6). Across species, increases in insulin-like peptides signal satiety and inhibit food intake. However, in the honeybee, IIS-related whole brain gene expression is lower in foragers, which have larger lipid stores (6). Furthermore, pharmacological inhibition of the target of rapamycin (TOR) pathway, which is typically upregulated by IIS, delays the onset of foraging in a seasonally related manner (6). In Pogonomyrmex harvester ants, whole body expression of IIS-related genes is involved in the development of new queens following a period of hibernation (81). The feeding-related genes neuropeptide F (NPF), the invertebrate homolog of NPY, and malvolio, a manganese transporter, are also associated with the transition from nursing to foraging behavior in honeybees (7, 12). Interestingly, the NPF pathway is also implicated in modifying foraging and social behavior in Drosophila larvae (175). These conserved feeding-related pathways appear to serve as prime substrates for the evolutionary modification of social behaviors.

Reproductive Physiology–Related Pathways

The reproductive ground plan hypothesis suggests that the genetic and hormonal networks regulating reproduction in a solitary ancestor have been co-opted in the evolution of social behavior
In support of this theory, several different genes and hormones have been found to have pleiotropic effects on reproductive traits and social behaviors (5). For example, in honeybees, a pleiotropic network seems to control a suite of traits related to foraging behavior, reproductive physiology, and sucrose responsiveness, suggesting that a reproductive circuit in solitary bees may have been co-opted to regulate social foraging behavior (3, 4). In the solitary akali bee, *Nomia melantheri*, a link was also identified between sucrose responsiveness and the size of Dufour’s gland, a conserved exocrine gland with reproductive functions (64). Support for the reproductive ground plan hypothesis also comes from ants, where the hormonal and reproductive networks became decoupled from reproduction, and yet an association remains between brood care and ovary activation in workers (117). In addition, in the harvester ant, *P. barbatus*, the gene for vitellogenin (Vg), a yolk precursor protein that transports carbohydrates, lipids, and nutrients to the ovaries, was duplicated and underwent subfunctionalization to acquire caste- and behavior-specific expression associated with reproductive and nonreproductive functions (32). Interestingly, expression of Vg and its receptor has also been linked to parental behavior in the burying beetle *Nicrophorus vespilloides* (134), suggesting widespread co-option of this gene in regulating diverse aspects of behavior.

Juvenile hormone (JH), secreted from the corpora allata, acts as a gonadotropin in most adult insects but also has wide-ranging pleiotropic effects on social behavior (reviewed in 18). For example, in the bumblebee *Bombus terrestris*, reproductive status and JH titers are tightly correlated. In addition, the presence of a queen has a powerful inhibitory effect on reproduction; after her removal, the rate of biosynthesis of JH increases within a day (17). In contrast, in honeybees, JH does not appear to influence adult reproduction, but rather affects age-related behavioral maturation (151). Interestingly, JH may play a role similar to that of androgens in vertebrates by mediating social responses to the environment (157). The actions of JH are mediated by its receptor, Methoprene-tolerant (Met) protein, which belongs to a family of transcription factors (10, 59). Conclusive evidence for Met (and in some species its paralog) as the JH receptor has only recently been shown (59). Further study of Met, as well as closer examination of the JH pathway (20), will likely greatly facilitate our understanding of the diverse actions of JH.

Similarly, ecdysteroids, a class of steroid hormones produced in the gonads, have broad effects on physiology and social behavior (118). Depending on their binding location within a cell, steroids can exert both slow-acting and rapid effects on behavior (1). Levels of ecdysteroids are typically high in reproductive individuals and low in workers of species with reproductive division of labor (118). In the ant *Pogonomyrmex californicus*, independent of age, foragers have lower whole body levels of ecdysteroids than within-nest workers that lay sterile nutritive eggs (39). Notably, there are significant interactions between the feeding-related pathways and the reproductive physiology–related pathways in mediating behavior. Elucidating the specific and causal actions of these circuits on social behavior remains an ongoing challenge.

**Other Pathways that Mediate Behavior**

Neurochemical systems are highly conserved across species. In insects, the biogenic amines serotonin (5-HT), dopamine (DA), and octopamine (OA) function as neurotransmitters and neuromodulators in the central nervous system and can also be released in circulation. Numerous receptors are distributed in various parts of the body, with high density in the brain, resulting in diverse effects to physiology and behavior. A well-studied example of the role of biogenic amines in mediating social behavior is in the phase transition from the solitary state to the gregarious state in the desert locust *Schistocerca gregaria*. The level of 5-HT in the thoracic
ganglion is positively correlated with the extent of gregariousness, irrespective of the behavioral stimulation used to invoke the gregarious state (9). In the migratory locust, Locusta migratoria, 5-HT is sufficient to induce gregarious behavior, but brain levels of 5-HT do not differ between solitary and gregarious states. In this species, the DA pathway has been implicated in modulating the phase shift (95). Interestingly, the shift from the gregarious state to the solitary state is mediated by microRNAs (miRNAs) that suppress DA synthesis (177). In insects, the biogenic amines have also been well studied for their role in mediating aggressive behavior (150). Interestingly, in the vertebrate brain, the location of neurochemical ligand-producing cells is more evolutionarily labile than is the receptor location (112). Further study of neural circuits and the spatial location of ligand-producing cells and their receptors in social insects will allow us to address how neurochemicals are modified in the evolution of insect social behavior.

In contrast to these highly conserved pathways, olfactory and chemosensory receptors are the fastest-evolving neuronal genes in animal genomes. Modifications to these receptors can lead to rapid shifts in behavior. In ants, communication is largely based on pheromone detection. A recent study suggests that a large and novel clade of olfactory receptor genes evolved in the ancestor of all ants, potentially enhancing pheromone communication (99).

**Network Architecture**

Broadly defined, a gene network is a group of structurally or functionally related genes. Although many of the studies outlined above have demonstrated the importance of single genes or hormones in mediating behavior, these genes are acting within larger regulatory networks to affect behavioral phenotypes (49). Gene networks can be highly conserved, as exemplified by the concept of orthologous phenotypes, or phenologs, whereby functionally coherent and conserved gene networks underlie unrelated phenotypes in different species, even predating the animal–plant divergence (98). The position and connectivity of genes within networks can make them more or less evolutionarily labile, and this has been shown to influence the evolution of phenotypic plasticity and insect social behavior in a variety of ways.

In the honeybee brain transcriptional regulatory network (27), highly connected transcription factors and their target genes are under stronger negative selection, as are proteins in the core of the network (105). In contrast, connectedness and structure apparently do not affect selection on regulatory sequences (105). Similarly, comparison of the level of expression of taxonomically restricted and conserved genes in several different honeybee tissues reveals that genes under positive selection are the least connected within the network (58).

Several studies have also begun to examine differences between castes to investigate the role of network architecture on selection. A whole body transcriptome study of the pharaoh ant Monomorium pharaonis revealed that, compared with the rest of the genome, genes upregulated in foragers are more highly connected and conserved in gene expression networks than genes upregulated in nurses, which are less connected and less conserved (103). A recent study examined whole brain profiles in the paper wasp, Polistes canadensis, and the dinosaur ant, Dinoptera quadriceps, two species where individuals retain the ability to switch between reproductive and nonreproductive phenotypes. Few transcriptional differences were found between these two phenotypes, instead there was nonrandom gene expression network organization between phenotypes in both species, containing both conserved and taxonomically restricted genes (119). Furthermore, an additional study comparing gene networks from whole body transcriptomes of 16 ant species revealed consistent network differences between queen and worker castes. Gene network (module) expression
seems also to correspond to specific phenotypes across species, including worker sterility, queen number, and invasiveness, again suggesting a role for conserved genetic elements in the evolution of behavioral phenotypes (109).

**SOCIAL ORGANIZATION**

Social organization refers to the number of individuals in a social group, their behavioral and genetic relationships, and the way in which reproduction is partitioned among them (132). It has become increasingly evident that social organization varies not only among species but also within species or even populations. This is best exemplified by variation in the number of reproductive queens per colony. Although many species invariably have a single queen per colony (monogyny), others can form multiple-queen (polygynous) colonies. Polygyny is particularly common in ants, where it may be the predominant social structure (65). Variation in queen number is typically associated with differences in a suite of reproductive and social traits, including queen phenotype, queen lifespan, breeding strategy, mode of dispersal, and method of colony founding (66, 132). Interestingly, many species exhibit intraspecific variation in queen number and other associated traits, and recent studies suggest that such variation has a genetic basis.

The fire ant *Solenopsis invicta* is the best-studied socially polymorphic ant (132). In this species, colony acceptance of multiple queens is directly linked to allelic variation at the *Gp-9* locus (*B* and *b* alleles), a gene that codes for an odorant binding protein (68, 75). Colonies with homozygous *BB* workers will accept only a single *BB* queen. Colonies with both *BB* and *Bb* workers, conversely, will accept multiple queens, but only if they are heterozygous (133). Recently, *Gp-9* was shown to be part of a supergene comprising a large nonrecombining chromosomal region with more than 500 genes in tight linkage (163). This genomic rearrangement results in coordinated effects on multiple social traits and allows for the maintenance of divergent social phenotypes. Interestingly, a variation in social organization in the Alpine silver ant, *Formica selysi* (126), has also been shown to be under the control of a single supergene. The chromosome location and specific content of the supergenes in *S. invicta* and *F. selysi* differ, indicating that they evolved independently. It is likely that other cases of social polymorphisms will be found to be under the control of supergenes (139). Similarly, other social polymorphisms where multiple traits are linked together also seem to be controlled by supergenes in plants (80), birds (78, 156, 161), cichlid fish (130), and other invertebrates (77). Because supergenes allow the cosegregation of adaptive variation within species, they may facilitate the spread of complex phenotypes across species boundaries and be more common than usually realized (139).

**SOCIAL PHENOTYPES**

Insect species with reproductive division of labor are extraordinary examples of phenotypic plasticity. Individuals with highly similar genomes can exhibit striking differences in morphology and behavior. To understand the proximate mechanisms regulating division of labor requires identifying the factors regulating both reproduction and behavior. Reproductive division of labor usually arises from a developmental switch during larval stages leading to the alternative development of queens and workers. However, in some species, reproductive status can be determined at the adult stage, either through a dominance hierarchy or through the temporal alternation of reproductive and nonreproductive phases. Similarly, in social insect species with polymorphic workers, worker behavior is usually associated with morphology, but behavioral variation also occurs in species with morphologically uniform workers. Thus, both reproductive and behavioral division of labor
The phenotype of an insect is determined by an interaction between parentally inherited factors, environmental factors, the social environment, and the individual’s state. These factors act at several different levels of gene regulation and transcription to modify the ultimate morphology, physiology, and behavior of an individual. A critical challenge is to integrate data sets across levels in a way that reveals how genome-wide changes translate environmental and genetic factors into adaptive behavioral phenotypes. Photographs © Alex Wild; used with permission.

can be affected by environmental, social, and genetic factors during larval development and at the adult stage (Figure 1).

**Queen-Worker Differentiation at the Adult Stage**

In some species, all individuals are monomorphic, and reproductive division of labor stems from social interactions among colony members. For example, in both the paper wasp *P. canadensis* and the dinosaur ant *D. quadriceps*, females compete to form a dominance hierarchy. The highest-ranking females engage in reproduction, whereas the lowest-ranking ones forage for food and nurse the brood (106, 167). Similarly, when the queen dies in colonies of the jumping ant *Harpegnatbus saltator*, the workers engage in ritualized combat resulting in dominant workers (gamergates) mating with their brothers and becoming the only reproductive individuals in the colony (121).

In these species without morphological castes, the extent to which gene expression varies between reproductive and nonreproductive individuals appears to be species specific, with current results showing potential effects of experimental design. In *H. saltator*, reproductive individuals show an increase in whole body expression of genes involved in sensory perception, neuronal...
function, and chemical communication (22). In contrast, very few genes are differentially expressed between the brains of reproductive and nonreproductive individuals in *D. quadriceps* and *P. canadensis* (119). Interestingly, in both species as well as in the clonal raider ant, *Ooceraea biroi*, Vg is upregulated in the brains of reproductive individuals (119). In addition, genes involved in the production of JH, which affects the expression of Vg in social insects (33, 81), are downregulated in reproductive individuals of *O. biroi* (116). Interestingly, JH content does not differ between gamergates and workers in *H. saltator* (123) but is positively correlated with ovarian activity in *Belonogaster longitarsus* (69), another paper wasp species with a dominance hierarchy. Thus, in different species of social insects, JH can have opposite roles in regulating adult reproductive division of labor.

The gene regulatory mechanisms that modulate the changes in gene expression associated with adult caste differentiation remain to be understood, as DNA methylation and miRNAs appear not to be associated with reproduction. Whole brain DNA methylation does not show any variation associated with changes in reproduction and is mostly associated with genes showing strong and stable expression in *O. biroi* (83), whereas DNA methylation patterns are very similar between reproductive and nonreproductive colony members in *H. saltator* (21), *D. quadriceps*, and *P. canadensis* (119). Gamergates and workers show similar whole body expression of miRNAs in *H. saltator* (22), and miRNAs do not preferentially target genes that are differentially expressed between the brains of reproductive and nonreproductive individuals in *D. quadriceps* and *P. canadensis* (119).

Finally, although *H. saltator* workers and gamergates exhibit similar branching patterns of dopaminergic and serotonergic neuronal systems (53), gamergates have higher brain DA levels than do workers (122), and genes involved in biogenic amine metabolic processes are differentially expressed between gamergates and workers (22). This suggests that biogenic amines such as DA may be involved in regulating reproduction-associated behavior in adult social insects.

**Queen–Worker Differentiation During Development**

In most species of social insects, queens differ morphologically from workers. Because all growth occurs during the larval phase in holometabolous insects (e.g., ants and bees), differences in morphology between queens and workers are determined during development. Usually, caste differentiation occurs in early larval stages, and multiple environmental and social factors are known to affect this process. The first factor is nutrition during larval development. Variation in food quality and quantity strongly affect caste differentiation in honeybees (169), wasps (15), and ants (146), but not in the ant *Myrmica rubra* (23). The second factor is the social environment during larval development. The production of new queens is favored when the queen is absent in ants (23, 67) and when the brood is absent in wasps (148). The third factor is the temperature experienced during larval development, as in ants, for example (23, 26). In addition, the temperature experienced by the queen can also affect caste determination: Only hibernated queens produce new queens in *Pogonomyrmex* harvester ants (137).

Several studies revealed that the likelihood of developing into a queen or worker also has genetic components (reviewed in 140). The first line of evidence came from the finding that different patrilines (i.e., subsets of workers sharing the same father) in colonies headed by one multiply mated queen have different probabilities of developing into queens and workers (54, 146, 158). These findings were first interpreted as evidence of single genetic elements influencing caste determination. However, the use of controlled crosses revealed that genetic effects on caste determination are unlikely to stem from simple direct genetic (additive) effects and are most consistent with nonadditive genetic influences (e.g., genetic compatibility effects between the
Some ant species have a strict genetic caste determination, whereby the caste fate is already determined at the egg stage (24, 45, 74, 114, 162). Overall, it is increasingly clear that the relative influences of genotypic and environmental effects on caste vary among species, ranging from largely environmentally controlled phenotypes to almost purely genetic systems (140).

The IIS pathway, a highly conserved pathway that links nutrition and physiology in insects (111), is involved in detecting variation in nutrition to regulate caste differentiation (31). In honeybee larvae, the link between the ingestion of royal jelly and caste differentiation involves not only IIS (38, 169, 174) but also other associated nutrient-sensing pathways such as TOR (28, 110, 120) and epidermal growth factor receptor (Egfr) (63). In the fire ant S. invicta, queen- and worker-destined larvae show differential expression of two insulin receptors (91). Nutrient sensing by the extended IIS pathway then affects the production of JH, which plays a critical role in caste differentiation (25, 29, 71, 110). JH affects the production of Vg (11, 125), and both JH and Vg regulate the alternative development of queens and workers through different influences on growth and tissue differentiation (31). Interestingly, some of the pathways regulating caste differentiation in larvae also regulate maternal effects on caste determination in Pogonomyrmex harvester ants. In this species, the interplay between IIS, JH, and Vg translates the low temperature experienced by the queen during hibernation into the development of new queens in its offspring (81).

In addition to genes involved in IIS, JH, and Vg pathways, gene expression studies have identified many other gene pathways that are differentially expressed between queen- and worker-destined larvae [e.g., Cytochrome P450 (25), Hexamerin70b (25), MRJP, and yellow genes (28)]. Interestingly, in the ant Cardiocondyla obscurior, genes involved in the sex differentiation cascade, including the conserved gene doublesex, were differentially expressed between queen and worker pupae, suggesting that this conserved pathway was co-opted to regulate caste differentiation (73). Gene regulatory mechanisms that mediate changes in gene expression may involve DNA methylation, as downregulation of the expression of the DNA methyltransferase Dnmt3—responsible for de novo DNA methylation—affects the caste fate of honeybee larvae (76). However, this finding awaits confirmation because the effect of the Dnmt3 knockdown on actual DNA methylation was assessed not genome-wide but for only ten CpGs of a single gene. In addition, there currently are no properly controlled and replicated bisulfite sequencing studies of differential DNA methylation between queen- and worker-destined larvae (83) (see the section titled Methodological Concerns). miRNAs could be another gene regulatory mechanism mediating gene expression changes associated with caste differentiation, as miRNAs involved in pathways regulating caste differentiation (e.g., IIS, TOR) are differentially expressed between honeybee queen- and worker-destined larvae (143).

**Worker Polyethism**

In most social insects, workers are morphologically uniform in size and shape (115). Task specialization results from different response thresholds to environmental and social signals (155). For example, the discovery of a new food source by an ant colony will prompt some—but not all—workers to become foragers, whereas artificially removing all nurses from a beehive will result in some—but not all—workers switching to nursing tasks. Several factors influence the likelihood of a given worker to perform a particular task in response to colony needs. The first is age, as evidenced by workers specializing in different tasks as they become older. Typically, young workers are more likely to stay inside the nest to care for the queen(s) and nurse the brood, whereas old workers tend to leave the nest to defend the colony or forage for food (101, 141). Such age-induced changes in behavior are strongly associated with the spatial location within the nest, leading to preferential interactions between workers performing similar tasks (101). The second factor is...
individual experience, which has been shown to influence task preference both theoretically (155) and empirically (128). Finally, the genetic background of workers affects their likelihood of undertaking different tasks. The first line of evidence suggesting that there are genetic components to worker behavior in species without worker polymorphism came from artificial selection successfully leading to strains with high and low foraging activities in the honeybee (8). Later studies also showed that workers produced by different fathers in honeybee colonies differ in their likelihood of foraging for pollen or nectar, scouting new nest sites, grooming nestmates, guarding the nest, and removing corpses from the colony (16, 45, 46). Similar differences among patrilines (or matrilines when colonies contain several queens) were reported in many other species of ants (30, 42), bees (16), wasps (113), and termites (62).

These genetic effects on worker behavior have typically been assumed to stem from additive genetic effects of many genes. However, studies comparing patrilines (or matrilines) do not allow one to discriminate between additive genetic effects and more complex genetic influences (e.g., compatibility effects, parent-of-origin specific effects) because for both additive and nonadditive genetic effects, an association between behavior and patriline (or matriline) representation is expected (82, 85, 138). To address these confounds, Libbrecht & Keller (82) performed controlled crosses to quantify the relative importance of the paternal and maternal genetic backgrounds on worker behavior in the Argentine ant *Linepithema humile*, thus discriminating between additive and nonadditive genetic effects. This study revealed genetic compatibility effects between the parental genomes, as well as parent-of-origin specific effects, on the workers’ efficiency at feeding larvae and collecting pupae, on their foraging propensity, and on their distance within the nest to the brood (28). This confirms that assessing the relative contributions of the paternal and maternal genetic backgrounds is crucial for investigating genetic effects on worker behavior.

Reproduction and behavior are often interconnected in animals. Accordingly, nurses and foragers differ in ovary activity, *Vg* expression, and JH levels in bees (4, 131, 151) and ants (32, 39, 40, 123). Interestingly, phylogenetic analyses revealed the existence of multiple genes encoding *Vg* in most of the ant genomes sequenced (32). The *ant Vg* genes cluster in two paralogous gene families that show caste- and behavior-specific expression in *S. invicta* (176), *Pogonomyrmex rugosus* (32), and *O. bironi* (116), suggesting that *Vg* in ants regulates not only reproduction but also worker behavior (32). DNA methylation may be one of the gene regulatory mechanisms that mediates behavior-specific changes in gene expression, as whole brain DNA methylation differs between nurses and foragers in honeybees (50). Biogenic amines produced in the brain may also regulate worker behavior, as DA, 5-HT, and OA levels in the antennal lobes differ between honeybee nurses and foragers, irrespective of age (136).

**Worker Caste Polymorphism**

In some species of social insects, the worker caste is subdivided not only in terms of behavior but also morphologically. In approximately 15% of ant genera, in addition to standard workers there are larger individuals, often with enlarged heads and mandibles, that function in nest defense, seed milling, foraging for large prey, or food storage (52). These large individuals can be distinguished either as a worker subcaste (majors), whose members follow the same growth pattern as standard (minor) workers, or as a separate soldier caste with a distinct size distribution (104, 168). Within bees and wasps, workers generally do not differ morphologically; however, a soldier caste has been identified in one species of eusocial bee (47). In lower termites, workers are totipotent and can differentiate into either a sterile soldier caste or a reproductive caste.

Similar to queen–worker differentiation, worker size and caste are determined during development. Worker caste determination is affected by the rearing environment, including nutrition,
hormones, and social interactions. In the ant genus *Pheidole*, larvae fed a protein-rich diet develop into soldiers. This development appears to be linked to JH, as application of a JH analog can also induce soldier development (86, 170). Interestingly, JH can also induce the production of supersoldiers even in *Pheidole* species that lack this subcaste (127). In termites, which are hemimetabolous, the presence of soldiers lowers JH titers in totipotent workers, affects caste-regulatory genes, and suppresses the formation of more soldiers (97, 154). The hexamerin proteins Hex-1 and Hex-2, which function as JH binding proteins, suppress the JH-mediated differentiation into soldiers (178). In addition to the rearing environment, it is also becoming clear that genetic effects play a role in worker caste differentiation. For example, in ant species where queens are multiply mated, different patrilines within colonies vary in their propensity to develop into minors or majors, although all patrilines produce at least some proportion of both subcastes (55).

Several studies in adult ants have identified DNA methylation and histone posttranslational modifications (PTMs) as factors contributing to behavioral plasticity in workers, which likely serve to mediate the response to environmental cues. In samples from head and thorax, as well as whole brains, small minor workers can be discriminated from large major workers on the basis of patterns of histone PTMs, particularly for the acetylation of Lys27 on histone H3 (145). Histone acetylation relaxes the structure of chromatin and allows for increased levels of gene transcription. Interestingly, the acetyltransferase and transcription coactivator CREB binding protein (CBP) shows differential recruitment to enhancer sequences between minors and majors (145). Blocking the acetyltransferase activity of CBP inhibits scouting behavior in minors. Remarkably, young majors injected in the brain with histone deacetyltransferase inhibitors begin foraging and scouting at levels similar to those of minors. This behavior is suppressed if they are co-injected with CBP inhibitor (144).

**METHODOLOGICAL CONCERNS**

The large quantity of sequencing data collected over the past decade has offered new insights into the evolution and molecular regulation of insect societies. However, most genomic studies of social behavior in insects have overlooked the potential importance of quantitative genetic theory in understanding the molecular evolution of social behavior (see sidebar titled Indirect Genetic Effects). In addition, many findings have to be met with caution until they can be confirmed in future independent studies. Skepticism is necessary because methodological issues are very
**Biological Replication**

Until recently, RNA-seq and bisulfite sequencing studies have typically been performed by pooling many individuals rather than conducting biological replication (Figure 2). This approach is problematic for several reasons. First, it prevents the measurement of interindividual variation. In RNA-seq studies, for example, one gene may appear highly expressed if its transcript is very abundant in one individual of the pool, thus biasing interpretation of the data. Second, pooling individuals does not necessarily suppress random variation. In a recent study in ants, most DNA methylation was sample specific although samples consisted of pools of 20 brains dissected from genetically identical individuals of the same age (83). Third, to conduct proper statistical analyses of the data and prevent false positives, it is imperative to measure interindividual variation rather than to reduce it to an unknown extent by pooling individuals. Social insect bisulfite sequencing studies comparing queens and workers provide a good illustration of this problem. Studies with one or two biological replicates detected differential methylation between castes (21, 44, 46, 94), whereas studies with three or more biological replicates did not (50, 83, 119). This may be because comparing one queen sample to one worker sample does not allow the discrimination between caste-driven differences and random (sample-specific) variation (83). In genomic studies as in any scientific studies, biological replication is the only way to show that observed differences between groups are true differences that do not arise from random variation. In addition, biological replicates must be independent. For instance, two worker samples collected in a single ant colony are only pseudoreplicates because they are genetically related and share the same environment. We argue that future social insect RNA-seq and bisulfite sequencing studies should include sufficient
Figure 3

Tissues and brain regions of the ant likely to play central roles in mediating social behavior. Rather than using whole body or whole brain approaches, we suggest that specific tissues and/or cell types should be investigated in studies of gene expression and regulation.

numbers of independent biological replicates and that such numbers should be clearly stated in the main text.

Tissue Specificity

So far, most social insect studies using RNA-seq have been performed on whole individuals. This is problematic because individuals from different castes frequently vary in both size and allometry (i.e., the relative proportion of different organs or tissues). For example, queens have much larger abdomen proportions than do workers. Thus, using whole bodies results in comparing different mixes of body parts, and tissue- or organ-specific gene expression is then likely to be falsely interpreted as caste-specific gene expression (107). For example, a transcriptome study of eight different tissues in the honeybee found that most regulatory genes show caste-specific expression in only a single tissue (60). Due to increasingly sophisticated technology, RNA-seq now requires very low input levels, as demonstrated by the growing popularity of single-cell sequencing. Furthermore, there are sufficient tools available for isolating specific tissues or cell types in nonmodel organisms, such as laser-capture microdissection (100), although these are more labor intensive. In Figure 3, using ants as an example, we point to several tissues and brain regions that require detailed study in order to gain a more complete picture of how social behavior is regulated.

Other Confounding Factors

Several other factors should be carefully controlled in RNA-seq and bisulfite sequencing studies comparing social insect reproductive and behavioral castes (Figure 1). The first factor is age, which affects gene expression and DNA methylation patterns (56, 89, 90). Controlling age is important because queens are usually older than workers, and foragers are older than nurses. Thus, if age is not actively controlled for in the experimental design, any molecular changes associated with
Caste may actually stem from age differences. The second factor that should always be controlled is the genetic background because it could independently affect phenotypic traits and molecular mechanisms. Genetic effects on caste determination and worker behavior are common (84, 140), and DNA methylation patterns have been found to be associated with the genetic background in bees (165). Additional factors that should be controlled because they affect gene expression include the social environment (89, 92, 96), temperature (79), and nutrition (171). Finally, experiments must be carefully designed so that all samples are collected at the same time of the day (56) and that all groups or treatments are equally represented in each batch of library preparation and sequencing (83).

CONCLUSION
The study of insect social behavior has significantly contributed to our understanding of general principles of molecular evolution and phenotypic plasticity. The application of genomic tools to the study of social insects, in particular, has deepened our appreciation of the complex processes driving the evolution of this remarkable group of animals. But there remain many methodological problems that need to be overcome. In particular, we predict that shifting the focus from whole body and whole brain gene expression studies to more specific tissue and cell types will greatly facilitate our understanding of the genetics and evolution of social behavior in insects.

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