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Leveraging technological innovations to investigate evolutionary transitions to eusociality

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The study of the major transition to eusociality presents several challenges to researchers, largely resulting from the importance of complex behavioral phenotypes and the shift from individual to group level selection. These challenges are being met with corresponding technological improvements. Advances in resource development for non-model taxa, behavioral tracking, nucleic acid sequencing, and reverse genetics are facilitating studies of hypotheses that were previously intractable. These innovations are resulting in the development of new model systems tailored to the exploration of specific behavioral phenotypes and the querying of underlying molecular mechanisms that drive eusocial behaviors. Here, we present a brief overview of how methodological innovations are advancing our understanding of the evolution of eusociality.

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Introduction

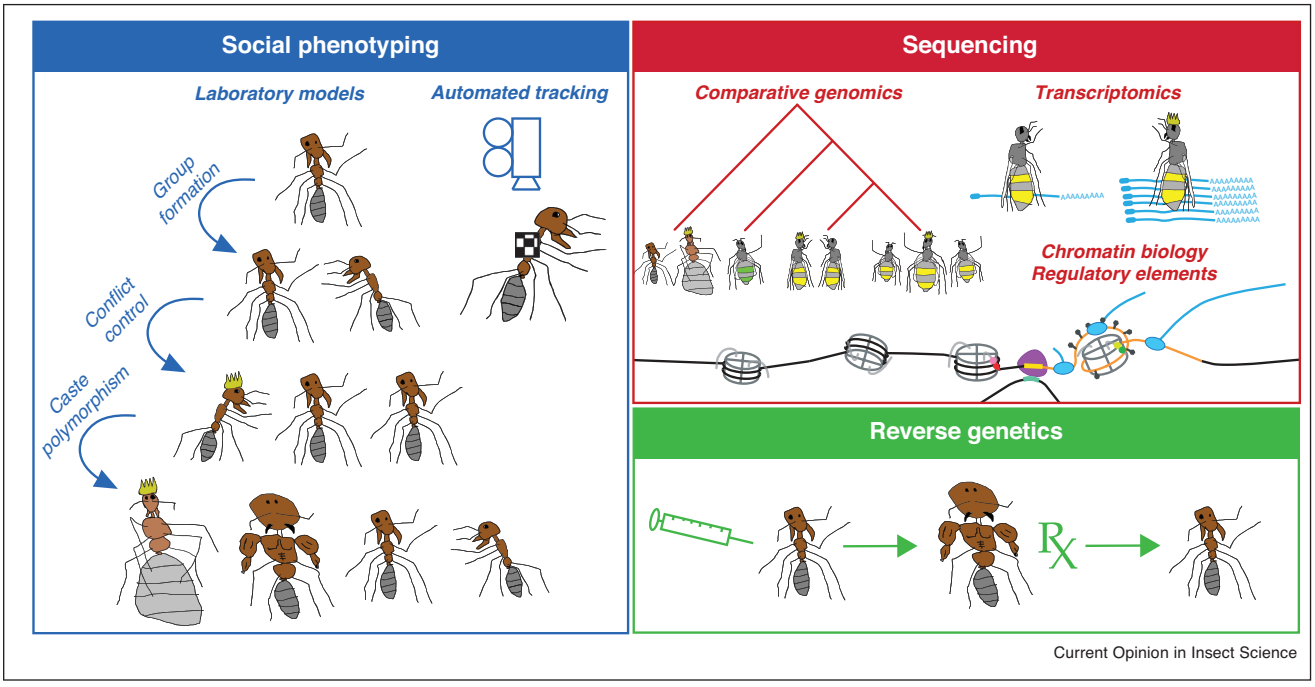
The history of life is marked by a series of major evolutionary transitions [1,2], from independent replicators to group-dependent replicators, as exemplified by the origins of protocells, multicellular organisms, and eusocial animal societies. These transitions are unique among adaptations as they change the unit of selection from the individual replicator to a group of linked replicators [3]. Synergistic fitness effects [3] and gene regulatory evolution [4–6] have been proposed as drivers of major evolutionary transitions. However, substantial barriers to testing such hypotheses include the complex nature of relevant phenotypes and the gulf between molecular genetic resources available for traditional model organisms versus those most relevant to understanding major evolutionary transitions. Fortunately, advances in molecular technologies and development of new model systems are facilitating exploration of the causes and consequences of major evolutionary transitions in ways previously considered impossible. The conceptual and technological challenges and advances facing researchers of eusocial insect biology have been subject to numerous recent reviews (e.g. in Refs. [7–9]). In this article, we complement those efforts by highlighting how particular technological innovations have advanced, and will continue to advance, our understanding of eusocial evolutionary transitions, with a particular focus on recent studies of the order Hymenoptera.

Social phenotyping

The evolutionary transition to eusociality proceeds through some combination of the following stages: the genesis of societies, the evolution of conflict control mechanisms, and the shift to dimorphic castes (Figure 1) [3]. Social insects exhibit great diversity in the extent to which each of these stages have proceeded, likely dependent on lineage-specific ecological and evolutionary constraints.

Some social species exist with monomorphic castes that are subject to ongoing conflict control, as seen in many bees [10,11], paper wasps [12,13], as well as ant taxa that have experienced an evolutionary reversion away from caste dimorphism [14••]. Other species exhibit polymorphism in social organization, with populations spanning multiple stages of the major transition [15••,16,17]. In contrast, some taxa exhibit elaborations of the eusocial phenotype beyond the framework laid out above, including origins of worker caste polymorphism [18•] and transitions to colonies comprised of multiple families (polygyny) [19,20]. This taxonomic variation in traits related to eusociality allows for fine-tuned investigation of the mechanisms underlying the major transition itself. For instance, ants that exhibit losses of an obligate queen caste have been used to explore the molecular mechanisms of conflict management and social cohesion [21••,22••,23••,24••]. In contrast, eusocial organisms with extreme caste dimorphism and worker subcastes are ideal models for understanding the evolution and maintenance of developmental polyphenisms [14••,18•,25•,26•,27]. In this regard, the diversity of emerging social insect model systems has the potential to elucidate many crucial details in the major transition to eusociality.

Figure 1



Innovations advancing our understanding of the evolution and maintenance of eusociality. New model systems are being developed to explore various aspects of the origins of eusociality, and the use of automated tracking is providing quantitative data on relevant behaviors. Low sequencing costs are fueling the generation of increasingly expansive comparative genomic and transcriptomic studies of insect taxa displaying different social phenotypes, and new molecular genetic protocols are making it easier to assay specific tissues and investigate gene regulatory mechanisms. Reverse genetics techniques are becoming increasingly accessible for utilization in non-model organisms, with the potential to provide evidence for genetic elements playing a causal role in phenotypic variation linked to eusociality.

A key element in the development of model systems for social transitions is understanding the phenotypes such systems exhibit. This is particularly challenging in social insects because the phenotypes in question are often complex behaviors that can be difficult to quantify. Advances in automated behavioral tracking are beginning to yield fine-scale and high throughput analyses of social behaviors [24••,28,29•]. For example, while it would be a titanic effort to track hundreds of colonies of varying sizes with manual observation, automated behavioral tracking allowed for such an experiment, providing new insight into how colony size affects behavioral specialization [24••]. A detailed understanding of behavioral phenotypes, and the use of technology to enhance high throughput phenotyping, enable more informed experimental designs when exploring the molecular underpinnings of division of labor [21••,22••], as well as a better framework for developing hypotheses about the evolution of these complex behaviors.

Sequencing

Comparative genomics offers one of the most accessible avenues for investigating the molecular basis of complex trait variation (Figure 1). Genomes are being sequenced from an unprecedented number of taxa, which have in turn been used to identify genes that exhibit common shifts in selective pressures [[33•],30] or expansions in gene families [[33•],31] during parallel transitions to eusociality. Similar approaches have been used to investigate the evolution of genes in ants [31,32] and termites [33•] as compared to distantly related non-eusocial outgroups. One of the common findings of these studies is that changes in the gene content related to chemical communication tend to co-occur with eusocial evolutionary transitions [31,33•].

Innovations in sequencing that reduce costs and simplify sample preparation are increasing the accessibility of genetic mapping and population genomic data generation for investigating genetic contributions to caste dimorphism and other forms of complex trait variation. For example, variation in colony queen number (and a variety of accompanying traits) in the fire ant *Solenopsis invicta* has long been known to be mediated by a single Mendelian element [34], but recent implementations of genome resequencing and genetic mapping revealed the locus mediating colony social form is not a single gene, but rather a large chromosomal rearrangement containing hundreds of genes [19], an observation made in another ant linage as well [20]. This illustrates how the genetic

architectures of complex traits associated with major transitions or, in this case, their elaborations are being investigated at increasingly higher resolution. Nonetheless, current short-read genome assemblies are highly fragmented and annotated incompletely, which limits studies of gene family evolution and results in incomplete lists of candidate genes (an issue exacerbated by the use of limited transcriptome evidence to inform gene models [35]). Long-read sequencing technologies are now beginning to fulfill the promise of better genome assemblies [36], which will facilitate more robust investigations of molecular contributors to phenotypic variation in non-model systems. Such advances also promise to improve the annotation of noncoding genetic elements and the utility of whole genome alignments to investigate the evolution of such elements [32,37]. We should note, however, that none of the technologies presented thus far will necessarily improve annotations of gene functions.

Decreasing sequencing costs have led to an increasing prevalence of RNA-sequencing studies in non-model systems. Many of these studies have focused on exploring the molecular basis of the transition from non-eusocial to eusocial lifestyles (Figure 1) [12,14••,16,17,23••,25•,26•,38,39], with studies increasingly sampling multiple taxa in search of commonalities [23••,25•,38,39]. Such gene expression studies have emphasized the importance of genes such as those involved in synthesizing and degrading juvenile hormone [25•,40,41], corazonin [23••], and insulin-like peptide 2 [14••] to caste determination and behavioral plasticity in social insects. Recent advances in nucleic acid sequencing serve to yield an even more nuanced perspective on gene regulation. Circularized long-read sequencing has been used to quantify alternative splicing of gene transcripts with a high degree of accuracy, while new library preparation and microfluidics technologies have allowed for single-cell transcriptome sequencing [42]. These advances are crucial for understanding the downstream effects of gene regulation in the case of tissue-specific and splicing-based effects, both of which have been implicated as important factors in the evolution of eusociality [23••,43].

In conjunction with differences in gene expression, the evolution of gene regulatory elements is an important facet of the transition to eusociality [32,44]. When it comes to exploring mechanisms of gene regulation, there are two main classes of noncoding DNA elements of interest: proximal and distal regulatory elements [45]. Although both classes operate by binding regulatory proteins, proximal regulatory elements are primarily comprised of promoters, while distal regulatory elements often interact with target genes through DNA looping and include enhancers, repressors, and insulators. Because of the lack of spatial concordance with coding sequence, distal regulatory elements have traditionally been challenging to study. Hi-C [46] and Hi-ChIP [47] address this particular challenge by linking interacting genomic loci together even when they are on disparate regions of the chromosome. Additionally, STARR-seq [48] allows the user to quantitatively test the effect of noncoding regulatory elements on gene expression with high throughput, but relies on the use of cell lines. The lack of immortalized cell lines for eusocial insects represents an impediment to fully utilizing the molecular resources developed for model organisms and is an area that warrants investment.

Many new technologies have focused on exploring gene regulation by profiling the genomic landscape of transcription factor binding. ChIP-seq has traditionally been used to investigate transcription factor binding and the localization of histones with specific post-translational modifications by precipitating the regions of the genome that are bound by particular proteins [49]. This technique has been refined further in recent years, as illustrated by Cut&Run [50], which has lower input requirements and is easier to execute, making it more amenable to use in non-model systems. ChIP-seq and Cut&Run can be used to assess the binding and gene regulatory effects of a specific transcription factor, but antibody development can be a non-trivial hurdle to this approach. Fortunately, histone proteins are exceptionally highly conserved and antibodies developed for mammals can be used to investigate histone modifications in insects. Profiling specific histone modifications can aid in the annotation of DNA regulatory elements, and comparing histone modifications among biological contexts offers a tractable way to explore the regulation of complex phenotypes [26•] and, consequently, major transitions (Figure 1) [51–53].

Determining which particular gene regulatory events are important to variation in a trait of interest (e.g. which transcription factors or histone modifications are worth querying) is often a challenging process. However, more general techniques exist to explore the gene regulatory landscape. ATAC-seq [54], for instance, quantifies accessibility of chromatin without the use of specific antibodies. Chromatin accessibility is fundamental to gene regulation: most transcription factors cannot bind inaccessible regions, and active loci are typically characterized by an open chromatin (accessible) state, which facilitates the identification of putatively active distal regulatory elements by ATAC-seq. Similar to Cut&Run, ATAC-seq has low input requirements and is comparatively tractable for use on non-model organisms [54]. Thus, ATAC-seq is well-suited for studying the evolution of putative regulatory regions across taxa.

Reverse genetics

Fundamental to exploring the gene-phenotype-society axis is the ability to perturb loci of interest through reverse-genetic techniques that can bridge the gap

between correlation and causation (Figure 1). These techniques also provide a crucial utility in elucidating the explicit function of genes that lack or have incomplete functional annotation. In recent years, RNA-interference (RNAi) technologies remain one of the primary toolkits for researchers working in non-model insect systems [23••,55], due to the lengthy, arduous and sometimes inaccessible process of classical reverse genetic techniques. RNAi involves the introduction of an exogenous dsRNA, usually via microinjection, complementary to a gene of interest, which, when incorporated into the innate RNA interference system present in most eukaryotic organisms, results in partial knockdown of the target mRNA. In this way, the expression of a single gene can be lowered *in vivo*. This has been used to great effect in social insect systems; recent examples include investigating the role of corazonin in foraging behavior [23••] and vestigial in imaginal wing disc-mediated worker caste differentiation [18•]. The effects of mRNA knockdown are often transient, and the magnitude of knockdown is often gene, tissue, and organism specific [56]. That being said, advances have been made with regards to delivery of dsRNA, allowing for a more sustainable knockdown [57]. Apart from RNAi, some studies have utilized pharmaceuticals to target epigenetic regulators [52] as well as synthetic peptides to alter organismal behavior [14••,23••].

Recently, a tool has emerged that allows for targeted deletion of a specific locus and holds great promise for researchers working in non-model organisms. The CRISPR/Cas9 system, which was first discovered as a bacterial immune system, allows for targeted editing of genomic DNA, utilizing specific guide RNAs. Delivery of the Cas9 nuclease in complex with a synthetic guide RNA complementary to a target genomic sequence results in a single or double stranded break at the targeted locus [58,59]. Previous methods of site-directed mutagenesis often involved laborious cloning and protein engineering (ZNFs and TALENs) [60]. However, with the advent and proliferation of the CRISPR/Cas9 system, the power of genomic knockouts has become accessible to researchers working in non-model systems. For example, this system has already been utilized in several hymenopteran species [21••,22••,61], showcasing the role of chemoreception in social behavior in ants [21••,22••]. Furthermore, this system is subject to ongoing development, with at least one alternative nuclease to Cas9 (Cpf1 [62]) recently being exploited to improve target specificity and allow for repeated editing – something prevented by the mechanisms of Cas9 cleavage – and emerging variants of Cas9 lacking nuclease activity allow for directed silencing or activation of target loci [63]. Developments have also been made regarding the delivery of Cas9 to particular tissues using peptide tags [64].

Despite the promise of CRISPR/Cas9, there are still many areas that require further development to address questions related to the major transitions. For one, most Cas9 implementations must occur in the germline or, as is the case with mosaic mutations, at early developmental time points. Because of this, most knockouts are constitutive, meaning that many genes which may be of interest but are essential to development cannot be easily perturbed. Furthermore, sustained lines of knockout animals must reproduce in the lab, which limits applications in many social insect species. Finally, the efficiency of CRISPR/Cas9 is relatively low per-injection and embryo mortality rates are often high, requiring that many embryos must be injected to attain even a few knockout animals [21••,22••].

Conclusion

Investigations of proximate and ultimate features of the major evolutionary transitions to eusociality present unique challenges to researchers. Many of these challenges are being met with the development of new model systems, advances in organismal tracking, nucleic acid sequencing, and molecular genetics techniques. An important element of such methodological advances is not what the methods do *per se* but their applicability to traditionally non-model organisms. Nevertheless, while much has been discovered about evolution and development in eusocial organisms, many questions remain. For example: (1) Is there an ancestral genetic module that was coopted by novel regulatory elements to generate features of the eusocial phenotype, as predicted by the genetic toolkit hypothesis [25•,39]? (2) Does an increase in gene regulatory complexity accompany the evolutionary transition to eusociality [6]? (3) Among the genes linked to caste determination in genome-wide screens, which are causative versus correlative? (4) Are there commonalities among the selective landscapes of organisms that have undergone full or partial reversions in the transition to eusociality? These and many other outstanding questions will continue to drive innovative research into eusocial evolutionary transitions.

Conflict of interest statement

Nothing declared.

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