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Special Collection: The Road to Sociality: Integrated Concepts of Social Behavior in Insects

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# What Can Mechanisms Underlying Derived Traits Tell Us About the Evolution of Social Behavior?

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## Abstract

Social behavior, although rare, is a highly successful form of living that has reached its most extreme forms in eusocial insects. A tractable framework to understand social evolution is the study of major transitions in social behavior. This includes the transitions between solitary to social living, from species exhibiting intermediate degrees of sociality to species exhibiting true sociality, and from primitive to advanced eusocial species. The latter transition is characterized by the emergence of traits not previously found in primitive eusocial species, such as fixed morphological differences between castes and task specialization within the sterile caste. Such derived traits appear to exist in a binary fashion, present in advanced eusocial species, and absent or rare in primitive ones, and thus do not exist in a gradient that is easily tracked and compared between species. Thus, they may not be viewed as valuable to explore ultimate questions related to social evolution. Here, we argue that derived traits can provide useful insights on social evolution even if they are absent or rare in species with a lower social organization. This applies only if the mechanism underlying the trait, rather than the function it regulates for, can be traced back to the solitary ancestors. We discuss two examples of derived traits, morphological differences in female castes and primer pheromones regulating female reproduction, demonstrating how their underlying mechanisms can be used to understand major transitions in the evolution of social behavior and emphasize the importance of studying mechanistic, rather than functional continuity of traits.

**Key words:** sociality, pheromone, caste difference, social insect

The evolution of complex social behavior has been the subject of enormous attention, ever since Darwin first presented his difficulty with the puzzling phenomenon of sterile workers (Darwin 1859). Social behavior, although rare across animals, is a highly successful form of living and has reached its most extreme form in insects where it has evolved independently multiple times. In Hymenoptera alone, eusocial behavior (the most derived and elaborate form of sociality) has evolved 8–11 times and reverted several times in groups like halictid bees (Hughes et al. 2008, Danforth et al. 2013), with many other species showing intermediate degrees of social behavior (e.g., sub-social or semi-social) (Michener 1974). Social species achieve tremendous ecological success compared with other species, in terms of their biomass, diversity, dominance, and evolutionary longevity of a clade (Wilson 1971, 1990). Ants, for example, can be found anywhere between the Arctic circle and the Equator, occupy diverse habitats from deserts to rainforests, and, together with termites, compose a third of the entire animal biomass of the Amazonian rain forest (Holldobler and Wilson 1990). This success is attributed to many factors, including collective behavior, enhanced defense capabilities and exceptional reproductive power (Deneubourg and Goss 1989, Camazine et al. 2003), regulation of internal conflicts (Ratnieks et al. 2006), and control of diseases (Schmid-Hempel 1998), raising the question what mechanisms facilitated this success and how they evolved.

## Transitions in Social Evolution

Social behavior can be best understood by studying major transitions in the evolution of sociality (Maynard Smith and Szathmary 1995, Rehan and Toth 2015). A simplified view of evolutionary transitions from solitary species to complex superorganisms includes three main transitions: 1) from solitary to social insect groups, 2) from social groups to primitively eusocial insect groups, and 3) from primitively to advanced eusocial insect groups. Solitary species found a nest alone and their transition to social living is typically characterized by facultative

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cooperative breeding without caste differences (Crespi and Yanega 1995, Boomsma and Gawne 2018). Primitively, eusocial species are defined by reproductive division of labor, mutual care of offspring, and overlap of generations (Wilson 1971), and are characterized by obligate cooperative breeding with some plasticity in castes. Finally, advanced eusocial species are characterized by obligate cooperative breeding with fixed caste differences, where workers remain unmated for life. They are further characterized by the emergence of traits, not previously found in primitive or solitary species. For example, fixed morphological differences between castes, task specialization among the sterile caste, the elimination or emergence of caste-specific organs (e.g., spermatheca and pollen collecting organs), and the regulation of reproduction and social behavior via highly specific primer pheromones (further examples are listed in Table 1). Such derived traits appear to exist in a binary fashion, present in advanced eusocial species, and absent or rare in primitive ones. Thus, they do not exist in a gradient that is easily tracked and compared between species and are viewed as not valuable for understanding social evolution. For the same reasons, these traits are rarely used to define level of social organization (Wilson 1971), since they vary across species and are often species specific. For example, the loss of spermatheca in some social insect workers is not a criterion for defining a species as a primitive or an advanced eusocial, although this is clearly a trait that is observed only in advanced eusocial insects. While transitions during social evolution are often characterized by increased complexity (Wilson 1971, Michener 1974, Theraulaz et al. 1998), productivity and overall higher ecological success, it should be noted that they do not necessarily reflect a ‘ladder of sociality’ in the simplest sense, as the evolution of social complexity has not proceeded, necessarily, in a predictable stepwise manner from solitary species to primitive and then to advanced eusocial species (Linksvayer and Johnson 2019).

## Using Shared versus Derived Traits to Study Transitions in Social Evolution

Much of the research to understand transitions in social behavior has focused on traits shared by all eusocial insect lineages, whereas traits that are considered unique to advanced eusocial groups are often viewed as not valuable to explore ultimate questions related to the evolution of sociality. However, although derived traits are not shared by all insects with different social organization, they are often regulated by conserved mechanisms that are more valuable for understanding transitions in social evolution compared with the examination of specific social traits. For example, morphological differences between female castes are a relatively ‘new’ trait in the evolution of social behavior and are found in species where reproductive skew is relatively large. Likewise, the use of primer pheromones to regulate worker reproduction (as opposed to behavioral means) is thought to be a characteristic of advanced eusocial species (though see Steitz and Ayasse 2020). Although they are very different traits, both caste differences in females and primer pheromones mediating reproduction in social groups are regulated by highly conserved hormone pathways that also regulate ovarian development (Pankiw et al. 1998), highlighting the importance of studying mechanisms over function to explain transitions in social behavior. Natural selection can only act on existing traits, and social behavior is no exception to this rule. However, while the functions of traits are likely to be lineage-specific or to shift as selective pressures change between solitary and social species, the underlying mechanisms regulating the trait are more likely to be conserved.

**Table 1.** Examples of derived traits in eusocial species

| Trait | Definition | Examples |
|---|---|---|
| Morphological caste differences | Morphological differences between reproductive and nonreproductive females in social insects that go beyond differences in body size | Honey bee female castes are irreversibly determined during larval development (Hartfelder and Engels 1998)<br>Queen caste in harvester ants is determined genetically (Julian et al. 2002) |
| Task specialization | Workers specialize in a specific helping behavior such as nursing, foraging, and guarding | Honey bee workers exhibit temporal age-based division of labor (Seeley 1982)<br>Size-based division of labor in leaf cutter ants (Wilson 1980) |
| Loss of spermatheca | The loss of the female organ where sperm is stored after mating in the sterile caste of social species | Lack of spermatheca in workers of the honey bee and several other ant species (Gotoh et al. 2013, 2016) |
| Loss of corbicula | The loss of the pollen basket located on the hind leg of bee species | Loss of the corbicula in the honey bee queen (but not in workers) (Bomtorin et al. 2012) |
| Regulation of worker reproduction via primer pheromones | The regulation of worker ovary activation and egg laying via specific chemical signals that act directly on worker physiology | Queen mandibular pheromone produced exclusively by the queen in *A. mellifera* regulates worker reproduction (Hoover et al. 2003)<br>Royal pheromones in termites regulate caste determination (Matsuura et al. 2010) |
| Defense-related morphologies | Morphological differences between castes that are associated with the task they exhibit | Termite soldiers have large mandibles (Deligne et al. 1981) |
| Outstanding morphologies | Lineage or species-specific morphologies that characterize the sterile caste | Honey pot ants serve as honey larders (Conway 1986) |
| Dance behavior | Worker communicate information about food resources via a dance | Unique to honey bees (Wilson 1971) |

A good example to demonstrate this principle is the production of the queen mandibular pheromone (QMP) in *Apis mellifera* Linnaeus (Hymenoptera: Apidae). This blend of chemicals mediates many of the social behaviors in the hive including worker reproduction and retinue behavior, worker division of labor and the development of new queens (Hoover et al. 2003). The production of QMP is a derived eusocial trait, highly specific to honey bees, and currently, the unique set of compounds have not been identified outside of Apis species (Shearer et al. 1970, Plettner et al. 1997, Nagaraja and Brockmann 2009). The only exception is one of the pheromonal components, phenylethanol, a common plant volatile that was also identified in termites, though its role is not clear yet (Himuro et al. 2011). If one focuses on the trait itself, no meaningful insights can be gained from comparing the trait across insects, simply because it is absent outside the honey bees. However, focusing on the biosynthesis of the QMP compounds, or in their perception across insects is much more useful. Indeed, several recent studies have focused on female response to

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QMP across insects, showing that *Drosophila melanogaster* (Matsumura) (Diptera: Drosophilidae) females are also reproductively inhibited by QMP (Carlisle and Butler 1956, Nayar 1963, Camiletti et al. 2013, Galang et al. 2019, Lovegrove et al. 2019). These results are perplexing since *D. melanogaster* and honey bees do not share the same habitats and are separated by hundreds million years of evolution (Lovegrove et al. 2019), but may suggest that there is a conserved mechanism in the perception of QMP, or of certain compounds within the QMP blend that are shared more broadly across insects.

## How Did Novel Traits Evolve?

The emergence of novel traits that are unique to social living may result from changes to the genome's coding sequence, regulation, and function. Genes may change their sequence resulting in a new function (novel genes), may change their pattern of regulation (via either pre-existing regulatory mechanisms that were co-opted during the evolution of sociality or via novel regulatory mechanisms), or genes may evolve a new function due to processes such as alternative splicing or post-transcriptional modifications to generate novelties. Evidence for all changes were demonstrated in social insects. For example, novel genes were shown to be involved in generating caste-specific phenotypic innovation (Feldmeyer et al. 2014, Sumner 2014), and play an important role in the evolution of odorant receptors (ORs; McKenzie et al. 2016, Brand and Ramírez 2017), and genes such as vitellogenin and hexamerins show novel social functions in bees and ants (Fischman et al. 2011). Much of the research, however, has focused on changes to gene regulation rather than to gene sequence, providing evidence that some novel traits rely on redeployment of existing mechanisms, and that novel genes may not be an absolute requirement (Robinson and Ben-Shahar 2002, Toth and Robinson 2007, Bloch and Grozinger 2011, Fischman et al. 2011). An example for changes to gene expression leading to novel traits is the genetic toolkit idea, suggesting that there are shared patterns of gene expression across social insect lineages (Rittschof and Robinson 2016). Some examples for this idea include the co-option of genes regulating maternal behavior in solitary insects to regulate sibling care in eusocial species (West-Eberhard 1987, Linksvayer and Wade 2005), genetic toolkits regulating division of labor in social species that evolved from genetic pathways regulating foraging behavior in solitary species (Toth et al. 2010), and genetic and physiological pathways regulating female castes that evolved from similar pathways regulating diapause (Hunt 2007, Santos et al. 2019, Treanore et al. 2020).

These approaches greatly advanced our understanding of the genetic and genomic mechanisms underlying novel traits but also suffer from some limitations. For example, it is not always easy to distinguish between a new gene and an old gene with a new regulation, and some genes may have multiple functions, making it harder to define a new function. Additionally, the function of novel genes is often unknown; thus it is not clear whether these are truly novel genes regulating social traits or not, and finally, studies based on the toolkit idea are limited by the fact that it is easier to show an overlap than distinction when comparing two genomic data sets. Regardless of these limitations, there is overall strong evidence for conservation in genomic mechanisms regulating novel traits that can aid in comparing trait mechanisms (rather than comparing trait function) across species.

## Derived Traits Can (Also) Be Valuable for Studying Social Evolution

Here, we would like to argue that although derived traits are understudied in the context of social evolution, they can be informative to address both ultimate and proximate questions in social evolution, for example, they may provide insights into the genetic regulatory mechanisms that led to the emergence of novel eusocial traits, or for understanding how primer pheromones regulating reproduction evolved from solitary ancestors. This, however, can be accomplished under certain conditions, namely that 1) the trait in question is unique to advanced eusocial species and is absent or rare in other insect groups, 2) the mechanisms underlying it can be traced back to solitary ancestors, and 3) the study focuses on the conserved mechanisms rather than on the derived function of the trait.

To do so, we discuss in depth two examples of derived eusocial traits, morphological differences between castes, and the use of primer pheromones to regulate worker reproduction, demonstrating how the mechanisms regulating derived traits of eusociality can be used to understand major transitions in the evolution of social behavior and emphasize the importance of studying mechanistic continuity underlying traits over their function. We argue that whether these traits are useful for understanding the origin, maintenance, or elaboration of sociality depends on the mechanistic continuity of the trait between the focal species and its ancestors and not by the continuity of the trait itself.

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## Morphological Differences Between Female Castes

### Caste Morphological Differences Across Insects

In groups of social insects, a caste can be loosely defined as a group of individuals performing a specific behavior over an extended period of time (Oster and Wilson 1978). At the broadest level, female individuals are grouped into two castes: reproductives (queen/s) and helpers (workers). These castes typically show behavioral and physiological differences, according to their role in the colony (reproductive queen/nonreproductive helpers), but do not always exhibit morphological differences. In primitively eusocial insect species, these castes are behaviorally delineated but are either lacking morphological differences or exhibiting morphological differences that are limited to body size. For example, in the Stenograstrine (Hymenoptera: Vespidae) social wasps, worker-queen dimorphism is limited to behavioral (e.g., aggressiveness) and physiological (e.g., ovary activation) differences (Turillazzi 2013), but otherwise cannot be visibly distinguished. In other groups, such as some species of Polistes L. (Hymenoptera: Vespidae), Bombus Linnaeus (Hymenoptera: Apidae), and halictid bees, morphological differences between castes are limited to body size, with queen typically two to three times larger than workers (West-Eberhard 1969, Alford 1975, Richards and Packer 1996). In these species, queens have increased fat body reserves, allowing them to survive the winter-diapause and found a nest the following spring. However, in more advanced eusocial insect species, morphological differences between female castes are more extensive and often irreversible (Wilson and Hölldobler 2005) For example, *A. mellifera* and *Apis cerana* Fabricius (Hymenoptera: Apidae) workers show degeneration or a lack of spermathecae (Gotoh et al. 2013) and queens have lost their pollen collecting organs (Michener 1974), and in some ant genera, workers lack their ovaries entirely (Villet et al. 1991, Khila and Abouheif 2010). Irreversible morphological differences between female castes are considered derived traits of eusociality because they are absent in species showing lower levels of social organization. These differences allow further task specialization and increased efficiency in performing of activities (Oster and Wilson 1978, Gordon 1996). Moreover, irreversible morphological differences are considered to be at the 'point of no return', where reversion to solitary living is improbable (Wilson and Holldobler 2005).

### Gaps in Our Knowledge and Outstanding Questions

While caste morphological differences are well described in many species, their molecular regulation, and the way they evolved is still poorly understood. Studies have focused mostly on a few model species (See following paragraph) and even within these, there is a great variability between species in the morphological traits that differentiate female castes, the factors determining caste development and the developmental phase at which caste determination occurs. Even less understood is how these traits evolved to play such an important role in shaping social behavior in societies of advanced eusocial species.

Caste differences among females are determined during development. However, the majority of studies looking at the molecular and genetic mechanisms underlying morphological caste differences in females have focused on gene expression differences in fully developed adults [selected examples: Schwander et al. (2010), Woodard et al. (2011), Harrison et al. (2014), Corona et al. (2016), Morandin et al. (2016)]. An exception is the extensive studies of caste differentiation in *A. mellifera*, where both developing and fully developed adults were investigated (Evans and Wheeler 1999, 2001; Barchuk et al. 2007). Naturally, most studies have focused on advanced eusocial insects where caste differences are significant, and very little work was done to extend these studies beyond eusocial insect species. Thus, the data available to investigate morphological differences across insects is limited. Across insects, the regulators of caste differences seem to be highly species-specific with a mix of both environmental and genetic impacts for caste determination (Schwander et al. 2010). However, the mechanisms impacted by these regulators seem to be more conserved. Within these, genetic pathways rather than individual genes have been shown to be more conserved across species and levels of social organization (Berens et al. 2015) and provide a robust framework for examining the evolution of morphological caste differences in insects.

## Genetic Mechanisms Regulating Caste Differences

Caste differences in social insects have been primarily linked to metabolic pathways that also regulate growth and development in solitary insects. Key pathways and gene families regulating caste differences in ants, bees, wasps, and termites include the insulin-signaling (IIS), glycolysis/gluconeogenesis, argenine/proline metabolism, target of rapamycin, epidermal growth factor receptor (Egfr), and hexamerins (Woodard et al. 2011, Berens et al. 2015, Corona et al. 2016). These pathways are regulated by lineage-specific nutritional, genetic/maternal, or environmental factors to generate phenotypic plasticity (Schwander et al. 2010, Libbrecht et al. 2013, Corona et al. 2016). An example demonstrating the mechanistic link between morphological differences in advanced eusocial and nonsocial species is the effect of royalactin on growth in the honey bee and Drosophila. Royalactin, the major protein component in the royal jelly, influences the development of honey bee larvae into queens through activation of the Egfr pathway in the fat body (Kamakura 2011). The same pathway regulates a similar phenotype in a solitary insect (*D. melanogaster*) that exhibits an increased body size, cell size and fecundity, and longer lifespan after being fed on a royal jelly medium (Kamakura 2011). Another example is the IIS pathway, a regulator of insect growth, development, and metabolism in many insect species (Brogiolo et al. 2001, Goberdhan and Wilson 2003, Wu and Brown 2006, Mirth and Shingleton 2012, Chole et al. 2019), which also serves acts as a key player in caste differentiation in eusocial insects, linking nutritional and hormonal gene networks (Corona et al. 2016). For example, the expression levels of IIS genes were up-regulated in queen-destined vs. worker-destined larvae in *Polistes metricus* (Hunt et al. 2003, 2010; Berens et al. 2015). The conserved function of the IIS and the broader network of metabolic pathways with which it interacts enables comparisons across major lineages of both social and nonsocial insects for studying social evolution.

## Hormonal Mechanisms Regulating Caste Differences

Hormones associated with development offer another level of mechanistic continuity across insects. Among these are some of the most well-studied hormones in insects such as prothoracicotropic hormone, ecdysteroids, and juvenile hormones, which regulate a suite of developmental, reproductive, and physiological processes (Nijhout 1998). In adult insects, caste-specific, key life events, such as reproductive maturation and regulation of diapause are controlled by a number of hormonal pathways, and many morphological traits in insects such as horns on male dung beetles, are regulated via hormones (Emlen and Nijhout 1999, Hartfelder 2000, Denlinger 2002). In larval stages, lowered JH levels in the hemolymph induce the release of the neurohormone, prothoracicotropic hormone, which then regulates ecdysteroid levels, thereby inducing significant morphological changes associated with development (Harrison et al. 2012, Niwa et al. 2014). These hormones, ultimately regulate the timing of feeding, molting, and metamorphosis, which will predict adult body size (Nijhout et al. 2006, Harrison et al. 2012). Much of the research examining the role of these hormones in insects has been done in model insects, most of which are solitary. For example, topical applications of JH analogs to *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) larvae resulted in delayed development (Gu et al. 1997). In *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae), it was shown that the inhibition of JH reduced developmental time and resulted in smaller adult size (Nijhout and Williams 1974).

The critical role hormones have in regulating body size and morphology make them well suited to regulate female caste morphological differences in social insects. This has been well-studied in termites (Miura and Scharf 2010), the honey bee (Hartfelder and Engels 1998), and ants (Libbrecht et al. 2013), where phases of larval development are characterized by JH-sensitive periods and levels of JH direct larva down a specific developmental caste trajectory (Wheeler 1986). For example, in the honey bee (*A. mellifera*), nutritional triggers during JH sensitive periods activate the IIS pathway in larvae, which in turn increases JH levels and results in the queen developmental trajectory (Mutti et al. 2011). In the bumble bee *Bombus terrestris* Linnaeus (Hymenoptera: Apidae), gyne-destined larvae in the second instar have significantly higher amounts of JH titer resulting in a longer developmental time compared with putative worker larvae (Cnaani et al. 2000). In termites, JH has the opposite role with low titers of JH and the absence of a JH peak during the molting time period resulting in alate production (Cornette et al. 2008). Caste differentiation is obviously complex and controlled by multiple interacting factors. In harvester ants (*Pogonomyrmex rugosus* Emery (Hymenoptera: Formicidae)), for example, application of methoprene, a JH analog, demonstrated that queen production is controlled by the interplay between JH and vitellogenin levels in the developing subsequent generations (Libbrecht et al. 2013). Comparative studies focusing on the shift in the role of hormones regulating development (Hartfelder 2000, Amsalem et al. 2014) can provide insights into how preexisting mechanisms have been co-opted to regulate social behavior during major transitions in social evolution.

## Integrative Approaches for Comparing Caste Differences Across Insects

The evolution of morphological caste differences in social species was also examined using an integrative approach, taking into account not only the mechanistic link between eusocial species and their solitary ancestors but also the selective pressures that may have acted on them in specific lineages. One such framework is the Diapause Ground Plan Hypothesis (DGPH). The DGPH proposes that the development of female worker and gynes castes in *Polistes* is based on the co-option of an underlying ground plan reproductive physiology of a solitary bivoltine ancestor (Hunt 2007). It is proposed that in a seasonal environment, caste differences were driven by differences in the sensitivity to nutrition during larval development, resulting in two trajectories: early-season, poorly-fed larvae that develop into adults exhibiting a reproductive-like physiology, and late-season, well-fed larvae that develop into adults exhibiting a diapause-like physiology who postpone reproduction until the following year.

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Empirical studies in support of this theory are limited, but in *Polistes* it was shown that late-season (gyne-destined) female larvae exhibited a diapause phenotype characterized by increased synthesis of hexamerin storage proteins and longer development time (Hunt 2007, Hunt et al. 2007). Additionally, a recent phylogeny demonstrated that an ancestral shift from developmental diapause (i.e., diapause as larvae or pupae) to adult diapause was a critical pre-adaptation in the evolution of sociality in bees (Santos et al. 2019). Indirect evidence for the DGPH includes a transcriptome analysis of *B. terrestris* queens showing a substantial overlap between genes regulating diapause in queens and genes previously identified to associate with the queen caste in bumble bees (in comparison to workers), as well as a shift in the function of two regulators of diapause, vitellogenin and JH, from that in Diptera. Increased JH levels were found to be unnecessary for diapause termination, and expression levels of vitellogenin and JH were not positively correlated, as is typically found in most insects (Hagedorn and Kunkel 1979, Ragland et al. 2010, Poelchau et al. 2013, Amsalem et al. 2015b). Furthermore, bumble bee workers emerging late in the colony life cycle were shown to have a diapause-like physiological profile compared with early-emerging workers, lending additional indirect support to this hypothesis (Treanore et al. 2020). This growing body of evidence suggests that the diapause genetic toolkit may have been co-opted to regulate female caste differentiation but needs to be examined on a broader scale. Such data can potentially be used to examine mechanism continuity, specific to a lineage that evolved in temperate regions and across insects exhibiting various social organizations.

Other frameworks for explaining how conserved mechanisms regulate caste-specific behaviors, such as the reproductive ground plan (RGPH; West-Eberhard 1987, Amdam et al. 2004) and the maternal-heterochrony (Woodard et al. 2014) hypotheses have been proposed. The RPGH proposes that dominance interactions in nest-sharing adult females led to a decoupling of the pre-existing reproductive cycle into reproductive tasks (e.g., egg laying) and nest maintenance tasks (e.g., brood care and foraging). The availability of genomic tools has made it possible to examine how reproductive regulatory networks in a solitary ancestor have been co-opted to regulate behavioral and physiological traits in workers of social species.

## Primer Pheromones Regulating Female Reproduction

The use of chemical signals to regulate female reproduction is widespread in social but not in solitary insects. These pheromones inhibit female reproduction by directly acting on female physiology (primer pheromones) and are more common in advanced eusocial species. Chemical molecules correlating with reproductive status have been characterized in wide range of species, both solitary and primitively eusocial (Caliari Oliveira et al. 2015, Smith and Liebig 2017, Billeter and Wolfner 2018) and are not to be confused with primer pheromones that have been identified only in a handful of species (Le Conte and Hefetz 2008, Hefetz 2019). In primitively eusocial species, primer pheromones often act in concert with behavioral mechanisms (Lommelen et al. 2010), while in advanced eusocial species, they may act alone (Le Conte and Hefetz 2008, Hefetz 2019). It is thought that pheromonal regulation (as opposed to behavioral means) is more common as the number of individuals in a colony increases, and the efficiency of behavioral means to maintain reproductive dominance decreases, as often happens in advanced eusocial species. However, the recent finding of a queen primer pheromone in a primitively eusocial Halictid with a relatively small colony size (Steitz and Ayasse 2020) provides at least one exception to this.

### Primer Pheromones Are Diverse

Primer queen pheromones vary substantially in their chemical structure and glandular origin (Keeling et al. 2004, Hefetz 2019). They have been identified in the form of oxygenated acids in the mandibular gland of *A. mellifera* (Slessor et al. 2005) and in the form of macrocyclic lactones on the cuticle (possibly from the Dufour's gland) of *Lasioglossum malachurum* Kirby (Hymenoptera: Halictidae) (Steitz and Ayasse 2020). Termites were shown to produce volatile esters and alcohols from an unknown glandular source that suppress the differentiation of new female neotenics and increase attractant to workers (Matsuura et al. 2010). Brood pheromones in the form of ethyl/methyl esters and the volatile isomeric hydrocarbon *E*-β-ocimene regulate worker reproduction in *A. mellifera* (Le Conte et al. 1990, Villalta et al. 2015), and cuticular hydrocarbons (CHCs) were shown to regulate worker reproduction in several species of ants, wasps, and bees (Holman et al. 2010, Van Oystaeyen et al. 2014, Oi et al. 2015, Smith and Liebig 2017). Evidence of queen primer pheromone was also found in the fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae), but the glandular source or the chemical identity of the pheromone remain elusive (Obin et al. 1988, Vargo 1999, Vargo and Hulsey 2000). These pheromones have been thoroughly discussed elsewhere (Howard and Blomquist 1982, Martin and Drijfhout 2009, Blomquist and Bagnères 2010, van Wilgenburg et al. 2011, Ingleby 2015, Smith and Liebig 2017, Blomquist et al. 2018, Otte et al. 2018). Here we only attempt to highlight their diverse characteristics and provide relevant examples for the possibly conserved mechanisms regulating their production and perception, and not to provide an exhaustive review of the literature. Because of these pheromones' diverse chemical structures and glandular source, they present another example for a trait that may be lineage- or species-specific that cannot be compared across insects, yet to share mechanistic elements related to the production and perception of the signals that are comparable across insects.

### Gaps in Our Knowledge and Outstanding Questions

Pheromones regulating reproduction, particularly in social insects, have been extensively studied within single species, but comparative studies across species are scarce. For instance, we still do not know how conserved or diverse the chemical structures of these signals are, and even within individual species we are still lacking basic information about the glandular origin of pheromones, their biosynthesis in the producer and perception in the receiver. The genetic mechanisms of pheromone production within species and the evolution of these regulatory mechanisms across species is even more severely limited. Thus, our understanding of the large-scale evolutionary processes underlying reproductive signaling is lacking.

### Mechanisms Underlying Production and Perception Of Primer Pheromones in Insects

The biosynthetic pathways, mechanisms of pheromone perception (olfactory receptors and neuroethology), and the genes regulating and regulated by pheromones are much less diverse compared with their chemical composition and glandular origin. Studying these processes is critical to understanding whether they can be useful to explain transitions in social behavior. However, there are only a few systems in which the molecular mechanisms of signal production, the compound's biosynthesis, and perception are studied concurrently. Two of these examples are CHCs (particularly in ants) and the QMP in *A. mellifera*. Another, esters from the Dufour's gland of bees, is less well characterized, specifically regarding their biosynthesis and perception. These examples, which, among others, we describe below, enable comparison of the many components of signal production and perception across social insect lineages, providing insight into whether signals arose de novo, were tied to previous biosynthetic processes in nonsocial insects, or were co-opted and elaborated from existing communication systems.

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## CHCs Across Insects

The diversity and ubiquitous nature of CHCs in insects provide a simultaneous challenge and opportunity for tracing the mechanistic continuity of a derived trait. As a class of compounds, they are present in all insects, but the composition of the CHC profile can vary widely between species, both quantitatively and qualitatively, preventing easy comparison. CHCs serve a basic function of preventing desiccation in both solitary and social insects and are found on the cuticle as blends of aliphatic hydrocarbons comprising various combinations of double-bond position and number, and methylations (Blomquist et al. 1987). When considered as a blend, the number of unique combinations is truly enormous, which would seem to facilitate their use in signaling multiple aspects of physiological state. In solitary insects, there is variation present in the form of contact sex pheromones produced by either males or females (Ginzel et al. 2003, Böröczky et al. 2009, Olaniran et al. 2013, Würf et al. 2020), or alteration of the hydrocarbon profile post-mating (Everaerts et al. 2010). In social insects, CHCs have been extensively researched as informative signals of species and colony identity, sex, age, caste, social, reproductive, and health condition (Blomquist and Bagnères 2010, Smith and Liebig 2017), as releaser pheromones regulating worker behavior (Funaro et al. 2018) and, in some instances, primer pheromones regulating worker reproduction (Holman et al. 2010, Van Oystaeyen et al. 2014, Oi et al. 2016), but see also (Amsalem et al. 2015a, Smith and Liebig 2017, Hefetz 2019). The signaling properties of CHCs are not limited to social insects, as CHCs were largely expanded to serve as social signals in advanced eusocial species and particularly in the context of reproduction (Orlova and Amsalem 2019), although the identity of the hydrocarbons serving as signals is species-specific. Almost all the chemical diversity seen in CHCs exists in solitary ancestors of social insects, providing a 'spring-loaded' platform for chemical communication (Kather and Martin 2015), and as the compounds involved in these signaling scenarios are no different from those found in social insects, it is likely that the receivers response to the compounds has changed, while the machinery of production and perception has been maintained. Similar to examining the mechanisms underlying morphologically distinct castes across insects, understanding how sensory systems have been elaborated from solitary ancestors can identify whether shared attributes evolved in eusocial lineages, or if the required perceptual systems evolved de novo. Thus, understanding the mechanisms of pheromone production and perception within and across lineages that evolved eusociality can be informative.

## CHCs Across Insects: Mechanisms Regulating Production

CHC biosynthesis proceeds by essentially the same process in all insects, regardless of social organization: formation of the straight chain precursors of alkanes or methyl-branched fatty acids, elongation into very-long-chain fatty acyl-CoAs, reduction of these compounds into aldehydes, and finally decarbonylation of the aldehydes into hydrocarbons (Blomquist and Bagnères 2010, Blomquist et al. 2018). This takes place in oenocytes, secretory cells found in clusters underlying the epicuticle, often associated with epidermal or fat body cells (Howard and Blomquist 2005). After synthesis, most evidence suggests that hydrocarbons are transported to the cuticle or other glands by lipophorins (Soroker and Hefetz 2000). Variation in the upstream elongases, desaturases, and reductases is what likely produce the specificity and variation seen in CHC profiles in insects, as the final step is carried out by an insect-specific subfamily of cytochrome P450 enzymes (CYP4G) that acts on all aldehyde precursors (Qiu et al. 2012). Each one of the penultimate enzymes is a candidate for tracing mechanistic continuity between levels of sociality. Most insects carry at least two CYP4G genes (Kefi et al. 2019), but in honey bees and bumble bees, the CYP4G1 gene was lost or reduced to a pseudogene (Feyereisen 2020). The upstream enzymes are also involved in fatty acid-derived pheromone biosynthesis, and some, such as reductases, have expanded greatly in the Hymenoptera and are thought to facilitate the complexity of chemical communication encountered there (Buček et al. 2013, Tupec et al. 2019). P450 enzymes are involved in two broad functions, metabolizing harmful foreign compounds (including pesticides, and plant and fungal secondary metabolites) and the biosynthesis and breakdown of the lipids and hormones that serve as signaling molecules (Feyereisen 2012). Whether solitary or social, most evidence points to CYP4G enzymes playing a role in the latter, specifically oxidative decarbonylation of hydrocarbon biosynthesis (Feyereisen 2020). This applies beyond CHCs, as they act on an intermediate of the mountain pine beetle aggregation pheromone exo-brevicomin, which shows certain CYP4Gs can accept a range of carbon chain lengths (Fischman et al. 2011, MacLean et al. 2018).

## CHCs Across Insects: Mechanisms Regulating Perception

Although both the production and the perception of CHCs in insects were extensively studied in recent years, more data pertaining CHC perception is available. In advanced eusocial insects where CHCs provide complex information related to social and physiological state, differentiating the signal from noise is a challenging task which requires specialized sensory capabilities. This is supported by genetic and neurosensory evidence that links eusociality with a higher investment in chemical communication, specifically olfaction (Robertson and Wanner 2006, Kapheim et al. 2015, Zhou et al. 2015, Wittwer et al. 2017). Ants have been extensively used as models to understand CHC perception. Evidence from leaf-cutter ants suggests that at least in this lineage, a large amount of the investment in olfaction is associated with CHCs, as CHC ORs were found to have expanded under positive selection (Engsontia et al. 2015). In *Harpegnathos saltator* Jerdon (Hymenoptera: Formicidae), a detailed characterization of a subset of ORs shows that they are narrowly tuned to individual compounds, including 3-methyl heptacosane, a candidate queen pheromone, as well as specific enantiomers of methyl-branched hydrocarbons (Pask et al. 2017). In single sensillum recordings with *Camponotus floridanus* Buckley (Hymenoptera: Formicidae), it was shown that female specific sensilla basiconica could be grouped into three categories based on the multiple ORs they housed, which were collectively capable of differentiating all CHCs tested, including caste-enriched blends (Sharma et al. 2015). In a study comparing genomes of ants and bees with flies, genes implicated in neurogenesis and olfaction were found to undergo increased positive selection before the evolution of sociality in Hymenoptera (Roux et al. 2014), and comparisons of olfactory circuits across insects and mammals show striking similarities in their sensory physiology and neuroanatomy (Benton 2006). Despite their scarcity, these studies suggesting that mechanisms underlying olfactory circuits evolved from pre-existing sensory mechanisms that served insects also in nonsocial context.

Very little work was done on specific chemoreceptors (particularly ORs) across insects, but substantial information regarding OR identity and characterization within species is available. Such 'decoding' is the first step in allowing mechanistic continuity to be traced, because comparative study requires a baseline level of information to be present in each individual case. Generally, OR complexes in insects are composed of an OR subunit (*orco*) that is necessary for the function of the unit and a specific OR that determines the specificity of the unit (Suh et al. 2014). While *orco* is highly conserved across species (Stengl and Funk 2013), ORs can be quite diverged with multiple events of gene gain and loss. For example, ORs show lineage-specific gene duplication patterns in both corbiculate bees (Brand and Ramirez 2017) and beetles (Mitchell et al. 2020), and in ponerine ants, a detailed electrophysiological study of the responses of OR subfamilies to a broad panel of ant semiochemicals, both CHCs and 'general odorants', found that ORs in each subfamily respond to multiple types of odorants, and their responsiveness to certain odorants is not predicted by their phylogeny (Slone et al. 2017). The essential nature of *orco* for olfaction and social behavior was demonstrated experimentally in clonal raider ants. Mutant ants lacking *orco* displayed

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reduced social behavior, did not follow pheromone trails, and had reduced antennal lobes (Trible et al. 2017, Yan et al. 2017). Further work on the conservation and functionality of specific odorant receptors including *orco* between solitary and social species can be used to demonstrate mechanisms continuity, and to discover the mechanisms underlying the evolution of traits unique to advanced eusocial species.

## Dufour's Gland Pheromones: Mechanisms Underlying Production

Exocrine glands are responsible for the production and dissemination of the vast array of insect semiochemicals (Billen and Morgan 1998, Billen and Šobotník 2015). Each gland often produces a complex mixture of compounds, the functions of which can vary between solitary and social insects. Two such examples are the Dufour's gland and mandibular glands. Across solitary and social Hymenoptera, the Dufour's gland (an exocrine gland present in females and associated with the sting complex) functions as a source of raw material for lining brood cells as well as chemical signals (Mitra 2013). Dufour's gland compounds have gained chemical communication functions in social lineages beyond their typical role in solitary ancestors to waterproof brood cells or preserve food provisions (Cane 1981, Hefetz 1987, Abdalla and da Cruz-Landim 2001, Mitra 2013). As such, it provides an excellent system to evaluate how communication systems evolved in transitions from solitary to social lineages.

In several solitary bee families, the gland secretes macrocyclic lactones and aliphatic, terpenyl, or terpenoid esters, in addition to hydrocarbons (Cane 1981, Hefetz 1987, Mitra 2013). There is evidence that these compounds function in kinship or nest recognition in andrenids (Ayasse et al. 1990), anthophorids (Shimron et al. 1985), megachilids (Pitts-Singer et al. 2012), and halictids (Wcislo 1992, Soro et al. 2011). As lining a brood cell is an activity linked to reproduction, these compounds have what might be thought of as a 'pre-adaptation' for becoming fertility-linked semiochemicals. Indeed, in social lineages, aliphatic esters in worker bumble bees have been linked to sterility signaling (Amsalem et al. 2009, 2013; Amsalem and Hefetz 2010). In *A. mellifera*, when queen-less workers develop ovaries, they also gain queen-like esters in their Dufour's gland which signal fertility (Dor et al. 2005). In a primitively eusocial halictid, macrocyclic lactones have been shown to function as a queen pheromone that elicits typical submissive behavior in workers and inhibits ovarian activation (Steitz et al. 2019, Steitz and Ayasse 2020).

Evidence from honey bees suggests that the Dufour's gland is the site of ester biosynthesis, while hydrocarbons may be sequestered from elsewhere (Katzav-Gozansky et al. 1997). Thus, the genes regulating pheromone production are likely active within the gland, and tissue-specific, gene expression studies could identify the key genes involved in their biosynthesis. Such information would facilitate the study of gene and pheromone evolution within and between lineages, to gain a better understanding of how pheromones may have impacted transitions in sociality.

## Mandibular Gland Pheromones: Mechanisms Underlying Production

The mandibular gland is the source of the most well-studied primer pheromone in social insects, the QMP of *A. mellifera*. In this highly eusocial species, QMP is a multi-component blend of aromatic compounds and derived 10 carbon acids, the major component of which is 9-oxy-2-decenoic-acid (9-ODA; Slessor et al. 1990, Engels et al. 1997, Plettner et al. 1997), biosynthesized from octadecanoic acid in the mandibular gland (Plettner et al. 1998). Workers and queens have similar 10 carbon acids, but during biosynthesis they are predominately functionalized at the last (ω) position in workers and the penultimate (ω−1) position in queens, resulting in caste-specific secretions (Plettner et al. 1998).

Gene expression studies of the mandibular glands in *A. mellifera* comparing queens with queen-less and queen-right workers have identified up to 46 candidate genes involved in castespecific pheromone biosynthesis in the glands (Hasegawa et al. 2009, Malka et al. 2014, Wu et al. 2017). Many of these are cytochrome P450 enzymes which regulate the site of hydroxylation (Malka et al. 2009), acting on a stearic acid-Acyl CoA precursor common to both workers and queens, and causing a divergence in the subsequent biosynthetic steps to produce queen and workerspecific substances (Plettner et al. 1998, Mumoki et al. 2019). Recent work in two subspecies of the honey bee showed that worker clones of *Apis m. capensis* Eschscholtz (Hymenoptera: Apidae), which act as social parasites, have queen-like expression of two cytochrome P450 genes involved in 9-ODA biosynthesis (cyp6bd1 and cyp6as8) that corresponds with the queen-like secretions they produce and allows them to gain reproductive dominance in their host colony (Mumoki et al. 2019). Queen-less workers of *Apis m. scutellata* Lepeletier (Hymenoptera: Apidae), on the other hand, maintain worker-like expression and pheromone production (Mumoki et al. 2019). This demonstrates that how small changes in gene expression can bias phenotypes and potentially lead to divergence in social lineages.

In *Bombus* species, the mandibular glands are also characterized by the presence of oxo-acids seemingly analogous to the compounds found in *A. mellifera* (Hefetz et al. 1996). However, they do not appear to be involved in regulating worker reproduction (Bloch and Hefetz 1999) as originally suggested (Honk van et al. 1980). Instead, they may function in sexual attraction of males to gynes (Krieger et al. 2006, Ayasse and Jarau 2014), a role common for mandibular gland secretions in solitary bees (McAuslane et al. 1990, Ayasse et al. 2001). For example, in the Andrenidae, males produce sex pheromone components that they use to mark substrates and attract females, but the females share many of the same compounds (Bergström et al. 1982).

## Mandibular Gland Pheromones: Mechanisms Underlying Perception

The machinery of exocrine gland pheromone perception offers another level at which to compare mechanisms across insects during transitions in sociality, even when the trait regulated by these gland-produced pheromones differs substantially across species. Functional characterization of the OR in *A. mellifera* antennae revealed a single odorant receptor tuned specifically to 9-ODA (AmOr11), but not of the other compounds in the QMP blend (Wanner et al. 2007). Functional imaging of the brain showed the compounds to be perceived primarily through the lateral antennal lobe in workers (Carcaud et al. 2015). Taken individually, any one of these components may offer limited insight into transitions in social behavior, but each aspect of this communication system (e.g., production and perception) could theoretically be followed to its solitary ancestor, to examine whether they were present prior to gaining social behavior, and if so, how they may have facilitated the transition. The depth of knowledge produced from the honey bee QMP pheromone system, from biosynthesis and genetic regulation of queen pheromone to olfactory receptors and how they affect worker physiology, provides an excellent foundation for comparison with solitary ancestors, but as of yet, this level of investigation in solitary bees is only just beginning. For instance, understanding whether QMP acts on physiology unique to honey bees, or a more conserved mechanism has been examined in experiments testing QMP exposure in *D. melanogaster*.

QMP reduces the number of mature oocytes in *D. melanogaster* females in a dose-dependent manner (3.25–26 queen equivalents), but long-chain CHCs proposed as ancestral Hymenopteran queen pheromones do not, nor do they augment the effect when presented in combination with QMP (Lovegrove et al. 2019). This approach could be expanded to test whether queen pheromones produce the same effect in the species they were

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identified from and target species which make more phylogenetic sense than *D. melanogaster*, like basal Hymenopterans, or solitary bees.

### Primer Pheromones in Termites

Two additional systems to trace a derived trait to a solitary ancestor are queen pheromones in termites and brood pheromones in social bees. Termite pheromones have the additional benefit of being phylogenetically distinct from Hymenoptera, where the vast majority of the information on social insect pheromones comes from. In the lower termite *Reticulitermes speratus* Kolbe (Blattodea: Rhinotermitidae), female neotenics are secondary reproductives that can differentiate from workers or nymphs after the death of the queen (Thorne et al. 1999). Matsuura et al. 2010 found that queens and their eggs produce a blend of volatiles (*n*-butyl-*n*butyrate and 2-methyl-1-butanol) which inhibits the differentiation of these new neotenic reproductives, similarly to the presence of a fertile queen (Matsuura et al. 2010). However, these compounds are not unique or novel insect semiochemicals, and mediate behaviors in distantly related groups such as sex aggregation in Coleoptera (Molander and Larsson 2018), and alarm in Hemiptera (Smith et al. 1991, Manrique et al. 2006). Knowing their glandular source and the presence in solitary cockroach relatives would facilitate study of their biosynthesis. Follow-up studies in a higher termite species *Nasutitermes takasagoensis* Nawa (Blattodea: Termitidae) found a different queen-specific volatile (phenylethanol) with as of yet undetermined role in inhibiting development of neotenics (Himuro et al. 2011), which highlight the lack of strict conservation of these compounds at least across termite groups. Outside of termites, phenylethanol was also identified as part of the queen-specific compounds of *A. mellifera* (Gilley et al. 2006), as a sex pheromone in male cabbage moth (Jacquin et al. 1991), as an alarm pheromone in the mandibular glands of ants (Wood et al. 2002), and as an aggregation pheromone in the cerambycid beetle *Megacyllene caryae* Gahan (Coleoptera: Cerambycidae) (Lacey et al. 2008). Examining whether these pheromones or the mechanisms regulating their synthesis are conserved across insect species is particularly valuable in termites, as a phylogenetically independent social lineage to compare with analogous processes in Hymenoptera (Korb 2018).

### Brood Pheromones Regulating Female Reproduction

One of the easiest systems in which to envision mechanistic continuity between solitary ancestors and eusocial descendants is the production of brood pheromones to regulate adult reproduction. Across insects, there is clear potential for conflict between females, who could invest more in egg-laying instead of brood care, and the brood, who benefit from direct care and not necessarily from having siblings (Trivers and Hare 1976). In some species of social insects, the brood regulates worker behavior such as foraging, brood care, and hygienic behavior (Maisonnasse et al. 2010, Wagoner et al. 2018). For example, in *A. mellifera*, the larvae produce a brood pheromone comprised of 10 esters, which among other functions, regulates worker reproduction (Le Conte et al. 2001, Slessor et al. 2005, Le Conte and Hefetz 2008). Two of the esters, methyl linolenate and ethyl palmitate, reduce worker ovarian development independently of the presence of the queen and brood (Mohammedi et al. 1998). The compounds are found on the cuticle, but the glandular origin of the compounds is not known. In bumble bees, larvae inhibit worker egg-laying but not ovary development (Starkey et al. 2019a, 2019b, Orlova et al. 2020). Whether the effect is pheromonal, or communicated through a different modality, is unknown. Identifying the glandular source of brood pheromones, their chemical composition and the mechanisms regulating their biosynthesis, when compared with other species, can provide valuable insight to the evolution of communication systems. Furthermore, understanding the mechanisms by which brood pheromones regulate worker behavior, and if they overlap with those regulating maternal behavior in queens and/ or solitary relatives could provide insight into the evolution of sibling care—an evolutionarily vexing phenomenon.

## Discussion

In this review, we argue that derived eusocial traits can be useful for studying major transitions in the evolution of social behavior if the underlying mechanisms of the trait can be traced back to primitively eusocial or solitary insects, regardless of whether its function is conserved across insect lineages. We discuss two examples of traits unique to advanced eusocial groups: morphological differences in female castes, and reproduction-regulating primer pheromones, both are absent or rare in primitively eusocial species, and discuss their underlying mechanisms and the merit in comparing their underlying mechanisms across insects. Mechanisms in different lineages are most often homologous, while functional adaptations are analogous (Hubbs 1944, Newman 2006). Therefore, examining mechanisms common to social and solitary insects is crucial for reaching meaningful conclusions about major shifts in key regulators during the evolution of sociality.

Transitions to sociality likely involved both novel genes and the co-option of conserved mechanisms to generate novel functions. These conserved and novel processes may interact or have complementary roles in generating unique or novel social traits. Conserved metabolic pathways, such as the IIS pathway (IIS) and Egfr, were shown to regulate female caste differences, providing a link between environmental and nutritional regulators and hormonal and metabolic networks. Ground plan theories, such as the DGPH and the RGPH, take an integrative approach for examining specific conserved mechanisms across insects that were subjected to lineagespecific selective pressures. Molecular mechanisms such as the insect cytochrome P450 enzymes underlying the perception and production of highly diverse chemical signals across insects, such as CHCs and other exocrine gland products, provide another opportunity to trace continuity from solitary ancestors to advanced eusocial species. Other examples not discussed here are task specialization in the worker caste, the loss of morphological organs associated with workers or queens (e.g., spermatheca and pollen collecting organs), specialized behaviors, and other outstanding morphologies (Table 1), all providing similar opportunity to trace mechanistic continuity of a trait that is unique to advanced eusocial species back to solitary ancestors.

While conservation of mechanisms is crucial for explaining transitions during sociality, so is the emergence of novel genes and novel regulatory pathways that can generate traits characteristic of advanced eusociality. Novel genes are previously undescribed gene sequences that do not have homology with any known sequences (Ding et al. 2012), and thus, can generate novel phenotypes. Novel genes associated with regulatory elements were found in various species including ants, bees, and wasps (Johnson and Tsutsui 2011, Ferreira et al. 2013, Simola et al. 2013, Jasper et al. 2015, Kapheim et al. 2015, Mikheyev and Linksvayer 2015). However, incomplete annotation of genomes and the lack of knowledge of their function make it difficult to understand which genes are truly novel. Better tools to increase annotation quality and to verify the functional role of these genes, and a clear understanding of the selective pressures under which different groups evolved may elucidate whether these novel genes are truly novel, why they arose and how they contributed to eusociality.

Novel eusocial traits can also be regulated by taxonomically restricted genes, which can be conserved within a lineage or across insects. A study in *A. mellifera* demonstrated that taxonomically restricted genes are more commonly found among worker-biased genes than among queen-related genes (Johnson and Tsutsui 2011), alluding that the reproductive individual employs the ancestral genetic toolkit, while the sterile helpers use the more derived one. An examination of the genes regulating age-based division of labor in ants (*Monomorium pharaonis* Linnaeus (Hymenoptera: Formicidae)) found that genetic modules regulating

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foraging and nursing behavior were made up of different fractions of conserved and taxonomically restricted genes (Mikheyev and Linksvayer 2015), indicating that novel and conserved elements may interact to generate unique behaviors. Comparison of seven ant genomes showed lineage-specific regulatory features linked to eusociality (Simola et al. 2013). However, little support was found for this idea in *Polistes*, a group with weaker levels of social organization (Berens et al. 2015). Recent comparative genomic studies across social lineages demonstrated that transitions in behavior and morphology that comprise current characteristics of eusociality involved genetic changes specific to individual lineages (Kapheim et al. 2015, Warner et al. 2019). Comparing transcriptomic changes across two major eusocial lineages with independent origins of eusociality showed that conserved genes associated with the RGPH, as well as younger lineage-specific genes, were both likely important in the evolution of eusociality. Likewise, reproduction regulating pheromones, or the glands in which they are produced can be lineage-specific, and yet to induce physiological responses that are conserved across insects in response to these semiochemicals. Our understanding of these processes in most social lineages is rudimentary (Hefetz 2019), which necessitates future research that can separate conservation from novelty specific to lineages.

## Limitations and Future Directions

Although numerous genetic pathways, individual genes, and hormonal networks have been identified as important in regulating caste differences or primer pheromones, there are several limitations to comparing these mechanisms across species. One of these is the limited availability of transcriptome- and genome-wide datasets in nonmodel organisms and across groups with varying levels of sociality. A recent estimate of available insect genomes registered with NCBI is 1219, with 137 of those belonging to Hymenoptera, yet only 47 of those were annotated (Li et al. 2019). Within Hymenoptera, there is a shortage of genomes of solitary species and poor representation of major families (Branstetter et al. 2018). Additionally, in transcriptome-wide studies, although gene expression varies with tissue, whole-body approaches are frequently used; limiting the specificity of information and strength of any conclusions made about molecular mechanisms. Mechanistic continuity is only tractable when enough empirical data exists across insect taxa.

Furthermore, although the relationship between hormones and female caste differences/primer pheromone production is understood at a basic level, less well understood are the molecular mechanisms governed by these hormones. For example, in the honey bee, it was shown that both JH and ecdysteroids regulate the expression of a nuclear hormone receptor, which then regulates the activity of downstream genes critical for development e.g., *vitellogenin* and cuticular genes (Mello et al. 2019). This line of research is still developing and restricted to a subset of social insects. Differentially expressed microRNAs have also been implicated in regulating caste differentiation in both honey bees and bumble bees (Shi et al. 2015), the latter was suggested to be regulated via the response to hemolymphatic hormone levels (Collins et al. 2017, Chole et al. 2019). The overlap in the role of individual miRNAs associated with specific castes between the honey bee and bumble bees was relatively modest. Thus, additional studies on the interplay between hormonal networks and molecular-level regulatory processes are sorely needed.

Future research efforts should focus on how conserved and novel mechanisms evolved in insects and how they facilitate the transitions in social behavior. For this to occur, continued efforts to identify the function of genes, genetic pathways, hormonal and neural regulators within individual species, as well as to characterize signaling pathways that govern physiological processes relevant to derived traits across insect taxa are required. However, such data may be limited. As we stated above, species-specific data about specific ORs responding to pheromone components or of specific enzymes regulating production of pheromones is still very limited. Even when these data are available, the comparison across species is challenging due to the focus on selected model organisms. For example, reference genomes of solitary species within Hymenoptera are scarce and most of the available genomes within this group are of parasitoid wasps with relatively poor representation of diverse families, and low depth of coverage even within the genomes that were sequenced (Branstetter et al. 2018). In addition, to make comparisons across species and identify mechanistic continuity, characterizing and identifying gene function will be essential. RNA interference is one promising avenue for establishing causality between genotype and phenotype as it has unique properties that make is highly useful for that purpose, it does not alter the germ line, it is transient and sound as gene expression, does not result in mutants and typically causes temporarily localized knock down of genes. As such, it is the go-to method for both wild and non-model organisms and could be especially effective for elucidating the function of master regulator genes that orcaster major phenotypic shift (Walton et al. 2020). Such progress was demonstrated in multiple species such as *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) and *A. mellifera* (Zhou et al. 2008, Liu et al. 2020, Walton et al. 2020).

In comparison, hormonal regulation of development and reproduction and neural regulation of pheromone olfaction in insects were much more extensively studied across both solitary and social species, providing a much more promising direction for understanding mechanistic continuity. However, these studies were largely focused on 'model' hormones like juvenile hormone and our understanding of the genetic pathways underlying hormonal and neural regulation is still limited.

Finally, although significant progress has been made in understanding shared mechanisms regulating caste differences and pheromones regulating reproduction in insects, fine-tuning of experimental approaches could reduce noise that may obscure potential conclusions. For example, studies focusing on tissue-specific, life stage-specific, and temporally regulated mechanisms may assist in generating more meaningful comparison between species. An example for the impact of such 'noise' was demonstrated in a transcriptomic comparison between foragers and nurses in harvester ants which found that certain gene expression differences were apparent only when workers were sampled during the middle of the day (Ingram et al. 2011).

### Conclusions

Altogether, identifying mechanistic continuity underlying traits associated with advanced eusociality can be a useful approach to address ultimate questions related to social evolution. These questions will require integrative, multi-disciplinary research spanning genomics, neurobiology, chemical ecology, and physiology. Promising genomic tools to improve annotation quality and advanced technologies for exploring the functional roles of genes are already in place to allow comparative studies to further our understanding of the fascinating phenomenon of social evolution.

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