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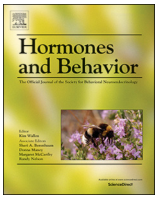

Review article

Distributed physiology and the molecular basis of social life in eusocial insects☆

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ABSTRACT

The traditional focus of physiological and functional genomic research is on molecular processes that play out within a single multicellular organism. In the colonial (eusocial) insects such as ants, bees, and termites, molecular and behavioral responses of interacting nestmates are tightly linked, and key physiological processes are regulated at the scale of the colony. Such colony-level physiological processes regulate nestmate physiology in a distributed fashion, through various social communication mechanisms. As a result of physiological decentralization over evolutionary time, organismal mechanisms, for example related to pheromone detection, hormone signaling, and neural signaling pathways, are deployed in novel contexts to influence nestmate and colony traits. Here we explore how functional genomic, physiological, and behavioral studies can benefit from considering the traits of eusocial insects in this light. We highlight functional genomic work exploring how nestmate-level and colony-level traits arise and are influenced by interactions among physiologically-specialized nestmates of various developmental stages. We also consider similarities and differences between nestmate-level (organismal) and colony-level (superorganismal) physiological processes, and make specific hypotheses regarding the physiology of eusocial taxa. Integrating theoretical models of distributed systems with empirical functional genomics approaches will be useful in addressing fundamental questions related to the evolution of eusociality and collective behavior in natural systems.

1. Introduction

Division of labor and collective behavior underlie the ecological success of eusocial species such as ants, termites, and honey bees (Ward, 2014; Korb, 2016). Eusocial species are those that live in colonies with multiple overlapping generations, with nestmates participating in brood care, nest building, foraging, and defense (Wilson and Hölldobler, 1988; Hölldobler and Wilson, 2009; Linksvayer, 2015). Eusocial species exhibit colony traits, such as collective foraging and defense (Wray et al., 2011; Gordon, 2013; Gordon et al., 2013), which are either absent or rudimentary in solitary insects.

Division of labor (DOL) refers to variation among eusocial insect nestmates in behavior, morphology, and physiology (Hölldobler and Wilson, 2009; Goldsby et al., 2014; Jeanne, 2016; Gordon, 2016a; Pasquaretta and Jeanson, 2018). In its most complex form, referred to as a caste system, the colony is composed of groups of nestmates that vary strongly in their reproductive potential and task performance. As Wilson, Hölldobler, and Seeley pointed out, the most elaborate forms of DOL lead to workers with limited information about the activities of the rest of the colony (Wilson and Hölldobler, 1988; Hölldobler and Wilson, 2009). Extensive signaling among specialized nestmates ensures a unity of purpose at the superorganismal (colony) level (Durand et al., 2019). Seeley referred to these signaling mechanisms among nestmates as “social physiology” (Seeley, 2009). Building on this concept of social physiology, Johnson and Linksvayer (Gordon, 2016a) argued that DOL is composed of two parts: **Social Anatomy** and **Social Physiology**. Social anatomy is analogous to specialized anatomy (e.g., organs, tissues) in a metazoan body, while social physiology plays the analogous role to organismal physiology in a metazoan body.

Social anatomy refers to colonies being composed of specialized parts with limited roles, like the organs of an animal organism (Fig. 1). Under certain conditions, this specialized colony anatomy allows for greater productivity and efficiency in the completion of many tasks (Waibel et al., 2006; Cooper and West, 2018; Gillooly et al., 2010;

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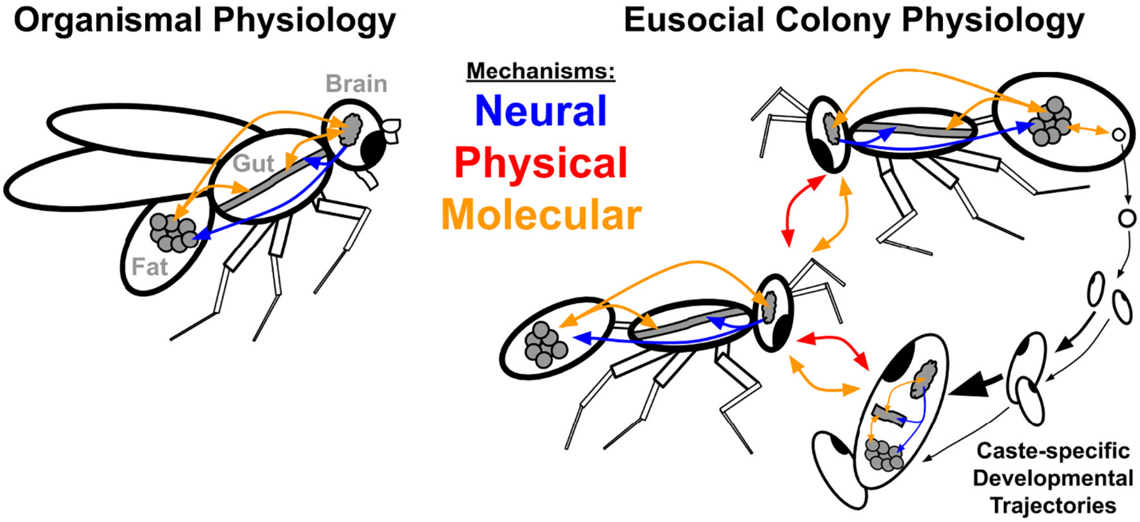

Fig. 1. Organismal and eusocial colony physiology. Organisms regulate their body physiology via neural and molecular mechanisms. In eusocial insect colonies, physiological mechanisms include these neural and molecular types, as well as the physical interactions among colony members. The regulation of nestmate and colony traits arises from interactions within and across castes, task groups, and developmental stages.

Burgess et al., 2017). Specialization exists at multiple levels within the colony. A primary distinction in the colony is between male and female nestmates (Beani et al., 2014). Queens are females with high reproductive output; workers are females with zero or very low reproductive output. Queen-worker reproductive skew allows for an increased reproductive output of queens alongside increased work output from workers, with both castes usually developing from the same diploid female genome (Ratnieks et al., 2011). Eusocial species vary widely in their total size, ratio of different classes of nestmates, and extent of subspecialization within the worker caste. Variation within and among classes of worker nestmates can manifest as variation in body size or allometry (Abouheif et al., 2014), and also is typified by temporal polyethism, the stereotyped process by which workers change in behavior and physiology as they age and in response to experiences (Gordon, 1989; Tripet and Nonacs, 2004; Johnson, 2010; Yan et al., 2014). Both morphological and temporal specializations among workers are reflected by extensive tissue-specific physiological changes that facilitate specialization by each age group (Walsh et al., 2018).

Social physiology is the set of dynamic mechanisms that coordinate the activity and development of the specialized parts of the colony (Fig. 1). The principles of colony physiology are broadly the same as the principles of organismal physiology (e.g. homeostasis, balance of anabolism/catabolism, nutrient partitioning among tissues), as colonies are complex adaptive systems that are targets of colony-level selection (Linksvayer, 2015; Birch and Okasha, 2015; Okasha, 2016). Whether colonies successfully grow and produce new queens and males that can start the next generation depends on coordination and function at the colony level. Thus it is not surprising that in many species, colony-level traits (e.g. architecture, foraging behavior) have been found to either directly influence colony-level productivity (i.e. production of workers and reproductives), or influence other ecologically-important aspects of colony function (Wilson, 1968; Anderson and Ratnieks, 1999).Colony physiological processes transfer information among nestmates and include both physical interactions (vibrations and tactile contact) and molecular signaling (Fielde and Parker, 1904; Johnson and Linksvayer, 2010; Leonhardt et al., 2016). Molecular signaling mechanisms are varied and include volatile and nonvolatile pheromones (Leonhardt et al., 2016; Bortolotti and Costa, 2014; Stökl and Steiger, 2017), as well as the direct transfer of bioactive compounds such as small RNAs, proteins, hormones, and nutrients (LeBoeuf et al., 2013; LeBoeuf et al., 2016; Maori et al., 2019).

Here we use a eusocial physiology framework to review empirical research on how organismal and colony-level physiological processes differ, interact, and co-evolve (Linksvayer, 2015; Seeley, 2009; Johnson and Linksvayer, 2010). The eusocial physiology framework considers how specialized components of the eusocial insect colony (social anatomy) interact to regulate key nestmate and colony traits (social physiology). We describe how the eusocial physiology framework leads to novel insights into how to best explore the genetics, evolution, and physiology of the eusocial insects.

2. Similarities between colony and organismal anatomy physiology

The concept of social anatomy (and similarly, the concept of the superorganism) has been criticized because in some eusocial insect species there is little queen-worker dimorphism, or because reproductive and physical castes demonstrate phenotypic plasticity at various scales (Jeanne, 2016; Gordon, 2016a; Gordon, 1989; Canciani et al., 2019). However there is a similar variation among taxa in the extent of germ-soma separation and plasticity in multicellular organisms, for example the extremely variable cases of plants, worms, sponges, and bilaterians (Winston, 2010; Gilbert et al., 2012; Neuhof et al., 2016; Colgren and Nichols, 2019; Sugden, 2000). Multicellular organisms vary considerably in size, degree of anatomical specialization, reproductive life history, and sophistication of physiological regulatory mechanisms. Similarly there is great variation among eusocial taxa in the complexity of their social anatomy (the extent of specialization among nestmates) and social physiology, and also their reproductive life history (Ward, 2014; Gordon, 2016b).

Colony and organismal physiology are both dynamic processes that play out via regulatory interactions involving neural and molecular mechanisms and various tissue types (Fig. 1). In organismal physiology, regulatory processes that are distributed among tissues coordinate behavior with cellular metabolism. For example in *Drosophila* neural and hormonal signals in the brain are in feedback with hormone signaling in gut and fat cells, leading to co-regulation of foraging behavior with fat cell metabolism (Liu and Jin, 2017; Musselman and Kühnlein, 2018). In the eusocial colonies, the coordination of foraging behavior with fat metabolism is also regulated by multi-tissue feedback loops. In the eusocial colony, larvae brain-fat-gut neurohormonal feedback loops are tightly linked to brain-fat-gut feedback loops in adult nestmates (Linksvayer, 2015; Johnson and Linksvayer, 2010). Multi-tissue physiological processes, whether in multicellular organisms or colonial superorganisms, are mediated by diffusible signaling molecules. In organismal physiology, internal fluids carry diffusible factors. These fluids include hemolymph (insects), as well as blood, lymph, and other fluids (mammals). In the eusocial insect colony, there is sharing of diffusible signaling molecules through the air (volatile compounds), through liquid solvents (through trophallaxis), as well as via the solid phase (deposition of long-lasting pheromone compounds on the ground allowing stigmergy). In sum, the hormonal and neurobiological mechanisms involved in the regulation of foraging in eusocial insects share many functional and genomic similarities with analogous systems in solitary insects (Friedman and Gordon, 2016; Kamhi et al., 2017; Nässel and Zandawala, 2019). The key difference is that the senders and the receivers of the information are in different metazoan bodies in the eusocial colony, as opposed to interactions among different tissues in a single metazoan body.

3. Anatomical and physiological innovations of superorganisms

In insects, physiological processes generally occur within single cells via signaling molecules (Tsuruyama, 2018), within multicellular organismal bodies via endocrine signaling (Rose and Mian, 2015; Stark and Theodoridis, 1973), and among insect bodies via exocrine signaling (Leonhardt et al., 2016; Stökl and Steiger, 2017). To facilitate social physiology (colony-level coordination of action) social insects have evolved radically new functions for conserved tissues and also novel tissues. For example, ant workers have more than 75 distinct exocrine glands, secreting hundreds of diverse molecular compounds (Hölldobler and Wilson, 2009; Billen, 1991; Hölldobler et al., 2014; Cerdá et al., 2014; d'Ettorre, 2016). Other eusocial insects show similarly complex exocrine systems, for example honey bees use hundreds of exocrine compounds; the physiological mechanisms and colony-level implications have been investigated for a small subset of these molecules (Bortolotti and Costa, 2014; Alaux et al., 2010).

The exocrine secretory mechanisms of insect colonies have become embedded within colony-level distributed physiological processes, meaning they are playing a fundamentally endocrine (internal regulatory) role within the colony. Whether one considers colony pheromones as exocrine compounds (from the perspective of the insect body glandular structure) or as colony-level endocrine compounds (from the perspective of the colony as an organism), there are key similarities between the influence of pheromones on colony members and hormones on organs. Both colony pheromones and organismal hormones result in large-scale behavioral changes via tissue-specific physiological manipulation, often acting in feedback loops, or at very low doses or slow time-scales (Stökl and Steiger, 2017; Khoury et al., 2013; Invernizzi and Ruxton, 2019). Reproductive signaling between the queen and workers are an obvious example of this, as is signaling from the brood to the foragers. In honey bees, for example, larvae secrete brood pheromones that stimulate foraging behavior and influence forager brain gene expression (Pankiw et al., 1998; Traynor et al., 2015; Ma et al., 2019). Essentially, foragers do not forage when they are hungry (mediated by conserved regulators of hunger signaling in solitary insects (Friedman and Gordon, 2016; Kaun and Sokolowski, 2009; Favreau et al., 2018)), but as a result of stimuli from other nestmates (Razin et al., 2013; Silberman et al., 2016; Davidson et al., 2016; Feinerman and Korman, 2017). The neurophysiological basis of reward, decision-making, and foraging are at least largely conserved across both eusocial and solitary insects (Friedman and Gordon, 2016; Perry and Barron, 2013; Søvik et al., 2015; Anreiter and Sokolowski, 2019), highlighting that evolutionary shifts in the relevance of different internal and external stimuli (e.g. internal hunger cues vs. brood signals) can lead to drastic changes in how the behavior (nursing or foraging) is deployed.

Colony traits, such as the elaborate foraging biology mediated by the dance language system in honey bees (Bortolotti and Costa, 2014; Perry et al., 2015; Lemanski et al., 2019) or the fungal agricultural practices of leaf cutting ants (Schultz and Brady, 2008; Aylward et al., 2012), are based on extensive signaling among nestmates. While many researchers have emphasized the importance of conflict within eusocial societies (Cooper and West, 2018; Birch and Okasha, 2015; Okasha, 2016; Quiñones et al., 2020; Almond et al., 2019), we suggest that these colony-level signaling mechanisms that underlie social physiological processes are largely honest in nature and have evolved mainly through colony-level selection. This is in contrast signaling mechanisms in simpler animal societies and solitary organisms, where conflict and deceptive signaling may predominate, as a result of individual-level selection. We suggest that the evolutionary elaboration of eusociality is an evolutionary trajectory that allows for runaway collaborative signaling rather than largely-adversarial tit-for-tat signaling games (Hölldobler and Wilson, 2009; Leonhardt et al., 2016; Holman, 2012). Even in the most conflict prone contexts, such as egg laying (where there is strong potential for conflict, i.e. within-colony selection, in some species between multiple queens or between queens and workers), socially complex societies likely practice largely honest communication, because dishonest communication is likely very costly at the colony level. Honest signaling of fertility is found in honey bees and several ant species (Oi et al., 2015; Villalta et al., 2018). In *Lasius niger* and other eusocial insect species, the queen(s) signals her fertility honestly even when that fertility is substandard and leads to her execution (Holman, 2012; Holman et al., 2013)(however in other ants and bees, see (Katzav-Gozansky et al., 2001; Liebig et al., 1999; Ratnieks and Visscher, 1989)).

4. Social physiology: hormonal mechanisms and evolutionary consequences

Exemplifying the distributed nature of colony physiology, some hormonal processes that are mediated internally in other insects may fall under the control of another task group within obligately eusocial colonies. While the basic players of the physiological processes may remain the same, there may be a spatial reorganization of signaling so that regulation is enacted across multiple insect bodies. For example, foraging behavior and fat metabolism are linked through integrated neurohormonal mechanisms in *Drosophila* (Kaun and Sokolowski, 2009), such that flies forage when hungry and stop feeding when full. Eusocial insect colonies must also balance foraging behavior with fat metabolism and food reserves, with an additional challenge: the foraging behavior is performed by an entirely disjoint set of nestmates (foragers) from those engaged in fat metabolism (larvae). These behaviorally- and physiologically-specialized components of the colony engage in cross-regulation using behavioral interactions (Davidson et al., 2016; Wainselboim et al., 2002; Rivera et al., 2015) and molecular signaling (Ma et al., 2019; Perry et al., 2015). The exact mechanics of the physiological distribution in the eusocial insect colony will depend on species-specific colony structure and life history. For example, stingless bees seal larvae into cells with provisions, while ants and honey bees feed brood continuously through the larval instars. These differences among eusocial taxa in the modes of nutrient processing should be associated with major adaptive shifts in larval and adult metabolism.

In social insects, there can be a fundamental rewiring and turnover of the physiological processes that were present in their solitary ancestors. The evolution of eusociality is often directly compared to other major lineage-specific transitions in evolution (e.g. prokaryote → eukaryote, unicellular life→ multicellular life (Wilson and Hölldobler, 1988; Szathmáry and Smith, 1995; Szathmáry, 2015)). Insect species that are eusocial reflect the outcome of basic insect physiology (e.g. ancestral body plan, conserved gene families) overlaid with novel superorganismal regulatory mechanisms (social anatomy and physiology). Simply by joining together into a social group, additional possible regulatory mechanisms can be enacted by non-eusocial insects at the group level (Ramdya et al., 2015; Ramdya et al., 2017), such mechanisms become refined and elaborated in the eusocial insects (Linksvayer, 2015; Leonhardt et al., 2016; Friedman and Gordon, 2016; Feinerman and Korman, 2017; Marshall et al., 2009). Here we consider several ways in which gene regulatory networks have been shaped during the transition toward eusociality, and during subsequent lineage-specific evolution of colony traits.

Several studies suggest that there is increased complexity of genomic regulatory mechanisms in eusocial insects (e.g., increased number of transcription factors or CREs (Yan et al., 2014; Simola et al., 2013; Shields et al., 2018)), as well as lineage-specific gains and losses of major families of transposable elements (Wissler et al., 2013; Rubenstein et al., 2019; Petersen et al., 2019). Over evolutionary time, genes and signaling molecules can be gained or lost from regulatory networks. Gene regulatory networks can evolve via the addition of signaling hubs from other ancestral signaling networks through new connections (more common as per EvoDevo model (Wagner and Zhang, 2011)), resulting in novel phenotypes (Eksi et al., 2018). Alternatively, gene regulatory networks can grow in social insect genomes by integrating novel (taxonomically-restricted) genes, facilitated by the fact that younger genes are apparently under less transcriptional coordination at the level of organs (Jasper et al., 2015; Johnson and Jasper, 2016) and nestmate caste distinctions (Mikheyev and Linksvayer, 2015; Warner et al., 2019a). It appears that gene regulatory networks evolve through both changes in the regulation of conserved loci and incorporation of new players: novel loci are more likely to be incorporated into distal parts of gene regulatory networks and be expressed in novel or secretory tissues, while conserved loci are more likely to undergo changes to transcriptional regulation in conserved tissues (Jasper et al., 2015; Feyertag et al., 2017).

The function of conserved members of physiological regulatory processes can be influenced by sequence changes, new regulatory connections, or other contextual changes. For example, the cGMP-dependent protein kinase G enzyme (known as *foraging* in *Drosophila*) is well-studied in the context of foraging and metabolic regulation across vertebrate and invertebrate taxa (Struk et al., 2019; Wang and Sokolowski, 2017; Anreiter et al., 2017). While the homology of the PKG locus is indeed deeply conserved, the action of PKG is cell-type specific and also probably depends on the identity of downstream phosphorylation targets. Hence there is not a consistent role or direction of effect for PKG even across just Hymenoptera (Wenseleers et al., 2008; Heylen et al., 2008; Lucas and Sokolowski, 2009; Ingram et al., 2011; Ingram et al., 2016; Malé et al., 2017). Thus while PKG may play a role in foraging-related physiological networks of diverse insects, there are species-specific changes to the inputs & outputs of PKG such that the function of over- or under-expression of PKG cannot be reliably predicted, even locally. Similar claims could be made for conserved gene families such as pigmentation/neurotransmitter-related genes that play roles in regulating worker behavior (Kamhi et al., 2017; Signor et al., 2016), and conserved neuropeptides that have gained task-specific functions (Gospocic et al., 2017; Chandra et al., 2018). Recent evidence suggests that genetic pathways involved in generating sexually dimorphic morphology and behavior in solitary insects (e.g. dsx/fru/tra (Verhulst and van de Zande, 2015; Millington and Rideout, 2018; Rice et al., 2019)), are involved in the caste differentiation in eusocial insects (Marshall et al., 2009; Trible and Kronauer, 2017; McAfee et al., 2019). This suggests that the gene regulatory networks that orchestrate variation among the physical castes in the eusocial insect colony may be as extensive as those underlying sexual dimorphism in solitary insects, potentially even reusing many of the same molecular components (Warner et al., 2019a; Sato and Yamamoto, 2019).

5. Tinkering with the toolkit may not be enough

The Reproductive Groundplan Hypothesis (Johnson and Linksvayer, 2010; Chandra et al., 2018; Pamminger and Hughes, 2017) (& other Toolkit-like hypotheses (Toth and Rehan, 2017)) posits that the plastic reproductive physiology underlying life history transitions in the solitary ancestors of social insects (e.g., between foraging and reproductive stages) is also used in social insect lineages to produce distinct worker- and queen- physiological states from the same genome (Friedman and Gordon, 2016; Favreau et al., 2018; Reinberg, 2017). This is a simple and potentially very powerful mechanism that allows for the evolution of colony phenotypes (i.e. social anatomy) without the complex genomic rewriting we emphasize in the present paper. We thus briefly explain why we think this idea is certainly true to some degree, but insufficient as a general and complete explanation for the evolution of increasingly complex social behavior leading to superorganisms (Johnson and Linksvayer, 2010; Warner et al., 2019a; Linksvayer and Johnson, 2019).

Simple modifications to the plastic state previously associated with reproductive life cycles may still be observed in queen-worker differences observed in some species considered to be “facultatively social”. However, in obligately eusocial insects, particularly the so-called “advanced” social insects, millions of years of evolution have shaped colony function such that tissue- and caste-specific specialization no longer exists within the bounds of any plausible ancestral phenotypic plasticity. In extant eusocial taxa, we observe behavioral and physiological extremes that are far beyond the range of any solitary species (e.g. 30+ year queen life in *Pogonomyrmex*, agriculture & discrete morphological castes of *Atta*, developmental scaling of *Pheidole*, workers without ovaries in *Monomorium* and *Brachyponera* (Gotoh and Ito, 2008), etc.). These extreme states are facilitated by strong alterations to the hormonal pathways involved in generating these phenotypes relative to the pre-eusocial ancestor or contemporary solitary insects, as well as the usage of conserved and novel social physiological processes used in novel contexts (Amdam and Page, 2005; Dolezal et al., 2012; LeBoeuf et al., 2018; Rodrigues and Flatt, 2016; Negroni et al., 2019). That is, while many conserved molecular mechanisms involved in basic insect reproductive physiology are certainly involved in generating both variation in reproductive and non-reproductive stages in the life cycle of solitary insects as well as variation in social insect reproductive physiology between queens and workers, the evolution of queen-worker dimorphism, and more broadly the evolution of colony-level social anatomy and social physiology truly involve phenotypic *innovation*, and not only simple modification of the expression of highly conserved insect “groundplans” or “toolkits” (Johnson and Linksvayer, 2010; Warner et al., 2019a).

6. Case studies in colony physiology: ancestral traits under colony control, and novel colony traits

There are broadly two kinds of phenotypes (measurable traits or characteristics) of eusocial insects. First there are phenotypes that can be measured from a single nestmate body, such as head width or ovariole number. Second, there are traits that are the outcomes of collective behavior and cannot be measured in single nestmates, for example, nest architecture or rate of brood production. Traits of the first kind, which manifest as variation in nestmate morphology or gene expression, bear direct homology to traits of solitary insects (Lemanski et al., 2019; Jandt et al., 2014). However, in eusocial insects, these bodily traits have fallen under extensive control of other nestmates via social physiology, so that even though these traits can be measured on a single nestmate, the traits are influenced by the physiological state of the colony during development, and properties of other group members as well (Linksvayer, 2015; Johnson and Linksvayer, 2010; Linksvayer, 2006; Linksvayer and Wade, 2016). The second kind of traits are not simply modifications of insect body physiology, as they reflect colony-specific traits that arise only in the context of group living, such as brood ratio or degree of reproductive skew. Additional examples of colony-level traits include variation in social immunity, royal jelly production, behavior, or nest architecture. These truly collective traits arise from the interaction of nestmates and the environment, and unsurprisingly the mechanisms that regulate these colony traits are likely largely unconnected or functionally absent in solitary insects.

7. Colony-level physiological regulation of worker and queen traits

Here we cover several case studies that reflect the broad range of physiological elaborations we see in eusocial insects, highlighting examples where physiological and functional genomic studies have elucidated mechanistic details about colony traits. Regulation of female fertility and reproduction is the crux of the eusocial colony lifestyle. Within a eusocial insect colony, the reproductive skew between queens (who can lay thousands of eggs in some species) and workers (who usually do not lay eggs, and may lack ovaries entirely) can be extreme. These differences in fertility are linked to morphological, hormonal, and transcriptomic differences in essentially every tissue of the body (Warner et al., 2019a). Diverse social mechanisms regulate the development and maintenance of these differences, including short-range molecular signaling (Trawinski and Fahrbach, 2018), control of nutritional intake (Vaiserman, 2014), and multiple modes of physical interaction such as piping in honey bees and drumming in paper wasps (Jeanne and Suryanarayanan, 2011; Schlegel et al., 2012; Tibbetts et al., 2018). In various ant and bee species, secretions passed among workers and queens can influence the fertility of all engaged actors, and thus influence colony productivity overall. In pharaoh ants and fire ants, queen fecundity is strongly affected by the presence of larvae, as well as the secretions made by larvae of specific stages (Tschinkel, 1995; Warner et al., 2016). Honey bee queens are stimulated to produce more eggs by being exposed to brood pheromone (Sagili and Pankiw, 2009), a positive feedback cycle within the colony where egg-laying stimulates more egg-laying. Another primary regulator of fertility in honey bees is queen mandibular pheromone (QMP). QMP is produced by active queens and has the effect of suppressing fertility and inducing other physiological changes in nearby workers, thus it is a negative feedback signal. The genes with expression responsive to queen pheromones are partially conserved among *Lasius* ants and *Apis* and *Bombus* bees despite vast evolutionary and ecological differences among these species (Holman et al., 2019). Interestingly, pharmacological treatment of *Drosophila* fruit flies with honey bee QMP seems to exert a similar phenotypic effect as in bees (e.g. repression of fertility in females), and also triggers behavioral changes in males (Croft et al., 2017). This is consistent with the notion that distribution of colony physiological may arise through the reuse of pheromonal mechanisms that are present in solitary insects, acting through novel use of inputs and outputs that have conserved for long evolutionary periods. A general caveat of pharmacological or genetic loss/gain-of-function experiments is that drastic changes to a hormone signaling axis may induce organismal or colony outcomes that do not reflect the natural physiological role of the hormone.

8. Hypotheses for social physiology

Here we present hypotheses regarding the evolutionary and functional genomics of behavior in eusocial insects. These hypotheses set a course for the integrated understanding of colony function as arising from nestmate specialization and coordination processes that have been shaped by colony-level selection. We stress that all hypotheses should be evaluated empirically using rigorous phylogenetic comparative methodology to disentangle the relative importance of genomic, ecological, and behavioral constraints, while explicitly accounting for evolutionary history (Kamhi et al., 2017; Blanchard and Moreau, 2017; Nelsen et al., 2018; Field and Toyoizumi, 2020).

9. Hypotheses for glands

We hypothesize that the distributed nature of the colony-level physiology of eusocial insect species means that the cumulative number of exocrine glands in all classes of nestmates in eusocial species will be larger when compared to solitary insects, and also that social insect glands will have more complex or voluminous glandular secretions. The increased repertoire of exocrine glands and complexity of exocrine glandular secretions likely evolves via duplication and neofunctionalization or subfunctionalization of conserved glands and underlying genes. Further, there may be patterns within eusocial taxa such that species with higher social complexity may have more specialized glandular structure present across nestmates. From an evolutionary signaling theory perspective, once a nestmate exocrine gland has become fully co-opted into colony-level regulatory networks, its dynamics and constraints will approximate that of organismal endocrine glands. Thus we hypothesize that molecular stimuli shared among nestmates have been selected for high-fidelity and rapid coordination of colony physiology to changing demands. We hypothesize that eusociality provides a new context for honest signaling systems to become elaborated such that the nature of the molecular signaling among nestmates can be more complex than social cues in non-eusocial species (Leonhardt et al., 2016). We hypothesize that the transfer of direct mediators of insect physiology among nestmates (microRNAs or chromatin remodelers in *Apis* royal jelly (Kurth et al., 2019), hormones in nurse feeding fluid (LeBoeuf et al., 2016)) will not induce antagonistic responses observed in solitary insects (such as sex conflict in *Drosophila* (Miller and Pitnick, 2002)), even when some of the same molecules may be used. We also hypothesize that recent findings in some eusocial species, such as carpenter ants, showing direct transfer of hormones via trophallaxis, will be found to be commonplace in eusocial clades (LeBoeuf et al., 2016; LeBoeuf et al., 2018).

10. Hypotheses for signaling pathways

We hypothesize that elaboration and partitioning of ancestral signals will occur such that receptors, signaling pathways, and metabolic pathways that were expressed over the course of the lifespan of the solitary ancestor, will be expressed synchronously, but distinctly by various castes, in the eusocial colony. The exocrine glands and chemosensory organs, (McKenzie et al., 2014; Hojo et al., 2015) in particular, can be expected to have strongly partitioned expression among castes and tasks (McKenzie et al., 2014; Hojo et al., 2015). One challenge for transcriptomic and epigenomic studies of brain function is that the brain undergoes many types of physiological changes for which gene expression changes are delayed, complex, or absent (e.g. topological changes in neural circuits, protein modification at synapses, time lags between neural transcription and translation). This can be contrasted with exocrine glands, for which the transcriptome can be expected to more closely approximate the instantaneous secretory function of the tissue due to rapid transcriptomic turnover (Jasper et al., 2015; Feyertag et al., 2017). We expect that neurotranscriptomic approaches involving single-cell profiling of eusocial insect brain tissue along with live-imaging and reverse genetic approaches will be required to reach nuanced understanding about the neurophysiology of nestmate behavior (Yan et al., 2014; Kohno and Kubo, 2019). Notably, collective behavior and other colony-level processes arise through interactions among nestmates, so that a second layer of organization above neurobiological mechanisms is fundamentally involved, though research on non-eusocial insects is still relevant (Gordon, 2016b; Feinerman and Korman, 2017; Dornhaus and Franks, 2008). Consistent with this decentralization of cognition across multiple nestmate bodies, colonies with increased size and specialization may have workers with proportionally smaller brains; however the strength of this trend is unclear (Gronenberg, 2008; Godfrey and Gronenberg, 2019). Another implication of increased physiological specialization in colonies is that genes with task- and tissue-specific expression patterns may be associated with non-linear changes in colony collective behavior, for example by altering worker response thresholds or sensitivity to interactions or ambient conditions (Muscedere et al., 2012; Friedman et al., 2018; Wu et al., 2019).

11. Hypotheses for functional genomics and gene regulatory networks

Gene regulatory networks of organismal colonies may be more complex than those controlling solitary insect physiology and behavior (Linksvayer, 2015; Johnson and Linksvayer, 2010; Warner et al., 2019b). Here we mean that eusocial regulatory networks are more complex in the sense that they allow for a broader range of functional connections among genes (through interactions among nestmates), increased spatial partitioning of expression (e.g. novel sex-, caste-, and tissue-specific expression patterns), and novel expression patterns through developmental time (e.g. age polyethism). Additionally, these eusocial regulatory networks can be considered more complex in that they allow the colony to exhibit emergent behaviors that are more developed or more efficient in large colonies as compared to smaller colonies or solitary insects. Several functional genomic studies have supported the hypothesis that eusocial species have increased regulatory complexity, reflected by unique patterns of transcription factors, cis-regulatory elements, and epigenetic regulatory mechanisms (Ma et al., 2019; Simola et al., 2013; Johnson and Jasper, 2016). This increased regulatory complexity of eusocial species may reflect the fact that a single genome sequence can give rise to divergent nestmate phenotypes via epigenetic plasticity. Coexpression network approaches to analyzing gene expression data have been used in many functional genomic studies of eusocial insects, in addition to the use of traditional differential expression statistics (Mikheyev and Linksvayer, 2015; Friedman et al., 2018; Morandin et al., 2019). Coexpression networks consist of genes that are co-regulated across tissues, nestmates, or colonies, thus potentially capturing sets of loci that are functionally linked over developmental (Walsh et al., 2018; Mikheyev and Linksvayer, 2015) and evolutionary timescales (Morandin et al., 2017; Qiu et al., 2018). Further research could explore how various mechanisms such as noncoding DNA (Simola et al., 2013; Rubin et al., 2019), small RNAs (Yan et al., 2014; Glastad et al., 2019), gene family evolution (Fontana et al., 2020; Brand et al., 2020), and mobile element activity (Koonin, 2016; Sanllorente et al., 2020) might contribute to these patterns. Key questions about the evolution of gene expression networks in the eusocial insects also include how ecological factors interact with ancestral gene regulatory network constraints in order to facilitate the transition to eusociality (Rubenstein et al., 2019; Linksvayer and Johnson, 2019; West-Eberhard et al., 2003), and in which ways these transitions toward eusociality are unique versus universal (Linksvayer and Johnson, 2019; Arsenault et al., 2019; Wright et al., 2019).

Eusocial insect regulatory networks can integrate new players over evolutionary time, especially in novel tissues and in positions peripheral to gene regulatory networks (Jasper et al., 2015). These new players in gene regulatory networks can arise via duplication followed by neo-functionalization, or via origination of novel coding sequences from non-coding sequences. In either case, these taxonomically-restricted genes could play a crucial role in establishing and cementing patterns of nestmate variation in physiology and behavior, for example, by allowing task-specific evolution of coding sequences in a task-biased paralog pair, as seen in the case of insulin (Chandra et al., 2018) and vitellogenin (Kohlmeier et al., 2018) signaling pathways. In mammals, it has been proposed that brain pathways can arise via duplication and subspecialization, and thus elaborate over evolutionary time analogously to gene duplication (Chakraborty and Jarvis, 2015). It would be interesting to consider whether exocrine glands in social insects may also undergo duplication and neofunctionalization or subfunctionalization over evolutionary time, potentially facilitated by expansions in families of transcription factors and enzymes involved in the production of gland secretions.

We hypothesize that novel gene regulatory networks will be formed from this decoupling of otherwise conserved pathways and traits. This is because the colony context allows for regulatory links to arise among nestmates in different developmental stages (e.g. signaling between larvae to adults (Warner et al., 2019b)), as well as utilizing physiological regulatory connections involving tactile and vibratory mechanisms (Razin et al., 2013; Hager et al., 2017). This means that there is the potential for diversified types of signaling and response in the eusocial insect colony, as well as elaboration of the molecular mechanisms underlying the response to stimuli. Functional genomic approaches that simultaneously consider multiple interacting nestmates, e.g., based on sequencing a time series of interacting nurse workers and larvae (Warner et al., 2019b), can begin to disentangle the molecular mechanisms of social signaling and the downstream physiological and developmental response. Exocrine gland and endocrine glands that are linked within the same physiological pathway in a colony (e.g. regulating foraging or reproduction) are unlikely to be functional in the same network in solitary insects, and this should be explicitly considered when performing pathway analysis or using other functional genomic approaches. Further, work on signaling pathways related to JH and Vitellogenin show that even the most fundamentally important conserved genes have different expression patterns in eusocial insects as compared to solitary insects, as well as expression variation between related eusocial species and among nestmates (LeBoeuf et al., 2018; Rodrigues and Flatt, 2016; Mello et al., 2019; Trumbo, 2018; Trumbo, 2019). The convention has been to act as if use of an ortholog constitutes conservation, but already for key cases such as PKG we know the same locus can be associated with a trait (e.g. "playing a conserved role") yet still have unpredictable patterns of expression or functional roles.

Holistic (i.e. colony-level) consideration of these issues is necessary to understand how selection acts to shape gene regulatory networks that play out across multiple insect bodies (Linksvayer, 2015). For example, a recent study in honey bees found that decades of artificial selection for increased royal jelly production was accommodated by changes in the expression of chemoreceptor proteins in nurse antenna (Wu et al., 2019). This can be understood from the perspective that nurse antennae are one of the multiple tissues that are involved in the emergent regulation of colony reproductive investment and royal jelly production. In other words, colonies may respond to evolutionary and ecological challenges in a non-linear fashion, via shaping the expression of genes that influence tissue-specific physiology of sensory organs and central processing in the brain (Lemanski et al., 2019; Kocher et al., 2018).

12. Future directions & questions

There are many opportunities for functional genomics to use eusocial insects as model systems to address general questions about hormones, development, and behavior. First, the epigenetic plasticity of eusocial workers situates them as tractable models to disentangle genetic and environmental influences on behavior (Yan et al., 2014; Chittka et al., 2012). The ecological diversity of the eusocial insects provides broad possible scope for understanding how colonies solve niche-specific challenges, particularly since many species can be kept in the laboratory so that genetic and environmental factors can be controlled. Second, new techniques can be integrated in eusocial insect taxa to bring about multidisciplinary synthesis. Recent and ongoing studies are combining natural history, automated behavioral analysis, DNA/RNA-sequencing, transgenic techniques, and pharmacological manipulations (Friedman and Gordon, 2016; Favreau et al., 2018; Kohno and Kubo, 2019; Arsenault et al., 2019; Friedman et al., 2017).

A key question is: How dramatic are the molecular changes necessary for the major transitions to eusocial colonial living from a solitary or social state? How are the initial steps toward eusocial colony living similar or different to later stages involving the expansion of colony size and elaboration of queen-worker dimorphism? Several previous authors have stressed that few molecular changes may be necessary for the initial transitions from solitary insects to small eusocial colonies (Michener, 1969; Linksvayer and Wade, 2005). We emphasize that in lineages with large and complex eusocial colonies, extreme molecular changes have likely occurred that obscure the traces of initial molecular inroads toward colony living (Warner et al., 2019a; Woodard et al., 2011). Thus, it is important to consider to what extent the multiple independent origins of eusociality converged on similar mechanisms versus how often they arrived at taxa-specific patterns (Linksvayer and Johnson, 2019).

New tools allow us to do many things in non-model systems that previously could only be done in model systems, and systems like *Drosophila* have proven helpful in broad strokes for elucidating insect physiological processes. However, millions of years of selection for colony function in eusocial insects means that even for conserved orthologs (e.g. PKG, biogenic amine receptors), gene functions may differ. This is a significant issue for Gene Ontology (GO) based analysis of functional genomic experiments in eusocial insects, as most GO terms in these species are directly transferred from *Drosophila*. Any analysis of eusocial insects that is templated off of a (distantly related) solitary insect species will systematically ignore the role of taxonomically restricted genes (Warner et al., 2019a), overstate the role of orthologous genes, and be unable to consider the implications of decentralized colony physiological processes. The challenges of colony living in eusocial insects have been accommodated through multiple types of genomic and epigenomic changes, and research should highlight these taxa-specific adaptations, not average over them. If the goal is to gain unbiased insight into the genetic changes that are most biologically important – as opposed to exploring just those genetic changes that involve highly conserved genes with more-or-less well-characterized functions in solitary organisms – then alternate approaches may be required. For example, analyzing genes that seem biologically important in eusocial insects, independent of whether they are found in other insect lineages.

Promising experimental approaches in the social insects could use RNA-Seq, proteomics, and metabolomics on the same tissue-specific samples across the classes of nestmates (e.g., developing larvae, adult nurses (Warner et al., 2019b)) involved in colony physiological processes. It is especially interesting to combine these functional genomic analyses with computational methods such as the automated tracking of behavior from video data (Davidson et al., 2016; Arsenault et al., 2019; Walsh et al., 2019). For example worker-level tracking can assess how worker heterogeneity leads to colony foraging performance (Beverly et al., 2009; Campos et al., 2016), or how trophallaxis networks provide robustness to variability in colony resource intake (Bles et al., 2018). Specifically, these types of studies in eusocial insect species could connect multilevel-network perspectives on animal behavior (Pasquaretta and Jeanson, 2018; Finn et al., 2019) with the molecular mechanisms of behavioral epigenetics and neurophysiology (Friedman and Gordon, 2016; Lemanski et al., 2019; Reinberg, 2017), in the context of a group of species with diverse ecologies and rich natural history.

Animal welfare statement

No animals were used in our research for this paper.

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Declaration of competing interest

None.

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