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REVIEW

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The *foraging* gene, behavioral plasticity, and honeybee division of labor

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Abstract In recent years, the honeybee has emerged as an excellent model for molecular and genetic studies of complex social behaviors. By using the global gene expression methods as well as the candidate gene approach, it is now possible to link the function of genes to social behaviors. In this paper, I discuss the findings about one such gene, *foraging*, a cGMP-dependent protein kinase. The involvement of this gene in regulating division of labor is discussed on two independent, but not mutually exclusive levels; the possible mechanisms for PKG action in regulating behavioral transitions associated with honeybee division of labor, and its possible involvement in the evolution of division of labor in bees.

Introduction

In his book "Sociobiology: the new synthesis", E.O. Wilson argues that sociality is rooted in biological processes, and hence associated with the function of genes (Wilson 1975). Nevertheless, sociality, like other complex behavioral phenotypes, is difficult to analyze in molecular terms, probably due to its polygenic nature as well as confounding epigenetic factors (Robinson et al. 2005). In spite of these difficulties, recent studies suggest that it is possible to identify key molecular components of social behaviors in a wide array of model organisms (Pennisi 2005). Such comparative and integrative studies suggest that social behaviors most likely evolved by acquiring new social roles for "old" genes rather than the evolution of entirely new sets of "social" genes (Robinson and Ben-Shahar 2002). This conservation of gene function is now catalyzing the ability to identify those genes that are important for complex social behaviors. The vast knowledge from molecular studies of basic behaviors and neuronal functions in genetically tractable models such as *Drosophila* can now be used as molecular building blocks for understanding complex behavioral traits such as social behavior (Ben-Shahar et al. 2004). Such prior data can be combined with the fast-growing knowledge about genomes and genetics of new social model organisms to advance "sociogenomics," the study of social behavior in molecular and genetic terms (Robinson 1999).

One model that has recently emerged as promising for sociogenomic studies is the honeybee. This insect, which lives in large social colonies, offers a unique combination of an obligatory social species with a relatively simple nervous system, and a good understanding of its sociobiology. Honeybee researchers can now use an array of genetic and molecular tools such as brain EST database (Whitfield et al. 2002), various cDNA libraries, microarrays (Whitfield et al. 2003), high-resolution genetic maps (Ruppell et al. 2004), RNAi and transgenic technologies (Kimura 2001; Farooqui et al. 2003), and most recently, the honeybee genome (http://racerx00.tamu.edu/PHP/bee_search.php).

While the recent introduction of genome-scale studies in social models promises to yield many new candidates for socially relevant genes (Pennisi 2005), we currently know little about specific genes or pathways that are important for the regulation of specific social traits in honeybees or other social models. In this review the author discusses the recent discoveries about one such molecular pathway, the *foraging* gene (*for*), a cGMP-dependent protein kinase (PKG), and its role in regulating division of labor among honeybee workers. The *for* gene is a good example of a major gene that is known to affect a nonsocial feeding behaviors in *Drosophila* and other species (Osborne et al. 1997), as well as socially regulated forms of feeding in a social insect, the honeybee (Ben-Shahar et al. 2002). This review discusses how changes in expression and activity of *for* contribute

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to the regulation of division of labor, most likely by regulating the behavioral response threshold to task specific stimuli such as light (Ben-Shahar et al. 2003), as well as the possibility that *for* has been one of the key evolutionary substrates in shaping social division of labor as exhibited by honeybees.

Honeybee division of labor as a model for molecular studies of a social behavior

One of the defining characteristics of social insects is division of labor among workers (Wilson 1971). This is an age-dependent process of behavioral development, a "temporal polyethism", in which bees at different ages perform different tasks (Winston 1987) (Fig.1). Typically, an adult bee will start her life maintaining the hive, and then gradually switch to nursing behavior, taking care of the brood and the queen. At about 1 week of age she will start performing other in-hive tasks such as comb-building or food-handling, and will keep performing these tasks until she gradually switches her behavior to foraging for pollen and nectar outside the hive at about 3 weeks of age. Some honeybees show further specializations. For example, some middle-aged bees can specialize as guards or in corpse removal prior to their final behavioral transition to foraging, traits that are affected by both genetic and environmental factors (Trumbo et al. 1997) (Fig. 1). In honeybees, division of labor is stereotypic and robust, making it an ideal model for mechanistic studies of social behavior. Although stereotypic, division of labor is also remarkably plastic, and according to colony needs, bees can accelerate their behavioral development, or even reverse it, indicating that all bees retain the potential to perform all the various tasks at every behavioral stage of their lives (Bloch and Robinson 2001) (Fig. 1).

Behavioral development in honeybees has been shown to be associated with various physiological and neural processes, which only now we are starting to understand in molecular terms (Fahrbach and Robinson 1995; Robinson et al. 1997). Studies have shown that changes in brain anatomy and chemistry are associated with division of labor (Robinson et al. 1997) as well as physiological and hormonal changes (Robinson 1992). In addition, recent work shows that in the brain, changes in global gene expression are associated with division of labor (Whitfield et al. 2003). Using microarray technology, gene expression analyses of single brains from bees that were identified behaviorally as either nurses or foragers revealed that each behavioral group has a signature gene expression profile. Studies of changes in global gene expression point to many different candidate genes for follow-up studies, which could lead to breakthroughs in our understand-

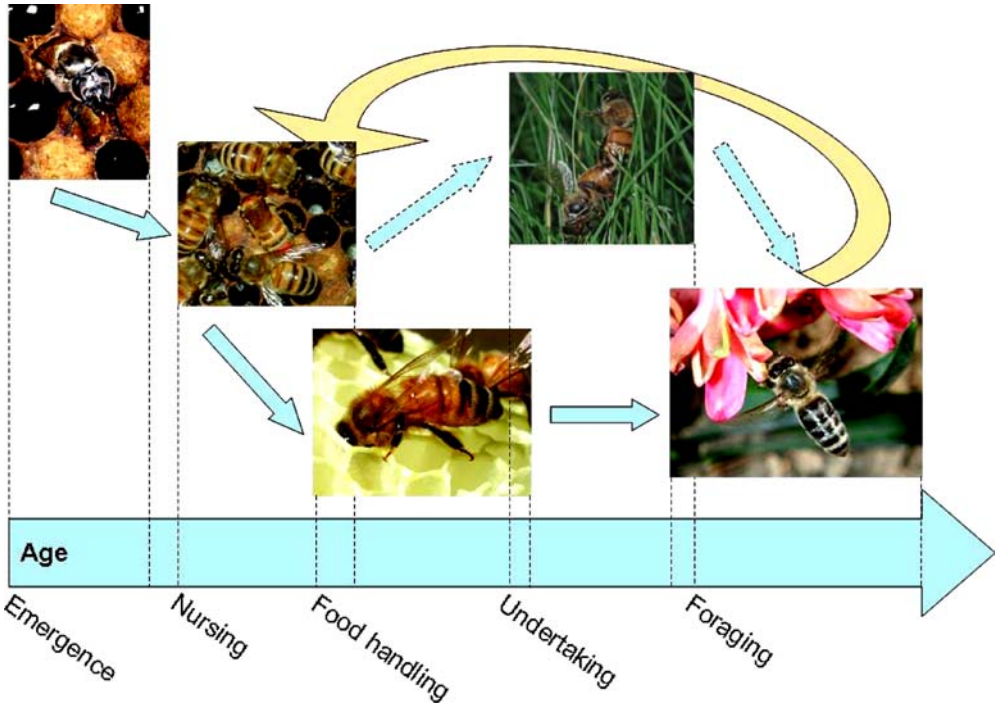

Fig. 1 Division of labor among honeybee workers. Honeybees show an age-dependent division of labor, in which each bee performs a stereotypic sequence of overlapping tasks (blue arrow). Division of labor is also very plastic. Under certain conditions, bees that are already engaged in foraging behavior can reverse back to nursing behavior, including the necessary physiological reversions (*orange arrow*). Under typical conditions, a bee will perform hive maintenance for 1–7 days, next she would nurse for about 7–14 days and then gradually switch to other in-hive tasks such as food handling or "undertaking". At about 3 weeks she will start foraging outside for nectar and pollen for the rest of her life. Under single-cohort conditions, some young bees will significantly accelerate their behavioral development, and will start foraging as early as 1-week old

ing of the genes that are important for social behavior, and enable us to narrow down the brain regions, and possibly neural circuits, where such regulation takes place. In addition, examining the function of such genes in social bees and related, nonsocial species will help us understand how such complex social behaviors could have evolved.

Genes that are likely involved in the regulation of division of labor have also been identified successfully by the "candidate gene approach" (Fitzpatrick et al. 2005). Temporal regulation of genes such as acetylcholine esterase (Shapira et al. 2001), the manganese transporter *malvolio* (Ben-Shahar et al. 2004), and *foraging* (Ben-Shahar et al. 2002; Ben-Shahar et al. 2003) were all recently shown to vary in expression and activity in association with division of labor in bees. Although the list of genes associated with division of labor is growing, to date only two genes are known to have a causative role, *foraging* and *malvolio*.

cGMP/ PKG and behavioral plasticity

The effects of cGMP and PKG signaling on behavior can be found in both the sensory and central components of the nervous system. These effects on nervous system function can be either long-term organizational, direct short-term effects on neuronal function, or both (Elekonich and Robinson 2000). Data from several model organisms suggest that cGMP can modulate the responsiveness of sensory neurons of various modalities as well as the proliferation and differentiation of such neurons (Firestein and Bredt 1998; Schmidt et al. 2002). In rodents, PKG affects the neurophysiological properties of chemosensory neurons in the vomeronasal organ (Kroner et al. 1996), and sensory neurons associated with pain (Qian et al. 1996). In the moth *Manduca sexta*, cGMP is a regulator of the male behavioral response to female sex pheromones, and in the mollusk *Aplysia*, cGMP and PKG signaling can modulate nociception-related sensory neurons (Lewin and Walters 1999). The lordosis behavior of female rats demonstrates how cGMP can also affect the central nervous system, suggesting that cGMP/PKG can mediate the effect of various steroids on behavior (Etgen et al. 1999).

Induction of feeding behavior is also closely linked to cGMP/PKG signaling in a wide variety of invertebrates. In the pond snail, feeding initiation is dependent on cGMP signaling (Moroz et al. 2000), and fictive feeding in response to sugar stimulation is blocked by inhibitors of the soluble guanylate cyclase, the enzyme that catalyze the synthesis of cGMP (Elphick et al. 1995). In *Aplysia*, cGMP was found to modulate the function of the metacerebral neuron, a serotonergic modulator of the feeding circuit in these snails (Jacklet and Tieman 2004). In addition, cGMP signaling affects feeding-related locomotion in the jellyfish *Aglantha digitale* (Moroz et al. 2004).

cGMP/PKG signaling can also regulate more complex aspects of feeding behavior. An ortholog of PKG (*egl4*) regulates feeding and growth in the worm *Caenorhabditis elegans* (Fujiwara et al. 2002). In response to hypoxic conditions, worms exhibit an aggregate feeding behavior, which is mediated by cGMP signaling (Gray et al. 2004). Finally, PKG signaling has been recently implicated in both natural behavioral polymorphisms and social behaviors in fruit flies and honeybees, the details of which are discussed below.

PKG signaling and division of labor

In honeybees, foraging is not an individual decision but rather is regulated on the colony level, suggesting that in social insects the initiation of food gathering behavior is independent of the physiological state of individual colony members (Schulz et al. 1998, 2002). That is, even under starvation conditions, with very little food in the colony, bees that are engaged in nursing behavior will not forage for food outside the hive (Schulz et al. 1998). Although we still know very little on how social foraging is regulated on the colony level, recent findings suggest that primary pheromones might be a part of the regulatory mechanism (Grozinger et al. 2003; Leoncini et al. 2004).

The *foraging* gene

In the search for genes important for the regulation of division of labor, the *foraging* (*for*) gene seemed a promising candidate. In addition to PKG-dependent regulation of the response to food-related stimuli in a variety of organisms, *for* has been shown to be involved in regulating foraging behavior in both larval and adult *Drosophila*. This gene, one of two PKG genes in the fruit fly genome, is naturally polymorphic with at least two major alleles in natural fly populations, for^R ("rover") and for^s ("sitter"). Flies carrying the rover allele exhibit longer foraging trails as larvae and adults relative to flies that carry the sitter allele (Osborne et al. 1997) (Fig. 2a). Analysis of *for* in these two natural behavioral variants revealed that rovers show both higher levels of *for* mRNA and higher levels of PKG activity in their central nervous systems relative to sitter flies (Osborne et al. 1997). These differences in PKG activity result in higher spontaneous neuronal activity at the larval neuromuscular junction. Interestingly, these patterns are also associated with increased ectopic entry points of motor neurons into muscles, suggesting that differences in PKG function can have both developmental and short-term neuronal consequences. The *for* locus also modulates the giant fiber circuit in adult flies, with adult sitters showing higher neuronal response decrements relative to rovers (Engel et al. 2000). More recently, it was shown

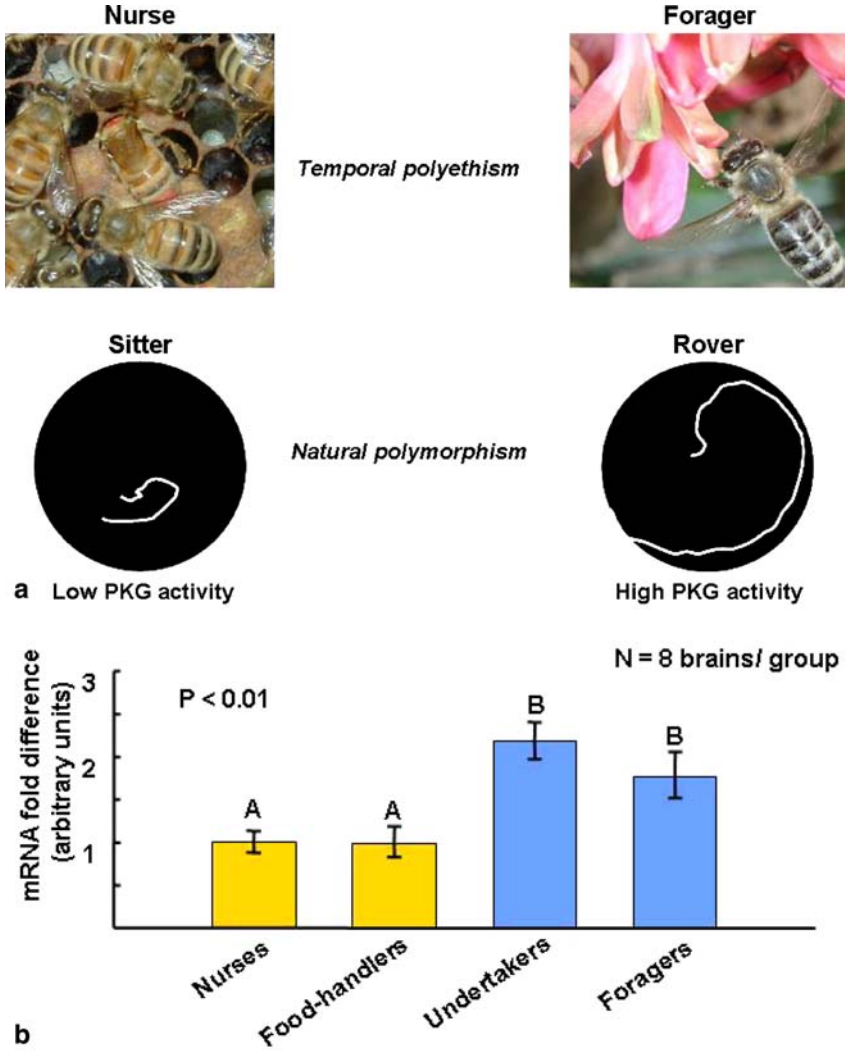

Fig. 2 Variations in the *foraging* gene and behavior. **a** The *foraging* gene can vary in expression and function due to either natural allelic variations between individual animals, "natural polymorphism"; or by being temporally regulated through the lifetime of a single individual, "temporal polyethism". Such species-dependent differences in gene function over varying time-scales, as represented by *Drosophila* and *Apis*, may resemble an evolutionary process from solitary to social regulation of feeding. **b** An increase in the expression levels of the *foraging* gene is associated with behavioral transition from "in-hive" behaviors to "outside" behaviors. Figure based on previously published data (Ben-Shahar et al. 2003)

that natural variations in *for* contribute to the adult response to appetitive sugar stimulus, with rovers showing higher sensitivity than sitters. This suggests that differences in PKG activity can affect foraging behavior in flies by modulating their sensitivity to appetitive stimuli (Scheiner et al. 2004).

The intriguing hypothesis that a gene can have conserved behavioral functions across distant species led us to suggest that *for* may affect similar, feeding-related behaviors in different species *on different time scales* (Ben-Shahar et al. 2002). We hypothesized that in flies *for*-dependent variations in feeding behavior are determined on a long-term evolutionary time scale with two behaviorally related alleles, while in bees the gene is affecting behavior on a shorter time scale by altering its activity during the behavioral development of an individual. This hypothesis is consistent with the behavioral analogies between the two behavioral morphs present in flies and bees. Hive-bound bees such as nurses feed locally on stored honey and are similar to flies carrying the sitter allele, which tend to show little locomotion while feeding. Alternatively, foragers search for food in the environment regardless of the of food already stored in the hive, a behavior similar to that of flies carrying the rover allele, which move extensively while feeding (Ben-Shahar et al. 2002) (Fig. 2a).

Amfor expression and honeybee behavior

The allelic variants in *Drosophila* suggest that variations in behavior are due, at least in part, to differences in the expression levels of *for*, which can be translated to differences in PKG activity levels (Osborne et al. 1997). We cloned a *for* ortholog from the Western honeybees *Apis mellifera* (termed *Amfor* after *A. mellifera foraging*), and showed that the protein encoded by this gene is more than 80% similar to the *Drosophila for* gene. We found that in honeybees, as in flies, differences in expression levels of *Amfor* can lead to differences in behavior (Ben-Shahar et al. 2002). As bees mature from nursing to foraging behavior, they show an increase in the expression of *for* in their brains as well as an increase in PKG

activity, representing a molecular homology to the allelic variations found in flies (Fig. 2b). The correlation between an increase in *Amfor* expression and the transition from in-hive behaviors to foraging behavior was also found in single-cohort colonies, artificial colonies composed of a group of same-age young bees. In these colonies, the lack of inhibition from older foraging bees results in accelerated precocious development of foragers, and permits the collection of bees that perform different behaviors at the same age (Leoncini et al. 2004). The results of these experiments indicate that the increase in *Amfor* expression and PKG activity are associated with task rather than age (Ben-Shahar et al. 2002).

It is also possible to activate PKG genes via pharmacological treatments. We performed oral treatments with the 8-bromo-cGMP analog of cGMP, the second messenger that activates PKG. This analog is membrane-permeable and is also resistant to phosphodiesterases, the enzymes that mediate the degradation of the endogenous cGMP. These experiments showed a dose-dependent activation of precocious foraging by cGMP, which is consistent with causative relationship between PKG activation and the initiation of foraging behavior (Ben-Shahar et al. 2002). Similar experiments with 8-Br-cAMP, an activator of the cAMP-dependent protein kinase (PKA), showed no increase in foraging activity above control levels, suggesting the effects of cGMP on behavior are specific.

The spatial expression pattern of *Amfor* in the bee brain indicates a possible place of action for PKG (Ben-Shahar et al. 2002). Although total mRNA expression levels are higher in foragers than nurses, in -situ hybridizations showed no obvious spatial differences between the two behavioral groups. This suggests that the overall increase in *Amfor* expression in foragers resulted from increased transcription in cells already expressing *Amfor* rather than recruitment of new *Amfor*-expressing cells. Although *Amfor* is widely expressed in the brain, several neuronal nuclei were more prominent than others (Ben-Shahar et al. 2002). We found that *Amfor* was expressed in a subset of the Kenyon cells, the intrinsic cells of the mushroom bodies, a brain region that is important for high-level processing of various stimuli, and learning and memory (Heisenberg 1998; Gronenberg 1999, 2001). *Amfor*-expressing Kenyon cells seem to be organized in a central column in each of the four mushroom body calyces. We also found high levels of *Amfor* expression in the optical lamina (Ben-Shahar et al. 2002). This expression pattern intrigued us since several studies suggested that interneurons in the hymenopteran optic lobe lamina project to Kenyon cells similar to those expressing *Amfor* in the mushroom bodies (Gronenberg 1999, 2001). These data suggested that the effects of PKG signaling on division of labor in honeybees might be, at least in part, by modulating the visual pathway. This hypothesis was also supported by anecdotal data showing that young bees treated with cGMP analogs were uncharacteristically present near the hive entrance suggesting a positive phototaxis (Ben-Shahar, unpublished data). Further more, previous studies showed that young bees tend to be negatively phototactic (Southwick and Moritz 1987), while in contrast, foragers are naturally positively phototactic (Menzel and Greggers 1985). Data suggest that in contrast to mammals, phototransduction in flies does not involve PKG signaling (Hardie 2001), indicating PKG is not likely to modulate visually related behaviors via direct alteration of photoreceptors function.

PKG signaling and phototaxis behavior

The possibility that the PKG signaling is affecting division of labor by modulating visually related behaviors fits well with the known physical partition in task space between foraging and hive bees; foragers are positively phototactic and are found mostly in the vicinity of the hive entrance, while younger bees such as nurses tend to be negatively phototactic and are found mostly in the inner parts of the hive (Seeley 1995). Foragers maintain their positive phototaxis behavior during foraging flight as well, showing foraging preference to well-lit places (Fry and Wehner 2002). Since the spatial expression patterns of *Amfor* in the brain support a visually related function, we tested the hypothesis that the increase in *Amfor* expression and activity during bee behavioral development is contributing to the regulation of phototaxis behavior that is associated with division of labor.

To test this hypothesis, we took advantage of a unique behavioral group of bees that specializes in the removal of dead bees from the hive (''undertakers''). These bees are at a preforaging stage, but are already performing their task outside the hive, as do the older foragers (Trumbo et al. 1997). We collected undertakers and compared the expression of *Amfor* in their brains relative to bees from other age and task groups. Our data indicate that undertakers have levels of *Amfor* expression similar to those found in foragers, and higher than the levels found in food-handlers, bees that are of the same age as undertakers but are usually found in the inner parts of the hive (Ben-Shahar et al. 2003). These data suggest that the increase in *Amfor* expression is strongly associated with tasks that are performed outside the hive, supporting the hypothesis that changes in *Amfor* expression are associated with age-dependent changes in phototaxis behavior of bees (Fig. 2b).

To further investigate the possible connection between PKG activity, phototaxis behavior, and division of labor, we again used cGMP analog treatments. Since treatments of bees with 8-Br-cGMP induce PKG activity and precocious foraging in bees (Ben-Shahar et al. 2002) we tested whether such treatments can also induce forager-like positive phototaxis, which may indicate the possible mode of action for cGMP in regulating division of labor. As predicted, bees treated with cGMP levels that can induce foraging behavior also show induced positive phototaxis, further enforcing the association between the

induction of foraging behavior and increased positive phototaxis (Ben-Shahar et al. 2003). The lack of expression of *Amfor* in photoreceptor cells as revealed by in-situ hybridization (Ben-Shahar et al. 2002) suggests that the effects of PKG signaling on phototaxis behavior are not by directly modulating visual perception. The effect is more likely to be indirect by affecting the behavioral response to a visual stimulus, downstream from the sensory level. To test this hypothesis, we used an electroretinogram analysis of 8-Br-cGMP-treated bees and showed that there were no obvious effects of the treatment on the electrophysiological properties of the photoreceptor cells; bees treated with cGMP have response curves that are indistinguishable from those found in untreated control bees. These findings were further supported by data indicating no obvious natural differences in photoreceptor activity between nurses and foragers of typical ages (Ben-Shahar et al. 2003).

cGMP/PKG signaling has a well-established role in the regulation of light input to the mammalian molecular clock, which drives the animal's circadian rhythms in both physiology and behavior (Gillette and Tischkau 1999; Golombek et al. 2003). In honeybees, division of labor is associated with a behavioral development of a circadian rhythm; young-age bees show no circadian rhythm, but as they grow older they start showing an obvious circadian behavioral pattern. This circadian behavioral shift was shown to be associated with an increase in the brain expression levels of *period*, one of the major molecular components of the clock mechanism (Toma et al. 2000; Bloch et al. 2001, 2004). Since it is not known whether PKG signaling also plays a role in the invertebrate clock system, we tested the possibility that cGMP treatments can alter the function of the central clock as another possible mechanism for the regulation of division of labor. By monitoring the activity of individual bees that were either treated with cGMP or no-treatment controls, we showed that cGMP treatments had no effect on circadian behavior, similar to what we found in untreated control bees of matched age and genetic backgrounds. We found no effects of the cGMP treatments on circadian behavior under either light/dark regime or total darkness (Ben-Shahar et al. 2003), indicating cGMP treatments did not affect either the light entrainment of the clock or its intrinsic function. We also showed that bees treated with cGMP were not more active overall, which dismisses hyperactivity as a possible explanation for the behavioral effects of cGMP (Ben-Shahar et al. 2003). In our experiments, bees were tested for circadian behavior at the same age that we tested other parameters such as PKG activity or foraging behavior. Although we found no effects of cGMP on circadian behavior, it is possible that PKG signaling is associated with noncircadian functions of clock-related proteins such as *period* (Sakai et al. 2004). In mammals, for example, expression of *period* can be directly regulated by PKG (Oster et al. 2003). A direct measure of the expression levels of *period* or other components of the molecular clock in response to cGMP treatments has not yet been performed in bees, and may reveal such interactions.

PKG signaling and the response threshold model

One of the most prominent theoretical models for the self-organizing nature of social insect colonies is the "response threshold model" (Beshers and Fewell 2001). In this model, it is assumed that although at any given time point, only a fraction of colony members is engaged in the performance of any specific task, all bees are capable of performing all the tasks required for colony survival. It is also assumed that the stimuli to perform these various behaviors are present at all times. To explain colony-level behavioral organization, the model predicts that bees at different developmental stages have different behavioral response thresholds to the task-specific stimuli, affecting the probability that a single bee will be engaged in a specific task at any given time point. The model does not exclude the possibility that some stimuli can have varying effects on bees, depending on their developmental stage, suggesting identical stimuli can lead to alternate behavioral outcomes as a result of differential processing. For example, nursing bees are sensitive to brood pheromones and will feed brood in the presence of these chemicals. In contrast, foragers are insensitive to brood pheromones in terms of nursing, but will show other behavioral modifications such as increased pollen foraging and delayed onset of foraging (Le Conte et al. 2001; Pankiw et al. 2004). These data suggest that all bees can detect brood pheromones, but respond differently to its presence depending on their developmental stage.

Empirical data for models of social insect division of labor, especially on the molecular and physiological levels, are still rare. I propose here that the association between PKG signaling and the behavioral transition from negative to positive phototaxis is one of the first cases to support the response threshold model in social insect colonies. Using this system, we showed causation between a change in a specific signaling pathway, PKG, and the response to a constant environmental stimulus, light. The striking difference in light conditions between the inside of the hive and the outside world provides a clear signal for bees in terms of task-space. Once a bee is ready to forage, light becomes the first cue for where foraging should be taking place. The light signal is likely to be perceived by all bees in the hive, and so serves as a stimulus-based barrier that defines where various tasks should be performed.

How does a PKG-dependent change in phototaxis affect division of labor? One possibility is that increased PKG activity is directly causing the transition to foraging behavior, and this behavioral state is directly associated with a change in the behavioral sensitivity to foraging-related stimuli such as light. Another related possibility is that the change in PKG activity induces higher affinity to light (positive phototaxis), which

results in the spatial positioning of foragers closer to the hive entrance where other foraging-related stimuli such as floral odors or dances of returning foragers can further increase the probability and motivation of a bee to perform foraging behavior. That is, increased sensitivity to light is not a direct cause for foraging behavior but rather a mechanism to increase exposure to other more direct foraging stimuli in a hierarchical manner. Future work should address such issues, whether changes in PKG activity can affect other sensory modalities such as olfaction or tactile sense. Based on what we know about its functions in other organisms, it is likely that PKG affects many other aspects of bee physiology and behavior with both short activational, and long-term organizational outcomes.

cGMP is only one of several molecules that is known to induce foraging behavior in bees. Factors such as juvenile hormone (Jassim et al. 2000) have been shown to induce division of labor as well, but the molecular mechanism underlying its effect is unknown. Although highly speculative, it is possible that JH may affect behavior in bees via a second messenger system (Wheeler and Nijhout 2003), possibly the PKG -signaling pathway. A similar mechanism for the activation of PKG by a steroid-like molecule, estradiol, was recently shown in mammals (Babiker et al. 2004). The relationship between cGMP signaling and other potent activators of foraging behavior such as octopamine (Schulz et al. 2003), and manganese (Ben-Shahar et al. 2004) is also not clear. Future experiments in which bees are treated with various combinations of these diverse compounds will help to sort whether these behavioral activators represent independent pathways or whether they affect similar molecular pathways.

Finally, a recent work now suggests that the *foraging* gene is involved in the regulation of division of labor in ants (Ingram et al. 2005) which further supports the key role for this gene in regulating feeding-related behavioral plasticity. Whether *foraging* has a universal role in regulating feeding-related behaviors as well as the evolution of division of labor in social insects is still an open question, which could be answered by understating its function in diverse behavioral systems.

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