The Physiology of Division of Labor in the Ant,

Pogonomyrmex californicus

by

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ABSTRACT

A notable feature of advanced eusocial insect groups is a division of labor within the sterile worker caste. However, the physiological aspects underlying the differentiation of behavioral phenotypes are poorly understood in one of the most successful social taxa, the ants. By starting to understand the foundations on which social behaviors are built, it also becomes possible to better evaluate hypothetical explanations regarding the mechanisms behind the evolution of insect eusociality, such as the argument that the reproductive regulatory infrastructure of solitary ancestors was co-opted and modified to produce distinct castes.

This dissertation provides new information regarding the internal factors that could underlie the division of labor observed in both founding queens and workers of *Pogonomyrmex californicus* ants, and shows that changes in task performance are correlated with differences in reproductive physiology in both castes. In queens and workers, foraging behavior is linked to elevated levels of the reproductively-associated juvenile hormone (JH), and, in workers, this behavioral change is accompanied by depressed levels of ecdysteroid hormones. In both castes, the transition to foraging is also associated with reduced ovarian activity. Further investigation shows that queens remain behaviorally plastic, even after worker emergence, but the association between JH and behavioral bias remains the same, suggesting that this hormone is an important component of behavioral development in these ants. In addition to these reproductive factors, treatment with an inhibitor of the nutrient-sensing pathway Target of Rapamycin (TOR)

also causes queens to become biased towards foraging, suggesting an additional sensory component that could play an important role in division of labor. Overall, this work provides novel identification of the possible regulators behind ant division of labor, and suggests how reproductive physiology could play an important role in the evolution and regulation of non-reproductive social behaviors.

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INTRODUCTION

Division of Labor in Social Insects

In most eusocial insects, members of the sterile worker caste respond differently to a common group environment, resulting in behavioral heterogeneity that builds a superorganism (Hölldobler and Wilson, 2009). This division of labor between workers allows social insect colonies to perform many tasks at once, and is a major factor in their widespread ecological success (Wilson 1971). For many eusocial species, task performance is temporally based, with younger workers performing in-nest tasks, such as brood care and nest maintenance, and older workers leaving the nest in favor of foraging-related tasks. This behavioral transition occurs over the lifespan of an individual worker, so that the majority of individuals perform different suites of tasks during their lives (Hölldobler and Wilson, 1990; 2009). Although these behaviors are usually age-dependent, changes in colony structure and condition can result in behavioral changes outside of this basic pattern. Since this division of labor is so important in the success of the social insects, understanding how this system of complex behavioral regulation evolved is a major question in the study of social insect biology.

There are several ultimate explanations for the course of insect evolution from solitary to eusocial species (Hamilton, 1964a, 1964b; Wilson and Hölldobler, 2005; Hölldobler and Wilson, 2009; Nowak et al., 2010). For the most part, however, these arguments do not address how this transition could have

occurred at a proximate, or mechanistic, level. Understanding this process is difficult, since the best studied social insect groups (honey bees and ants) are highly derived and have been eusocial for many millions of years (Moreau, 2006; Winston, 1987). However, by looking at how extant eusocial insect behaviors are regulated, it is possible to extrapolate how these behaviors may have evolved.

The Ground Plan Hypotheses

Research into the regulation of how individuals transition across behavioral phenotypes has provided insights into genetic, physiological and neural affecters of social behavior (e.g., Robinson et al., 1992; Ben-Shahar et al., 2002; Amdam et al., 2004; Rueppell et al., 2004; Amdam et al., 2006; Hunt et al., 2007; Amdam and Page, 2010). The data from these studies have been used to generate, and increasingly support, arguments referred to as the ground plan hypotheses (West-Eberhard, 1987, 1996; Amdam, 2004, 2006; Linksvayer and Wade, 2005; Hunt, 2005). These lines of reasoning argue that the building blocks of eusocial insect behaviors are derived from the reproductive regulatory structures of solitary ancestors: instead of evolving new genetic and physiological regulators for non-reproductive phenotypes, such as nurses and foragers, the mechanisms that controlled sequential reproductive events in solitary ancestors were co-opted via natural selection for social tasks decoupled from reproduction.

These arguments began with studies exploring how social behavior and physiology are linked in *Polistes* paper wasps that found variation in ovarian activation was associated with behavioral differences in wasp workers

(references). Similar associations within a variety of eusocial wasps, ants and bees support this idea: slight ovarian development is generally interlinked with worker activities such as cell initiation, brood-rearing and production of trophic eggs, while individuals with undeveloped ovaries perform tasks outside the nest, such as foraging (West-Eberhard, 1987).

In many solitary Aculeate wasps, the source of all eusocial hymenoptera, a parent forages for large quantities of food to provide for a group of offspring raised in a nest (Wilson and Hölldobler, 2005), and changes in these behaviors depend upon reproductive physiology. Therefore, it was argued that female worker behaviors are regulated by the same mechanisms that caused solitary ancestors to transition between stages of provisioning (foraging) and egg laying (nest-tasks). Instead of developing a new regulatory architecture to control worker behaviors, ovarian mechanisms were co-opted for regulating parallel behaviors in social workers—implying that the ovarian cycle, as a sequence of co-regulated physiological and behavioral events, provided a mechanistic basis for insect social evolution. This argument is referred to as the ovarian ground plan hypothesis (OGPH; West-Eberhard, 1987; 1996).

How could the ovary, or the reproductive system in general, be used to develop the complex, non-reproductive behavioral suites observed in eusocial insects? In eusocial Hymenoptera, colonies are predominantly made up of females: the reproductive queen and many sterile, female workers. Since the female workers represent a source of reproductive conflict (Bourke and Franks, 1995), which is deleterious to colony efficiency, selective forces have acted upon

the worker phenotype to prevent reproductive development and activation through a variety of processes (Khila and Abouheif, 2008; Hölldobler and Wilson, 2009). Therefore, the traditional view is that selective pressures have pushed towards workers with increasingly atrophied reproductive systems (Bourke and Franks, 1995). However, abolition of reproductive structures, even just the observable ovary, is quite rare, having occurred only in a few ant genera (Hölldobler and Wilson, 1990), and there are clear correlations between division of labor and the status of the reproductive organs in many social insects (Seeley, 1982; West-Eberhard, 1987; Hölldobler and Wilson, 2009). Therefore, the reproductive system could still play a role in functionally sterile workers.

Investigation of the foraging preferences of honey bee workers led to further understanding of the reproductive underpinnings of eusocial insect behaviors. Bee colonies naturally vary in their predilection for storing different types of food, differentiating between protein (pollen) and carbohydrate (nectar) sources. This variation was utilized in bi-directional selection programs to breed colony-level phenotypes that predictably differed in the amount of pollen stored in the nest. These programs produced two distinct genetic strains of bees: high pollen-hoarding strains, which store vast amounts of pollen, and low pollen-hoarding strains, which store mostly nectar, and very little pollen (Page and Fondrk, 1995). In addition to food-related behavior, however, the strains also diverged in traits associated with reproductive infrastructure and behavior.

In honey bee workers, levels of the yolk precursor protein vitellogenin change during adulthood, peaking during the nurse stage and decreasing

afterwards, which is followed by the onset of foraging. During the nursing phase, vitellogenin is used to produce proteinaceous food for developing larvae, implying that a normally reproductive protein, used to build eggs, could be co-opted for social uses (Amdam et al., 2003). Furthermore, in the artificially selected high- and low-pollen hoarding bees, strain-specific differences in both ovariole number and vitellogenin levels were observed. Compared to the low strain workers, those of the high strain have larger ovaries (i.e. more ovarioles), and *vitellogenin* expression increases more quickly and to a higher level. The subsequent drop in *vitellogenin* expression occurs earlier in high strain bees, and they also initiate foraging behavior earlier (reviewed in Amdam and Page, 2010), suggesting that high levels of vitellogenin predispose workers to become pollen foragers while a drop in vitellogenin signals the onset of foraging. In support of these ideas, RNA interference-mediated knockdown of *vitellogenin* gene expression in wild-type (unselected) honey bees triggered early foraging onset and biased foragers to collect nectar (Nelson et al., 2007). Additional studies confirmed that having better developed ovaries correlates with high initial *vitellogenin* expression and subsequent pollen foraging in wild type bees (reviewed in Amdam and Page, 2010). Taken together, these results breathed new life into the investigation of the reproductive system's role in social insect behavioral regulation.

By using an evolutionary developmental biology approach and viewing honey bee behaviors as modules, it was argued that these results provided evidence for a solitary ground plan on which natural selection acted to produce

highly derived worker traits. Thus, the associations identified in honey bees were compared with the cycle of reproduction and behavior previously studied in solitary insects (Finch et al., 1995; Lin and Lee, 1999; Miyatake, 2002). In mosquitoes, for instance, young females seek out protein-rich blood meals to fuel their reproductive systems. Protein foraging ceases as eggs mature inside the female, and once the eggs are laid, the mosquito begins the cycle over again (Klowden, 1997). Aspects of these relationships appeared to be paralleled in honey bee workers, where reproductive traits such as vitellogenin level and ovary size influenced the individuals' foraging preference for pollen —the major protein source of bees (Amdam et al., 2004). From such theoretical comparisons, the reproductive ground plan hypothesis (RGPH) was proposed.

The RGPH hypothesis, like the OGPH, argues that co-regulatory modules of reproductive activity and behavior provided a substrate that evolution could act upon to produce distinct behavioral phenotypes. But unlike the OGPH, the RGPH provides a framework to account for behavioral biases within worker task groups. Instead of maturing or cycling through reproductive states and changing behavior accordingly (like the mosquito), a worker could express a distinct behavioral bias throughout life based on her innate reproductive tuning. Thus, worker honey bees tuned for 'reproduction', observed with higher vitellogenin levels and ovary size, would hoard protein-rich food sources, similar to a reproductively active solitary ancestor provisioning her nest. (Amdam et al., 2004; Amdam and Page, 2010).

The work investigating these selected strains of bees, with their artificially extreme behavioral phenotypes, led to a broader evolutionary hypothesis

regarding a more generalized pathway to social evolution. However, while the strains with these extreme phenotypes are very useful for identifying the genetic and physiological links to behavioral differences, such a broad evolutionary hypothesis is best understood by investigation in multiple social insect lineages.

Assessing Cross-Taxa Applicability: *Pogonomyrmex californicus*

In order to broaden our perspective on how reproductive physiology is related to social insect behavioral regulation and evolution, it is necessary to investigate these factors in other social species.The most obvious group in which to expand this research is the ants. While the behavioral phenotypes in ants and bees are very similar, ant sociality evolved independently 115-135 million years ago (Brady et al., 2006, Moreau et al., 2006). Despite their widespread phenotypic diversity, all ant species exhibit the same temporal division of labor in the worker caste as observed in *A. mellifera*. Thus, the ants provide ideal systems for comparative studies with the regulatory systems previously described and evaluated in the honey bees. Although numerous evolutionary scenarios have been posited for the temporal division of labor between ant workers (reviewed in Hölldobler and Wilson, 1990, 2009), there have been few studies testing the physiological adaptations necessary to achieve such behavioral changes.

This dissertation research attempts to address these questions using the ant species *Pogonomyrmex californicus*. Although many ant species exhibit worker temporal polyethism (Hölldobler and Wilson; 1990, 2009), *P. californicus* was chosen for this research because of the behavioral variation observed in their

queens. First, the queens have a semiclaustral founding strategy, in which newly mated queens must forage for larval provisions before their first workers emerge. This is a temporal shift from nest-biased (non-foraging) to field-biased (foraging) behavior (Johnson, 2002), which parallels the behavioral transition observed in their workers. In addition, *P. californicus* populations can vary in the number of queens that found a nest. In some populations, aggression between founders is high, so queens initiate new colonies alone (hapolometrosis). In other populations, aggression is low and new colonies are founded by multiqueen associations (pleometrosis) (Cahan and Fewell, 2004; Johnson, 2004; R. Overson, unpublished data). Under pleometrotic conditions, behavioral biases can emerge between queens, so that one primarily performs nest tasks and the other forages (Dolezal et al., 2009). This is similar to the system of division of labor found in many ants, including normal *P. californicus* colonies, where the functionally sterile workers divide their labor between nest and foraging tasks based predominantly on age (Hölldobler and Wilson, 2009). In addition, this species is easily manipulated in the laboratory; even colony demographics can be manipulated, allowing for age to be decoupled from behavior. Therefore, this ant species presents an opportunity to investigate a social ant whose reproductive caste, during a short period of their lives, exhibit a system of division of labor similar to workers. Based on the RGPH, the behavioral transitions observed in both castes are predicted to be regulated by physiological factors generally linked to reproductive cycling, such as ovarian status and reproductive hormones.

Reproductive Hormones and Physiology

Two central classes of hormones that act as insect reproductive regulators are the juvenile hormones (JH) and the ecdysteroids. The JH family of sesquiterpenoid insect hormones is important in multiple developmental and behavioral functions across insect taxa, and has been implicated in reproductive maturation and behavior (Truman and Riddiford, 2002; Flatt et al., 2005) and the behavioral ontogeny of temporal polyethism (Robinson and Vargo, 1997, Hartfelder, 2000, Hartfelder and Emlen, 2005). Ecdysteroid hormones are key in developmental regulation of larvae, specifically during molting and metamorphosis (Hartfelder and Emlen, 2005, Truman and Riddiford, 2002). Ecdysteroids may also act independently or in conjunction with JH to produce organizational or activational effects on behavior (Hartfelder and Emlen, 2005) and gametogenesis (Klowden, 1997).

Unfortunately, neither of these hormones has been particularly well-studied in the majority of social insects. What has been discovered, however, is that, although these hormones show similar patterns across taxa, the actual relationships between hormones and behaviors can be quite different. JH influences guarding behavior and ovarian development in the primitively eusocial wasp *Polistes canadensis* (Giray et al., 2005), queen maturation and reproduction in the fire ant *Solenopsis invicta* (Brent and Vargo, 2003), and reproductive status and dominance in the queenless ants *Diacamma sp.* (Sommer et al., 1993) and *Streblognathus peetersi* (Brent et al., 2006) and in the bee *Bombus terrestris* (Bloch et al., 2000a). In contrast, there is a wealth of information on the functions

of JH in honey bee worker division of labor. JH levels increase as bees shift from within-nest tasks to foraging, such that foragers generally have much higher JH titers than nest bees (reviewed in Robinson and Vargo, 1997). Treatment of nest bees with JH or the JH analog methoprene affects the same suite of traits as well as behavior, causing precocious foraging (Jaycox, 1974; Robinson, 1987). These results may point to obligatory roles of JH in worker division of labor. However, bees that perform sporadic foraging flights during the winter season do not show elevated JH titers (Huang and Robinson, 1995), and ablation of the *corpora allata*, the glands that produce JH, does not block foraging onset (Sullivan et al., 2000). Thus, in honey bee workers, JH is a central integrator of forager-associated traits, and likely plays a role in behavioral reinforcement, but is not required for successful foraging to occur (Amdam and Omholt, 2003). Obviously, honey bees are the best studied social insect in this context; however, honey bees are highly derived insects, and much of their biology deviates from that of other insects (Winston, 1987). Therefore, there are inherent difficulties in using honey bee data to make evolutionary predictions in other insect groups.

Another important reproductive hormone group, the ecdysteroids, also appears to play important roles in dominance and reproductive status in bumble bees (Bloch et al., 2000b) and *S. peetersi* (Brent et al., 2006). However, the current role of ecdysteroids in division of labor is not clear. Though ecdysteroid titers are very low in adult social insects, changes in titers occur during the first days after eclosion, and priming effects on behavior are suspected (Velarde et al., 2009). Additionally, there is a suggested association between the ecdysone

cascade gene *HR46* and division of labor in honey bees: when ovariole number was artificially increased by ovary grafting, worker behavioral development was accelerated with increasing *HR46* transcript abundance (Wang et al., 2009). While the honey bee ovary is the likely source of ecdysteroids (Lafont, 20050, no explicit data support correlation or causation between ecdysteroid titers and division of labor in any social species (Hartfelder et al., 2002).

In addition to the effects of reproductive hormones, ovarian physiology can also be an important component of behavior in insects. The ovary produces systemic factors that regulate host-seeking behavior in mosquitoes (Klowden, 1997) and also affects lifespan and sensitivity to behavioral stimuli in *Drosophila* (Flatt et al., 2008). As previously discussed, the size of the ovary is correlated to the age of foraging onset in honey bees (reviewed, Amdam and Page, 2010), and experimental increases of ovarian mass can predictably change this phenotype, showing that the ovary has a causal role in honey bee behavior (Wang et al., 2010). Its effects can take the form of differential ecdysteroid hormone production (Amdam et al., 2010) or changes in gene expression (Velarde et al., 2009; Wang et al., 2009; 2010; 2012). Thus, the ovary itself could be a key mediator of behavioral development in social insects, even within the functionally sterile worker caste.

Reproductive physiology and behavior can be affected by a variety of other factors, including metabolic pathways that are involve in sensing the nutritional environment of the organism, such as insulin/insulin-like signaling and Target of Rapamycin (TOR; Oldham and Hafen, 2003). These pathways are

present in many eukaryotic organisms, and have effects on many different components of life history (Oldham and Hafen, 2003). Changes in TOR signaling, for example, can also affect JH levels in larval honey bees (Mutti et al., 2011) and cockroaches (Maestro et al., 2009); TOR is also a key player in the production of yolk proteins (and thus oogenesis) in mosquitoes (Hansen et al., 2004), and ovarian activity in *Drosophila* adults (Thomson and Johnson, 2010). Despite inconclusive results in regards to the role of TOR in honey bee behavior (Ament et al., 2008), the fact that both JH and ovarian physiology affect social insect behavior (Hölldobler and Wilson, 2009) suggests that the TOR pathway could be a component of the regulation of division of labor. Thus, TOR represents another possible source of behavioral regulation that could have been used to build social phenotypes.

Relationships Between Physiology and Behavior in *P. californicus* Queens and Workers

This dissertation research was driven by two main goals: 1) to build a better understanding of the proximate factors involved in division of labor in ants and 2) use the findings of these investigations to begin to evaluate the applicability of the RGPH outside of the honey bee lineage. Here, I describe how I investigated correlations between reproductive hormones and ovarian physiology in relation to behavioral division of labor in both queens and workers of *P. californicus*. This work provides novel information on the physiological

factors linked to behavioral differences in these ants and also provides data to better evaluate the RGPH.

In Chapter 2, published in 2009 in *Animal Behaviour*, I use the rare phenotypes of *P. californicus* founding queens to identify differences in JH and ecydsteroid levels between queens biased towards different types of tasks. As in honey bee workers (Sullivan et al., 2000), JH levels were elevated in queens biased towards foraging. Again, like other highly social species (Hartfelder et al., 2002), ecdysteroid levels were not correlated with behavioral differences.

In Chapter 3, published in 2012 in the *Journal of Experimental Biology*, I identify if the same relationships between reproductive hormones and behavior observed in queens are found in *P. californicus* workers. To identify relationships between hormones and behavior independent of age, I used colonies of age-typical workers, which transitioned to foraging as they grew older, as well as single-cohort colonies, where manipulations of colony demographics forced young workers to precociously forage,. This study showed that, as in queens, increased JH levels are associated with foraging, and, unlike in queens, increased ecdysteroid levels were associated with nest-biased tasks. In Chapter 4, I investigated the behavioral plasticity of *P. californicus* founding queens, using behavioral manipulations to show that established queens are able to respond to the loss of workers by reinitiating colony founding behaviors; when this occurs, queens divide labor into nest- and foraging-biased phenotypes, and JH patterns are similar to those found in Chapter 2.

To identify how other components of reproductive physiology were involved in division of labor, I then used the same systems of founding queens and single-cohort workers quantify the relationship between ovarian activity and behavior in Chapter 5. Here, I show that, independent of age, ovarian activity is decreased in foraging-biased ants of both castes, indicating again that reproductive physiology may be an important component of division of labor in this species.

Finally, in Chapter 6, I begin to evaluate how other internal factors are involved in regulation of *P. californicus* behaviors, specifically studying whether the nutrient sensing and growth regulation pathway TOR affects queen division of labor. This study showed that treatment with a pharmacological inhibitor of this pathway heavily biased treated queens towards foraging, suggesting that this pathway could be used to regulate behavioral changes in these queens.

These studies provide new information showing the linkages between reproductive physiology and division of labor in these ants and help to fill in the gaps in our understanding of what factors may be involved in these behavioral changes. I have also provided raw material with which to better evaluate if the ground plan hypotheses are applicable to the ants. In general, *P. californicus* division of labor appears to have multiple links to reproductive physiology, consistent with this hypothesis.

Chapter 2

ENDOCRINE PHYSIOLOGY OF DIVISION OF LABOR IN *POGONOMYRMEX CALIFORNICUS* FOUNDING QUEENS

ABSTRACT

The proximate controls of a behavior in extant species can inform us about the evolutionary route towards that behavioral phenotype. In social insects, different behavioral phenotypes often correlate with divergent hormone levels, and, in honeybees (*Apis mellifera*), this insight has lead to the hypothesis that behavioral biases, or division of labor, emerged via co-option of endocrine regulatory systems that paced behavioral change during the reproductive cycle of solitary ancestors. Founding queens of the California harvester ant *Pogonomyrmex californicus* show discrete behavioral changes during colony founding, with a dichotomy between nest-biased behavior and field-biased behavior. Additionally, a division of labor can develop if queens found nests together, with one queen being nest-biased and another being field-biased. To determine whether behavioral diphenism can be associated with reproductive endocrine regulators in an ant, we measured ecdysteroid and juvenile hormone (JH) content in (1) single-founding queens showing normal behavioral progression and (2) cofounding queens showing a division of labor. We found that ecdysteroid levels did not correlate with behavior. JH titers, on the other hand, were elevated during the foraging life stage of single-founding queens as well as in the cofounding queens with a behavioral bias towards foraging. Our

results suggest that JH affects the propensity for foraging task replication in *P. californicus*, and provide evidence for a common evolutionary route towards social behavior in ants and bees.

INTRODUCTION

A major factor leading to the ecological success of social insects is the evolution of a system of division of labor (Wilson, 1971). Although many studies have explored this aspect of social organization, it is unclear how complex labor systems evolve or whether the evolutionary route to various levels of social complexity is shared across insect taxa. One approach to understanding the evolution of division of labor is to study the proximate control of current systems of social organization (Robinson et al., 2005; Page et al., 2006). Research in this domain has focused primarily on the honeybee (*Apis mellifera*), a model with well-developed toolkits for molecular analyses, which provides insights into genetic, physiological and neural affecters of social behavior (e.g. Ben-Shahar et al., 2002; Amdam et al., 2004, 2006; Rueppell et al., 2004; Hunt et al., 2007). The evolutionary interpretations of the data are not always in agreement (Robinson, 1992; Robinson and Vargo, 1997; Page and Amdam, 2007), but one idea receiving increasing support is that complex social phenotypes emerged through co-option of gene networks and endocrine signaling cascades that were regulators of reproduction in solitary ancestors (the reproductive ground plan hypothesis) (West-Eberhard, 1987, 1996; Amdam et al., 2004).

Two endocrine factors shown to have important roles in regulating insect reproductive physiology and behavior are ecdysteroids and juvenile hormones (JH). In adult females, ecdysteroids are primarily produced in the ovary (Lafont et al., 2005). Changes in circulating ecdysteroid titers are correlated with ovarian development in the mosquito (*Aedes aegypti*) (Klowden, 1997). For some social insects, there is evidence that ecdysteroids may contribute to reproductive division of labor. In the primitively eusocial bumblebee, *Bombus terrestris*, differences in ecdysteroid titer appear to be linked to reproductive and social status (Bloch et al., 2000b). A similar positive correlation of reproductive ranking with ecdysteroid titer is also found in the queenless ant *Streblognathus peeters*i (Brent et al., 2006). However, this correlation does not hold for all social insects. For example, in adult honeybees, ecdysteroid titers remain quite low and have few, if any, phenotypic correlates in both queens and workers (Hartfelder et al., 2002).

JH appears to be important in determining behavior and reproduction in the solitary Drosophila melanogaster (Flatt et al., 2005) and *Aedes aegypti* (Klowden, 1997), as well as in some social insects. JH influences ovarian physiology in queens and guarding behavior in workers of a primitive eusocial wasp (*Polistes canadensis*) (Giray et al., 2005). It affects queen maturation and reproduction in *Solenopsis invicta* (Brent and Vargo, 2003), is correlated with reproductive status in the queenless ant species *Diacamma sp.* (Sommer et al., 1993) and *Streblognathus peetersi* (Brent et al., 2006), and JH titer and biosynthesis rate are correlated with social hierarchy and reproductive status in

bumblebee workers (Bloch et al., 2000a). JH determines ovarian morphology during honeybee larval development (Rembold et al., 1974; Schmidt-Capella and Hartfelder, 1998), and it is correlated with the onset of foraging behavior in adult honeybee workers (Jaycox et al., 1974; Robinson, 1987; Sullivan et al., 2003) and *Myrmicaria eumenoides* ants (Lengyel et al., 2007).

As is evident from just this small sampling of species, these two systemic hormones have a prominent but quite varied role in regulating physiological and behavioral processes pertaining to division of labor. To better understand how this social complexity might evolve, it is necessary to take a comparative approach, examining the proximate mechanisms regulating behavior both within and between closely related eusocial species. In this study we are taking the former approach, looking at the proximate mechanism underlying the varied behaviors shown by queens, the female reproductives in a colony that most often resemble their primitive solitary ancestor during their early life stages. Queens of the California harvester ant, *Pogonomyrmex californicus,* display some specific life-history traits that make them useful for such studies. First, they have a semi-claustral founding strategy, in which the newly mated queens are required to forage for larval provisions before their first workers emerge. Therefore, during the initial founding stage, the queens display a temporal shift from nest-biased (non-foraging) to field-biased (foraging) behavior (Johnson, 2002), which mimics the behavioral transition observed in their workers. In addition, *P. californicus* populations can vary in the number of queens founding a nest. In some populations, aggression between founders is high, so that queens initiate new

colonies alone (haplometrosis). In other populations, aggression is low and new colonies are founded by multi-queen associations (pleometrosis) (Johnson, 2004; R. Overson, unpublished data). Under pleometrotic conditions, behavioral biases can emerge between queens, so that one primarily performs nest tasks and the other forages. This variation in the behavior of these founding queens may be produced by differences in endocrine activity.

Here, we study changes in titers of ecdysteroids and JH coinciding with (1) the behavioral progression of single-founding *P. californicus* queens and (2) the behavioral biases shown by cofounding *P. californicus* queens. If the partitioning of labor within a nest results from the exploitation of these endocrine networks, we predicted that in-nest tasks and foraging behaviors would correspond to different endocrine states, and that the same hormonal dynamics that emerged sequentially in single-founding queens would be mirrored in the division of labor between co-foundresses.

METHODS

Queen Collection and Observations

We collected *Pogonomyrmex californicus* founding queens during and directly after their yearly mating flights in July 2006 in San Diego County, California. Queens were collected from two behaviorally and geographically discrete populations in which new queens founded colonies either by themselves (haplometrosis) or with one or more co-founders (pleometrosis). All were kept under laboratory conditions for the duration of the experiment (constant 28° C,

natural photoperiod). Haplometrotic queens were kept plastic nest boxes constructed of two discrete square arenas. One arena was filled with plaster and contained a water-filled test tube stoppered with cotton. A plastic tube connected this 'nesting' arena to a 'foraging' arena that was empty save for a small pile of grass seed.

We introduced a single haplometrotic queen into each nest box, which was observed four times daily. 'Nest-biased' queens were collected for hormonal assays upon the first observation of eggs. 'Field-biased' queens were identified by observing the first instance of foraging activity, as noted by the movement of seeds from the foraging area into the nest area, near the queen's eggs. Previous observations (unpublished, R. Overson) indicate there is approximately a 30 day span between egg laying and worker emergence, during which queens will forage. To ensure the queens had established a strong pattern of foraging activity prior to hormone analysis, individual behavior was monitored daily for 15 days following oviposition.

Although pleometrotic queens are willing to co-found new nests, each is still fully capable of individual colony founding, exhibiting the same polyethism observed in haplometrotic queens. To determine whether the behavioral biases that can occur between co-founding queens are associated with endocrine changes similar to those associated with the behavioral shifts in single founding queens, we paired two queens, each individually marked on the abdomen with enamel paint (Testor's), and placed them in a soil-filled jar to initiate a colony. 390 queens were used to create 195 associations. The soil was moistened and a small

quantity of seeds was added regularly. Care was taken to limit seed availability, ensuring a continued need to forage.

We observed the behavior of paired queens for 15 minute intervals, four times per day for 15 days. Each observation of a queen outside of the nest arena foraging for seeds was noted. Once there were at least 10 observed foraging events, the queen pairings were categorized as being either behaviorally biased or non-biased. Biased associations, of which there were 44, were those in which one of the queens foraged for at least 80% of the recorded events. Queens from these pairings were placed into either the 'nest-biased' or 'field-biased' category based on their frequency of foraging. Non-biased associations, of which there were 14, were those in which each queen performed approximately 50% of the foraging. Any pairing which failed to meet our strict criteria for categorization as biased or non-biased were not used for hormonal analysis.

Sampling and hormone assay

Due to limited availability, only one single-founding queen was used for each hormone titration. Co-founding queens were available in greater number, therefore two individuals from the same behavioral grouping were pooled for each sample to ensure a high resolution of the hormonal profiles.

We collected the queens of all groups in 0.5 ml of methanol on ice and then stored them at -80° C. Care was still taken to perform the terminal sampling as quickly and humanely as reasonably possible. The small body size of the queens necessitated whole body extraction of the hormones. A glass tissue grinder was used to thoroughly pulverize the bodies in methanol. Hexane was used to

extract out the JH , leaving the ecdysteroid in the methanol portion (Brent and Vargo, 2003). The methanol layer was lyophilized, resuspended in 250 µl methanol and stored at -80° C until analysis. Duplicate 10 µl aliquots of the methanol section of each sample were incubated overnight with 100 µl of [3H]-20-hydroxyecdysone stock (1mg/ml, NEN) in Borate Buffer, and 100 µl of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH) at 4ºC on an orbital shaker. The specific ecdysteroid form is not known for this species, but the antibody used cross reacts with ecdysone, ecdysterone, 20-hydroxyecdysone, and makisterone A (Warren and Gilbert, 1986). To minimize intra- and inter-assay variability, new standard competition curves were generated for each set of samples run, using 20-hydroxyecdysone Stock (Sigma) in quantities from 15.6-2,000 pg, a range which was well within the detection limits. After 18 hours, 20 µl of cleaned Protein A solution (Pansorbin, CalBiochem) was added to each tube to precipitate the complex during another hour of incubation at room temperature. Samples were then centrifuged at 5000g and the remaining pellet washed twice with 100 µl of borate buffer. The incorporation of microlabel was determined by a scintillation counter and ecdysteroid concentrations were estimated by nonlinear regression (Brent et al., 2006).

The hexane phase from the same individual samples, which contained JH, was then used to titer JH using the GC-MS method of Bergot et al., (1981) as modified by Shu et al., (1997) and Brent and Vargo, (2003). The homogenized samples were eluted through aluminum oxide columns with hexane, 10% ethyl ether-hexane and 30% ethyl ether-hexane. The sample was then suspended and

derivatized in a methyl d alcohol and triflouroacetic acid solution. The derivatized sample was resuspended in hexane and again eluted through aluminum oxide columns; 30%-ethyl ether was used to remove non-derivatized components and ethyl-acetate-hexane was used to collect the JH derivative. The sample was then dried in a Speedvac and resuspended in hexane. The purified and derivatized JH was then analyzed using an HP 6890 Series GC (Hewlett Packard, Palo Alto, CA, USA) equipped with a 30 m X 0.25 mm Carbowax Econo-Cap GC column (Alltech, Fresno, CA, USA) coupled to a HP 5973N inert MSD/DS. JH form was confirmed by first running test samples in SCAN mode for known signatures of JH 0, JH I, JH II, JH III, and JH III ethyl; JH III was confirmed as the primary endogenous form in this species. Subsequent samples were analyzed using the MSD/DS running in SIM mode. Helium was used as a carrier gas. The JH III derivative was monitored at *m/*z 76 and 225 to ensure specificity; total abundance was quantified against a standard curve of JH III. The detection limit of the assay is approximately 1 picogram.

Statistical Analysis

Due to a general lack of a normally distributed data, Mann-Whitney U tests were used to test for ecdysteroid and JH titer differences of the single-founding nest-biased queens vs. field-biased queens and co-founding nest-biased queens vs. field-biased queens. Significance values were adjusted by Dunn's Methods to compensate for the multiple comparisons between the three behavioral groups of queens from pleometrotic associations. All analyses were performed using Sigmaplot version 11.0 (Systat Software, Inc.).

Results

Single-founding (haplometrotic) queens

The comparison of queens in the nest-biased (non-foraging) stage with those in the field-biased (foraging) stage showed no differences in ecdysteroid titer between the groups (Mann-Whitney U test: U=185, N_1=25, N_2=15, P=0.94, Fig. 2.1a). This finding is similar to results from the honey bee, where ecdysteroid titers remain constant in queens and in workers going through the behavioral transition from in-nest tasks to foraging in the field (Robinson et al., 1991).

In contrast, nest-biased and field-biased *P. californicus* queens had significantly different JH titers. The concentration of JH was three times higher in the field-biased queens compared to the nest-biased queens (Mann-Whitney U test: U=18, N_{nest}=16, $N_{forager}$=19, P=0.000001, Fig. 2.1b). These data led us to predict that co-founding queens that partitioned labor between nest-tasks and foraging would have similar ecdysteroid titers but different JH levels, with JH being elevated in the foraging queens.

Co-founding (pleometrotic) queens

Our observations of behavior showed that among co-founding queens, those with a nest-bias foraged at a very low rate, which persisted throughout the observation period. Field-biased queens, however, showed a progressive increase in foraging activity during the period between founding and being sampled (Fig. 2.2a). As predicted from the results for haplometrotically founding queens, nest- and field-biased pleometrotic queens did not have divergent ecdysteroid titers (Mann-Whitney U test: U=381, N_1=N_2=22, P=0.85, Fig. 2.3a). However, field-

biased queens expressed three times the JH (Mann-Whitney U test: U=89, N_1=N_2=22, P=0.004; Fig. 2.3b), which was comparable to the mean titer observed in haplometrotic queens of the foraging stage (Mann-Whitney U test: U=89, N_1=22, N_2=21, P=0.330). Because the co-founding queens were of similar age, we can discount the possibility that the link between JH titer and behavior emerged simply because these traits co-occur independently as a consequence of increasing chronological age.

Queens from co-founding associations where no behavioral bias occurred exhibited decreasing foraging activity over time (Fig. 2.2b) but the frequency of activity remained within the same general range observed for the field biased queens (Fig. 2.2a). Further, despite a higher foraging more frequently than nest-biased queens, a post-hoc test showed that the queens in associations that did not partition labor (N=14) had JH titers comparable to that of nest-biased queens (Mann-Whitney U test: U=106, N_1=22, N_2=14, P=0.123), and significantly lower than field-biased queens (Mann-Whitney U test: U=28, N_1=22, N_2=14, P<0.001, Fig. 2.3b). These results suggest that a high JH titer may bias *P. californicus* behavior toward tasks in the field, it is not required for the onset of foraging behavior.

DISCUSSION

We have shown that the ecdysteroid titer is not significantly correlated with foraging behavior in *P. californicus* founding queens. It has been suggested that ecdysteroids lost a reproductive regulatory function in adults of highly social

insects in the course of becoming determinates of caste differentiation during larval development (Hartfelder et al., 2002). *P. californicus* biology may support this hypothesis, as this ant shows a relatively high degree of reproductive dimorphism between the queen and worker castes yet no apparent association between ecdysteroids and reproductive development in adults. Furthermore, we find a robust association between JH titer and behavioral biases in founding queens. Nest-bias is linked to a low JH level and field-bias to a high JH level. This association between hormone expression and behavioral bias is independent of age, as it occurs during the sequential behavioral development of single-foundresses (Fig. 2.4a) as well as during the concurrent division of labor of similarly-aged co-founding queens (Fig. 2.4b). Yet, when behaviorally biased queens were compared to co-founding queens that did not exhibit bias, it became clear that JH itself does not cause foraging behavior. Queens without behavioral biases showed little within-group variation in JH, and thus, they exhibited no discernable differences between individuals both in terms of observed behavior and endocrine status (Fig. 2.4c). Our conclusion is that JH is likely one component of a regulatory system that can establish a behavioral bias in queens of *P. californicus*, but the hormone is not required for the performance of foraging.

A very similar conclusion has been reached for the regulation of foraging behavior in honey bee workers. Foraging workers have elevated JH titers (Jaycox et al., 1974; Robinson, 1987; Sullivan et al., 2003), suggesting that JH may act as a releaser. However, workers will initiate foraging even after surgical removal of the corpora allata complex, which is the site of JH synthesis (Sullivan et al.,

2003). Another endocrine component that appears to regulate the pacing of foraging onset in honey bees, and possibly in *P. californicus*, is the expression of vitellogenin, a yolk precursor protein. As vitellogenin titers decline in maturing honey bee workers (Engels and Fahrenhorst, 1974), both foraging behavior and JH synthesis increase (Guidugli et al., 2005; Nelson et al., 2007). JH may reinforce the forager behavioral state by a suppressive feedback effect on its own regulator, vitellogenin, and by integrating changes in gene transcription and metabolism that result in a distinct forager phenotype (Amdam and Omholt, 2003). *P. californicus* queens do not begin foraging until after their first clutch of eggs have been produced, which would normally coincide with a decline in vitellogenin production. They also cease foraging around the time that they begin producing a second clutch of oocytes. This suggests that the *P. californicus* queens may rely on the same reproductively-linked double repressor mechanism as *A. mellifera* workers to regulate foraging behavior.

The results also suggest that it is possible to develop divergent behavioral phenotypes by simply varying the expression of endocrine factors normally associated with reproductive activity. The reproductive ground plan hypothesis suggests that co-option of this regulatory mechanism and its subsequent dissociation from gametogenesis may be the common route by which insect species have evolved greater social complexity (Amdam et al., 2004). If future research shows that the behavior of the effectively sterile workers of *P. californicus* is regulated by the same endocrine mechanism used to control queen foraging, then this proposed evolutionary pathway would be strongly supported.

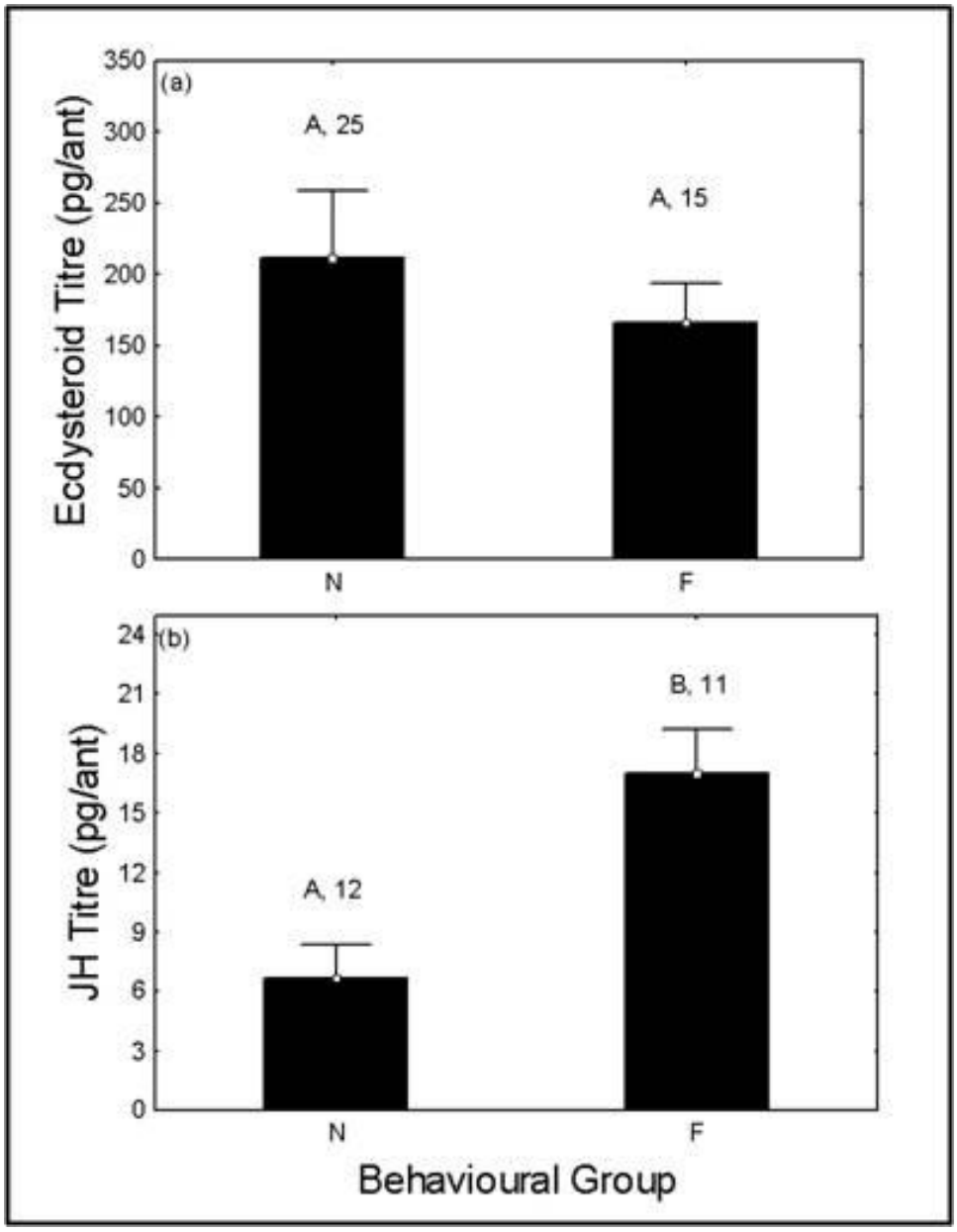

Figure 2.1: Ecdysteroid and JH content of singly founding *P. californicus* queens. Mean ± SE titre (pg/ant) of (a) ecdysteroids and (b) juvenile hormone (JH) of single-founding (haplometrotic) queens during nest-biased (nonforaging) (N) and field-biased (foraging) (F) stages. Letters denote significant differences between the groups. Sample sizes are indicated.

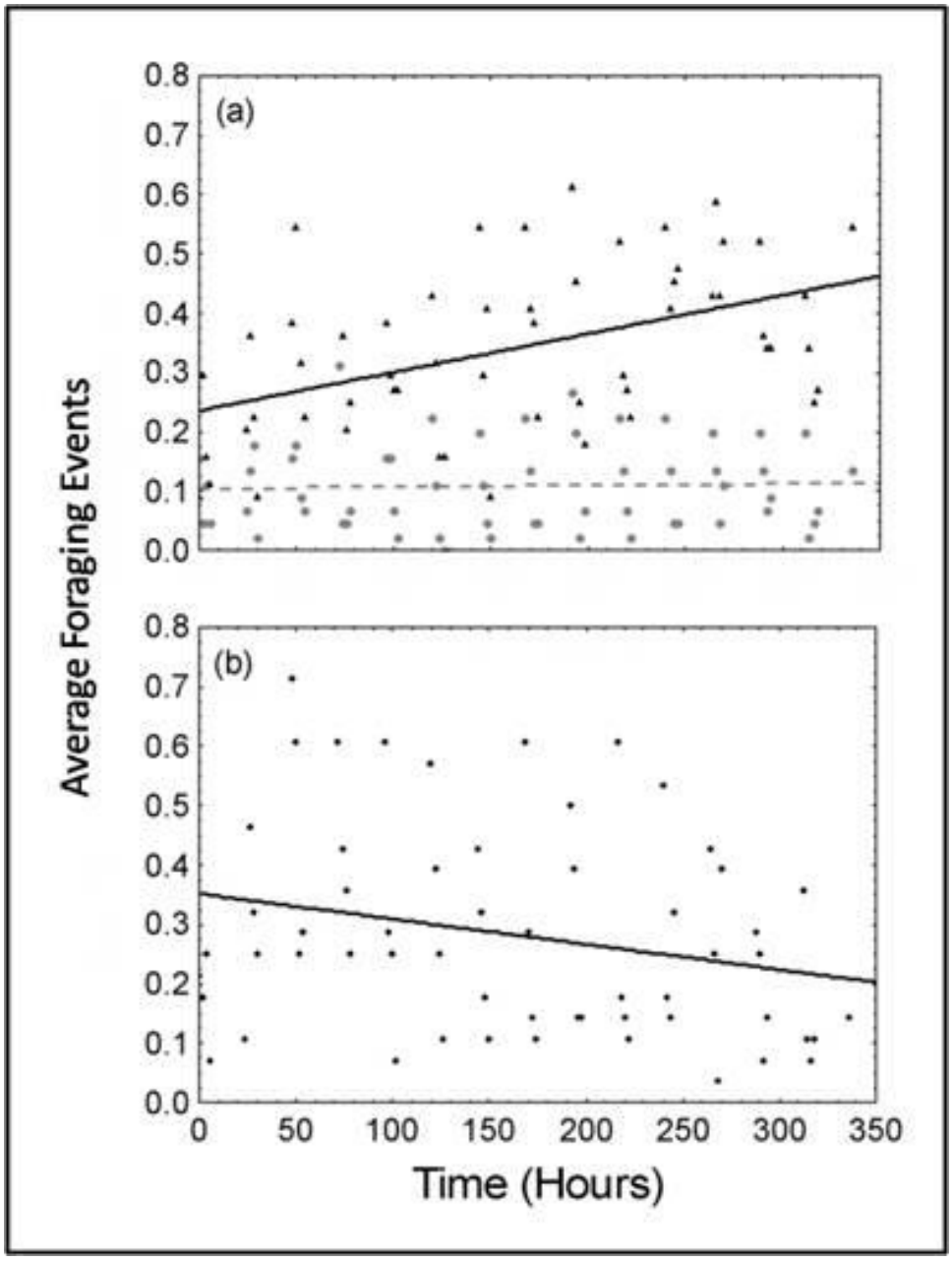

Figure 2.2: Co-founding *P. californicus* foraging events over time. Average foraging events over time for (a) field-biased (solid black line, black triangles) and nest-biased (dotted line, open circles) queens and (b) queens with no observed behavioural bias.

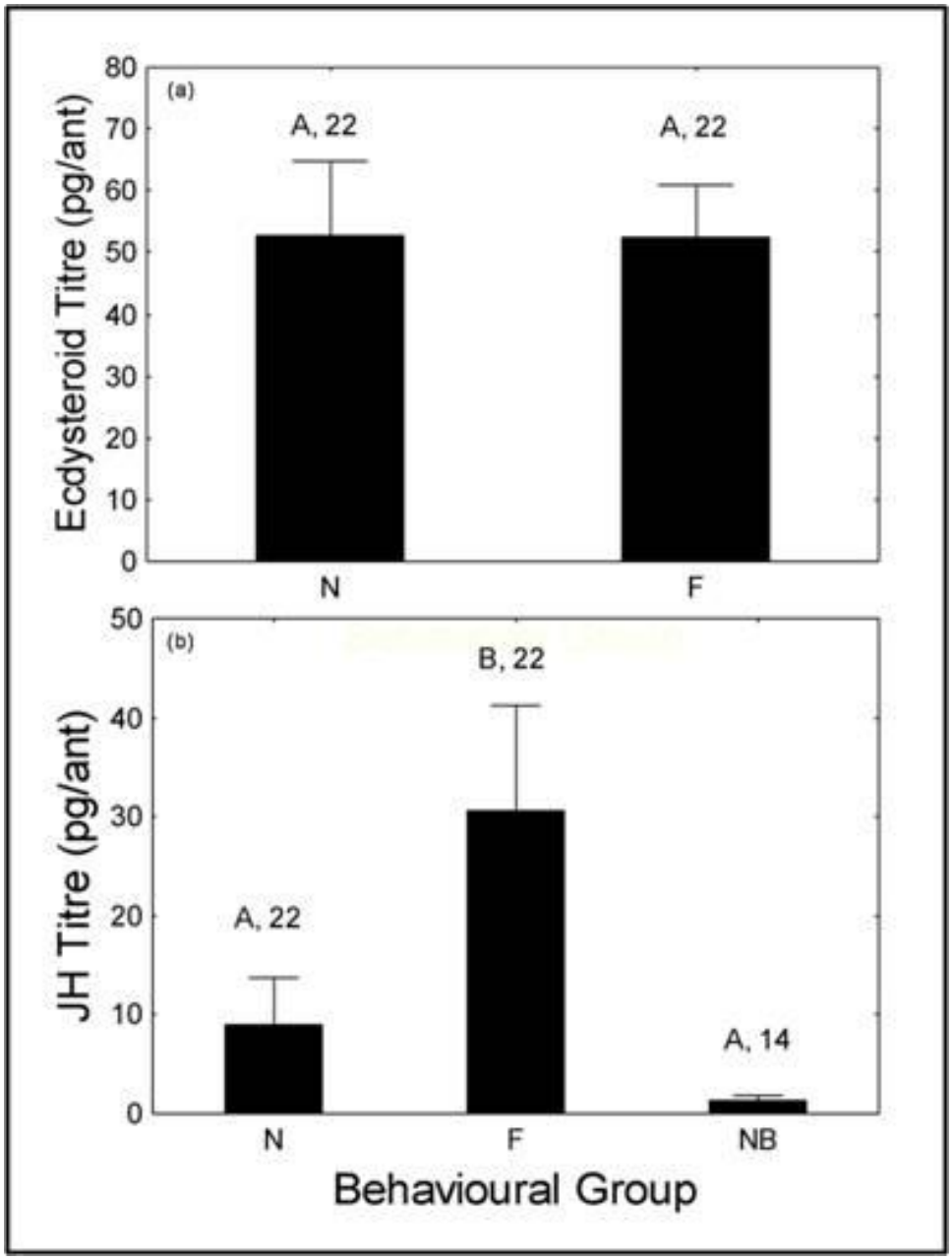

Figure 2.3: Ecdysteroid and JH content of co-founding *P. californicus* queens. Mean ± SE titres (pg/ant) of (a) ecdysteroids and (b) juvenile hormone (JH) in cofounding (pleometrotic) queens from different behavioural groups: nest-biased (nonforaging) (N), field-biased (foraging) (F) and nonbiased (NB). Letters denote significant differences between the groups. Sample sizes are indicated.

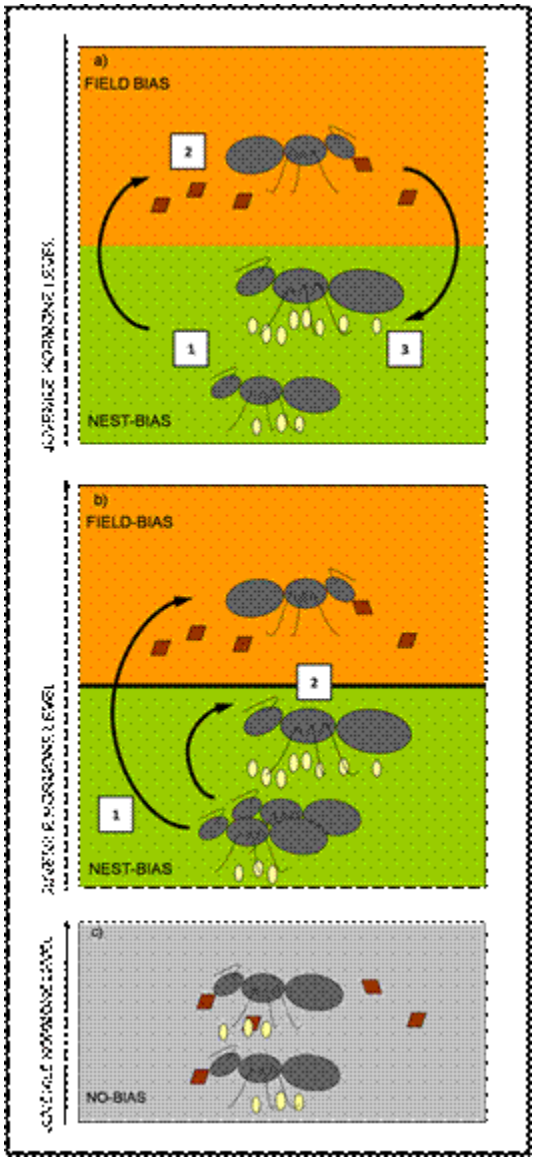

Figure 2.4: The hypothesized relationship between juvenile hormone (JH) and the behavioural bias of *P. californicus* queens. An increased JH titre does not cause the onset of foraging behaviour, but biases behaviour towards foraging/outside-nest activities. (a) Single-founding queen: the newly mated queen (1) has a low JH titre and remains in the nest laying eggs that she produced prior to colony founding. Subsequently, during the period of nest provisioning, the JH level is elevated, new eggs develop in the ovaries, and the queen's probability of foraging

task replication is increased (2). After the first workers emerge from the nest, the queen has a reduced JH titre and a low propensity to forage, and she begins to oviposit her second clutch of eggs. The presence of foraging workers increases the threshold stimulus necessary to induce foraging behaviour, ultimately confining her to the nest (3). (b) Cofounding queens that develop a division of labour: newly mated queens (1) have low JH titres and both remain in the nest. Subsequently, an increase in JH level (2) encourages foraging task replication, resulting in a correlation between JH and the behavioural bias towards outside-nest activities. (c) Cofounding queens that do not divide labour show no behavioural bias and no difference in JH level.

Chapter 3

WORKER DIVISION OF LABOR AND ENDOCRINE PHYSIOLOGY ARE ASSOCIATED IN THE ANT, *POGONOMYRMEX CALIFORNICUS*

ABSTRACT

In *Pogonomyrmex californicus* harvester ants, an age-associated division of labor occurs in the worker caste, in which young workers perform in-nest tasks and older workers forage for food. Here, we test whether this behavioral division is age-based or age-flexible, and whether it coincides with differential expression of systemic hormones with known roles in behavioral regulation. Whole body content of juvenile hormone (JH) and ecdysteroids were determined in workers from 1) colonies with a typical age structure (age-typical), in which workers transition across behaviors naturally, and from 2) single-cohort colonies, which are entirely composed of same-aged workers, facilitating the establishment of age-independent division of labor. Foragers from both colony types had higher JH and lower ecdysteroid content than workers performing in-nest tasks, suggesting age does is not the sole determinant of worker behavior. This association between hormone content and behavior of *P. californicus* workers is similar to that previously observed in founding queens of this species. Because these hormones are key regulators of development and reproductive behavior, our data are consistent with the reproductive ground plan hypothesis (RGPH), which posits that the reproductive regulatory mechanisms of solitary ancestors were co-opted to regulate worker behavior.

INTRODUCTION

The evolution of increasing social complexity in insects culminates in colonial species exhibiting a reproductive division of labor (Wilson, 1971). In addition, a further division of tasks within the non-reproductive worker caste often exists (Hölldobler and Wilson, 2009). The regulatory mechanisms by which such complexity are produced have received considerable attention. Much of this work has focused on the honey bee (*Apis mellifera*) (e.g., Robinson, 1992; Ben-Shahar et al., 2002; Amdam et al., 2004; Rueppell et al., 2004; Amdam et al., 2006; Hunt et al., 2007; Ihle et al., 2010). Emerging from these studies is support for a hypothesis that social behavior evolved through the co-option of regulatory factors that originally coordinated the progression of female reproductive physiology and behavior in solitary ancestors – the Reproductive Ground Plan Hypothesis (RGPH) (West-Eberhard, 1987; West-Eberhard, 1996; Amdam et al., 2004). Specifically, this hypothesis predicts that several non-reproductive behavioral traits that are observed in social insect societies are regulated by mechanisms normally associated with reproductive development and activity. While there is support for this argument in a few species of bees and wasps, testing in other social insect taxa is necessary to assess the broader application of the RGPH (Amdam and Page, 2010). Here we present the first such study for ants, a major taxon that independently evolved (Brady et al., 2006, Moreau et al., 2006) a level of social complexity comparable to that of *A. mellifera* (Hölldobler and Wilson, 1990). In addition, our investigations provide basic proximate information on how hormones correlate with behavioral transitions in ants.

Numerous evolutionary scenarios have been posited for the division of labor between ant workers (Hölldobler and Wilson, 2009), but few studies have investigated connections between physiology, specifically the reproductive system, and the regulation of behavioral differentiation. We recently showed that changes in the behavior of nest-founding queens (female reproductives) of the California harvester ant, *Pogonomyrmex californicus*, are coordinated with their endocrine state. This finding suggests that divergent behavioral phenotypes could be produced by differential expression of endocrine factors normally associated with reproduction (Dolezal et al., 2009), and is consistent with the predictions of the RPGH. If the RPGH is broadly applicable, then the behavior of *P. californicus* workers should be underpinned by the same reproductive physiology that was observed in founding queens. Task performance in these workers is naturally age-related (Hölldobler and Wilson, 2009; A. Dolezal, personal observation), with younger individuals performing in-nest tasks such as brood care and nest maintenance, and older individuals leaving the nest to perform foraging-related tasks; this temporal polyethism is one of the key factors contributing to the major ecological success of social insects (Hölldobler and Wilson, 2009). For many species exhibiting these behavioral transitions, it is possible to decouple behavior from age by manipulating the environment of the workers (Nelson, 1927; Hölldobler and Wilson, 2009), providing a useful tool for verifying the role of putative behavioral regulators.

The primary regulators of both behavior and ovarian activity in insects are the systemic endocrine factors juvenile hormone (JH) and ecdysteroids. Both have

organizational, priming and/or coordinating effects (reviewed by Raikhel et al., 2005). JH has been associated with division of labor among workers of the honey bee (reviewed by Robinson and Vargo, 1997), the bumble bee (Bloch et al., 2000a), the queenless ant *Streblognathus peetersi* (Brent et al., 2006), and *Polistes* wasp (Giray et al., 2005). Although no causal route from ecdysteroid content to division of labor has been demonstrated (Hartfelder et al., 2002), this group of hormones is suspected of having priming effects on honey bee worker behavior (Velarde et al., 2009; Wang et al., 2009; Amdam and Page, 2010), and has links to adult reproductive activity (Robinson et al., 1991). Ecdysteroids act via effects on the axis of brain/fat body/ovaries (Wang et al., 2010; Yamazaki et al., 2011), and ecdysteroid production is often linked to insect ovarian activation (Raikhel et al., 2005; Dong et al., 2009). These hormones are also implicated as a behavioral regulator in *S. peetersi* (Brent et al., 2006), paper wasps (Röseler et al., 1985) and bumble bees (Bloch et al., 2000b). In *P. californicus* founding queens, we found more JH in foragers compared to those performing in-nest tasks, while ecdysteroids had no apparent behavioral association (Dolezal et al., 2009). Queen ovarian activity (egg production) co-varies with JH and behavior, while the ovaries, a major source of ecdysteroids in insects (Raikhel et al., 2005), are invariably intact and presumably functional (A. Dolezal, unpublished data). Nurse workers of *P. californicus*, have functional ovaries that produce nutritional eggs; in contrast, foragers usually have degraded and presumably nonfunctional ovaries (Hölldobler and Wilson, 2009; A. Dolezal, unpublished data).

The genus *Pogonomyrmex* is a very suitable model species for studying the endocrinological parameters involved in the division of labor between nurse workers and foragers in ants. *P. californicus* is, in particular, relatively easy to culture in the laboratory under controlled conditions. In the current study, we anticipated that *P. californicus* worker division of labor would be associated with endocrine content, and that associations could be predicted from the physiology of queens, as suggested by the RGPH. Accordingly, we expected JH to be elevated in foragers, while ecdysteroids should be influenced by worker ovarian integrity, and therefore be reduced in foragers. To test these predictions, we examine the behavior and corresponding endocrine patterns in the monomorphic workers and foragers from both single-cohort colonies and age-typical colonies (those possessing a normal distribution of workers at all ages and stages of development). The similarly aged workers of the single-cohort colonies must divide labor between nest tasks and foraging, removing the influence of any age-based behavioral biases. Although this robust approach has been widely used in honey bee research (Nelson, 1927; Robinson et al., 1989; Huang et al., 1995), single-cohort experiments are rare in ant research (Gronenberg et al., 1996; Haight, 2006, 2008).

Our analysis, which provides one of the most complete investigations between ant division of labor and hormone physiology, reveals significant associations between ant reproductive endocrine physiology and social task performance independent of chronological age effects. The results highlight the behavioral and endocrine plasticity of worker ants and provide support for the

RPGH in a non-*Apis* eusocial insect species with an independent evolutionary origin of sociality.

METHODS

Division of labor among workers from colonies with a typical age structure

Mature colonies of *P. californicus* were maintained in the laboratory at the School of Life Sciences, Arizona State University, Tempe, Arizona, USA. Four colonies were chosen for the experiment, each reared under laboratory conditions (natural light cycle, ~25º C) for at least 3 years and stably exhibiting normal social structure. Late stage pupae from those, and five other laboratory-maintained colonies were carefully observed. When callow (young, light-colored) workers emerged, all individuals that eclosed on the same day were marked by tying a small colored wire around the petiole (modified from Tschinkel, 2006). Approximately 350 newly emerged workers were identified, marked, and introduced in several pulses to the four experimental colonies between July 15, 2007 and October 28, 2007. The new workers were readily accepted, and because they were marked with colors corresponding to their day of emergence, it was possible to determine their age at the point which their behavior transitioned.

Between July 15, 2007 and May 13, 2008, the colonies were observed one to three times per week, for 15-30 minutes per observation. After 15 days, we collected marked workers that had performed nursing behaviors; these were individuals that had not been observed outside of the inner nest and were collected from the vicinity of the brood pile. No marked workers had begun

foraging at this time. Colony observation continued and the day on which marked individuals were first observed to forage was noted. Ants handling food items or water outside the nest were defined as foraging. New foragers were marked with a small amount of colored fluorescent powder, to allow for repeated identification. The powder is normally observable for several days. When an individual worker had been observed foraging three times, it was designated as a confirmed forager. Although the marking powder may have been groomed off prior to confirmation of forager status, this would only result in some workers being observed foraging more than three times. For both identified nest workers and foragers, 89 individuals were surveyed for age and collected to determine hormone content.

Division of labor among workers from single-cohort colonies

Local field colonies of *P. californicus* were partially excavated in the summer and fall of 2008, and as many callow workers as possible were collected from each nest. Cuticle pigmentation was used as a marker to identify workers of close to identical adult age. Only the lightest colored, and thus youngest, workers were collected. The workers were brought back to the laboratory, and approximately 200 were added to each of six experimental colonies where they were readily accepted. The host colonies were 1.5-2.5 years of age, with approximately 300 workers each. After two days, all of the original members of the colony were removed, leaving only the queen, eggs, larvae, and 200 young, same-aged workers. Four out of the six colonies recovered successfully from the disturbance while the remaining two failed and were excluded from the experiment.

The four single-cohort colonies were observed 3-7 days per week, 2-3 times per day, for 5-10 minutes per observation. Foraging individuals were marked as described above, with fluorescent powder. Once a worker had been observed foraging on three occasions, she and an in-nest worker from the same colony were collected. These collections continued for the duration of the experiment, with all workers collected between the ages of 13 and 50 days. The experiment was ended after 50 days, at which point the population of each colony had dropped below approximately 25 workers, and foraging events were rare.

Sampling and hormone assays

The measurement of whole body hormone content was necessitated by the small size of the ants, but facilitated by the monomorphic (Johnson, 2000) bodies of these workers. Five workers from within the same behavioral group (ie: foraging or nursing) and colony type (i.e.: age-typical or single-cohort) were pooled to form each biological sample for hormone analysis. Workers were pooled between the four replicate colonies of each type, so each biological sample provided a representative cross-section of the sample material. Animals were collected directly into 0.5 mL of cold methanol (Sigma-Aldrich, St. Louis, MO, USA) to minimize the effect of handling stress on their endocrine state.

Hormone extraction and purification then followed the same processes utilized in *P. californicus* queens (Dolezal et al., 2009), as modified from Shu et al. (1997). JH was extracted from the homogenate using hexane and was then purified via elution through aluminum oxide columns with hexane, 10% ethyl ether–hexane and 30% ethyl ether–hexane (Sigma-Aldrich). The JH was

derivatized using a solution of methyl-d alcohol and trifuoroacetic acid, which was then resuspended and eluted through aluminum oxide columns with 30% ethyl ether and ethyl-acetate–hexane. JH was then quantified using an Agilent 6890 Series GC (Hewlett Packard, Palo Alto, CA, U.S.A.) equipped with a 30 m x 0.25 mm Carbowax Econo-Cap GC column (Alltech, Fresno, CA, U.S.A.) coupled to an Agilent 5973N inert mass selective detector/detection software (MSD/DS).

After JH had been extracted, the remaining homogenate in methanol was analyzed using a radioimmunoassay to determine ecdysteroid content. Standard competition curves were generated for each sample set using 20-hydroxyecdysone stock (Sigma-Aldrich). Duplicates of each sample were incubated overnight at 4º C on an orbital shaker with 100 µl of (3H)-20-hydroxyecdysone stock (1 mg/ml, NEN) in borate buffer, and 100 µl of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH). Subsequently, samples received 20 µL of cleaned protein-A solution (Pansorbin; CalBiochem, San Diego, CA, USA) and were incubated for 1 hour at room temperature. Samples were then centrifuged and washed with borate buffer. Microlabel incorporation was determined by a 2450 MicroBeta2 scintillation counter (Perkin-Elmer, Waltham, MA, U.S.A.) and ecdysteroid concentrations were estimated via nonlinear regression (Brent et al., 2006).

Statistics

The data for initiation of foraging, JH content, and ecdysteroid content in the single-cohort workers showed a general lack of normality, and did not pass the

assumption of homogeneity of variances (Levene's test; $p<0.05$). Therefore, non-parametric Mann-Whitney U tests were used to determine if there were significant differences in foraging age, JH content, and single-cohort ecdysteroid content between nest workers and foragers. The ecdysteroid content of the age-typical group met the homogeneity assumption (Levene's test, $p>0.05$), but did not fit a normal distribution as determined by a normality plot on the residuals. The data conformed to the assumption of normality after log-transformation and were subsequently analyzed with the parametric Student's *t*-test. A nonparametric Kruskal-Wallis one-way ANOVA was performed to identify any intercolonial differences in hormone levels. Spearman rank tests were used to determine if there were correlations between age and hormone levels under each colony condition. An alpha value of 0.05 was used for acceptable significance in all tests. The analyses were performed using Statistica 7.0 (StatSoft, Tulsa, OK, USA).

RESULTS

i) Timing and age of behavioral transitions

The observations of confirmed foraging by age-typical and single-cohort workers showed significant differences in age at foraging onset. On average, single-cohort workers initiated foraging five times earlier than the marked workers in age-typical colonies (Mann-Whitney U test: $U=0.0$, $N_{um}=88$, $N_{sc}=115$, $P<0.001$, Fig. 3.1). In addition, the variance in age at foraging initiation was reduced in single-cohort colonies ($Var_{um}=4600.506$, $Var_{sc}=141.75$; Levene's test,

F=164.76, p<0.001), reflecting the compressed timescale for the transition to foraging behavior.

ii) Age-typical colonies: hormone activity

Significant differences were observed in JH content between nest workers and foragers. Relative to nest workers, foragers from age-typical colonies contained six times the JH (Mann-Whitney U test: U=18, N_{nest}=16, $N_{forager}$=19, p<0.001, Fig. 3.2a) and half the ecdysteroids (Student's t-test: N_{nest}=16, $N_{forager}$=19, F=1.363, p=0.043; log transformed for normalization; Fig. 3.2a). There were no significant differences in JH (Kruskal-Wallis ANOVA: N=35, H=0.299 p=0.96) or ecdysteroid (Kruskal-Wallis ANOVA: N=35, H=3.938, p=0.268) content between the colonies. While each replicate colony had a sample size that was too small for reliable statistics, the data trend of each colony was the same as the overall result (Fig. 3.2c). Analysis also showed that there was no significantcorrelation between JH and ecdysteroid content (Spearman Rank Correlation: N_{nest}=16, $N_{forager}$=19 ρ = -0.19, $p>0.05$). In the age-typical colonies, however, there was a significant correlation between hormone content and age when all individuals were considered together. Increased age was correlated with increased JH (Spearman Rank Correlation: N_{nest}=16, $N_{forager}$=19, ρ= 0.84, $p<0.05$, Fig. 3.3a), and decreased ecdysteroids (Spearman Rank Correlation: N_{nest}=16, $N_{forager}$=19, ρ = -0.387, $p<0.05$, Fig. 3.3b).

iii) Single-cohort colonies: hormone activity

Relative to nest workers, same-aged foragers from the single-cohort colonies contained ten times more JH (Mann-Whitney U test: U=3, N_{nest}=17,

$N_{forager}$=18, p<0.001, Fig. 3.2b), and 50% less ecdysteroids (Mann-Whitney U test: U=74, N_{nest}=18, $N_{forager}$=18, p=0.005, Fig. 3.2b). There were no significant differences in JH (Kruskal-Wallis ANOVA: N=35, H=0.313 p=0.9576) or ecdysteroid levels (Kruskal-Wallis ANOVA: N=35, H=3.814 p=0.2822) between the colonies. While statistics were not calculated for each replicate colony, the data trends were consistent with the overall result (Fig. 3.2d). Unlike the age-typical colonies, single-cohort colonies exhibited a significant negative correlation between JH and ecdysteroid content (Spearman Rank Correlation: N_{nest}=17, $N_{forager}$=18, ρ = -0.569, $p<0.05$), and no correlation between age and JH content (Spearman Rank Correlation: N_{nest}=17, $N_{forager}$=18, ρ = -0.18, $p>0.05$, Fig. 3.3c) or ecdysteroid (Spearman Rank Correlation: N_{nest}=17, $N_{forager}$=18, ρ = 0.03, $p>0.05$, Fig. 3.3d) content. In addition, the JH content of foragers from single-cohort colonies was significantly higher than that of foragers from the age-typical colonies (Mann-Whitney U test: U=48, $N_{typical\ forager}$=18, $N_{SC\ forager}$=18, p<0.005).

DISCUSSION

Our observations of foraging onset times in age-typical and single-cohort colonies show that *P. californicus* worker behavior is very flexible, and can be accelerated substantially by modified colony demography (Fig. 3.1). This acceleration is well described in honey bees (Nelson, 1927; Robinson et al., 1989; Huang et al., 1995), and similar experiments in ants have shown that worker behavior can change quickly as task requirements change (Ehrhardt, 1931).

However, our manipulation of *P. californicus* worker age demography provides new evidence that extensive behavioral plasticity is possible in these ants. Workers in single-cohort colonies exhibited remarkably accelerated behavioral maturation and initiated foraging an average of one hundred days earlier than those in age-typical colonies (Fig. 3.1).

Regardless of whether the workers were raised in age-typical or single-cohort colonies, there was an association between endocrine patterns and behavioral phenotypes. JH content was consistently higher in foragers from both groups relative to content in the in-nest workers (Fig. 3.2a, b). Although age was correlated with JH content in the age-typical colonies (Fig. 3.3a), there was no correlation in the single-cohort colonies (Fig. 3.3c). This suggests that, while age may influence endocrine state to indirectly affect behavior, JH is the principal correlate of foraging activity. Another notable difference between these colony types was that JH was higher in single-cohort foragers than in age-typical foragers (Fig. 3.2a vs. 3.2b). Perhaps the very young workers of the single-cohort colonies have a higher threshold for foraging (Beshers and Fewell, 2001), and correspondingly, more circulating JH may be required for foraging onset to occur. The finding that JH was elevated in foraging workers is similar to results for *P. californicus* founding queens (Dolezal et al., 2009) and parallels information on the behavioral physiology of honey bees, where elevated JH corresponds to foraging activity in workers (e.g.: Jaycox, 1974; Robinson, 1987; Sullivan et al., 2000). While there is evidence that JH is not required for foraging activity in honey bees (Huang and Robinson, 1995; Sullivan et al., 2000) and *P. californicus*

queens (Dolezal et al., 2009), collectively, the data support the hypothesized role of JH as a behavioral reinforcer during and following the transition from nest tasks to field tasks (Amdam and Omholt, 2003), changing its role as a regulator of reproductive status and behavioral dominance in more primitive groups (Hartfelder, 2000).

Unlike JH, ecdysteroid content did not follow the same pattern as found in *P. californicus* queens, in which no clear differences were observed in foragers and non-foragers (Dolezal et al. 2009). Under age-typical (Fig. 3.4a) and single-cohort (Fig. 3.4b) circumstances, the onset of worker foraging behavior corresponded with both increased JH and, unlike in *P. californicus* queens, ecdysteroid content. As we speculated, ecdysteroid content was associated with the general trend in ovarian integrity and was thereby consistently elevated in nest workers compared to foragers (Fig. 3.2a, b). Active ovaries are the primary source of ecdysteroids in adult insects (Raikhel et al., 2005), and although *P. californicus* nest workers do not reproduce directly, their ovaries produce sterile nutritive eggs that are fed to developing larvae (Dolezal, personal observation). Because ovarian activation is often linked to ecdysteroid changes (Raikhel et al., 2005; Dong et al., 2009), we suggest that this activity elevates the workers' ecdysteroid level. As workers transition to foraging, they no longer produce nutritive eggs and their ovaries gradually degenerate (A. Dolezal, unpublished data; Hölldobler and Wilson, 2009). We suggest that these changes reduce the ovaries' production and release of ecdysteroids. Unlike workers, queens forage for only a short period before returning to large scale egg production in the nest, and during foraging, the

functionality of queen ovaries is maintained (A. Dolezal, unpublished data). These factors can explain why ecdysteroid levels remain steady in queens instead of declining, as they do in workers.

Despite a body of evidence that suggests that ecdysteroids may have lost their behavioral role in adults during the evolution of eusocial insect taxa (Hartfelder et al., 2002), there are examples of divergent ecdysteroid titers being associated with behavioral castes (Roseler et al., 1985; Bloch et al., 2000b, Brent et al., 2006), and there is emerging evidence of a link between ecdysteroids and foraging onset in worker honey bees (Velarde et al., 2009; Wang et al., 2010). Whether ecdysteroids impact the behavior of *P. californicus* workers remains to be determined. Lower levels in foragers might only reflect changes in ovarian physiology, and not be robustly tied to JH — the primary endocrine correlate of behavior. This lack of association is supported by our results; although JH and ecdysteroid levels were significantly correlated in single-cohort colonies, they were not in age-typical colonies (Fig. 3.3). Correlation in single-cohort colonies could be a consequence of the compressed transition that is taking place. Ecdysteroid-related processes are being downregulated at the same time that JH-related processes are being upregulated, when they would otherwise be on different schedules. While ecdysteroids may not directly influence the expression of foraging behavior in *P. californicus* workers, their role in ovarian activity makes them likely endocrine facilitators of nurse behavior. Ovarian ecdysteroids can stimulate the production of egg yolk precursors (vitellogenins) from the insect fat body — a tissue that is functionally homologous to the vertebrate liver and

white adipose tissue (Raikhel et al., 2005). This stimulatory effect on yolk production implies that ecdysteroids are not only markers of ovarian activity, but also a functional requirement for nutritive egg production, a nurse-specific trait in many ants, including other *Pogonomyrmex* species (Wilson and Eisner, 1957; Hölldobler and Wilson, 1990). Whether ecdysteroids influence the rate of nutritive egg production in workers of *P. californicus* can be addressed in future experiments.

Interestingly, the function and makeup of nutritive eggs in these ants bears a striking resemblance to secretions by the hypopharyngeal head glands of honey bees (Wilson and Eisner, 1957; Amdam et al., 2003). During the nest stage, these glands produce royal jelly that can be mixed with other secretions, nectar and pollen as a general food source for worker honey bee larvae and adult colony members, including foragers (Crailsheim and Stolberg, 1989). The hypopharyngeal glands use and store vitellogenin, the yolk protein that is essential for egg production. Metabolic consumption of vitellogenin by the bees' hypopharyngeal glands has been causally linked to their production of proteinaceous food secretions (Amdam et al., 2003). Thereby, both ants with nutritive eggs and honey bees have evolved mechanisms for exploiting vitellogenin in social nourishment. Such nourishment is crucial to colony integrity and development and is much-studied in honey bees (Engels and Imperatriz-Fonseca, 1990; Naiem et al., 1999; Amdam et al., 2003), while less work is done in ants (Hölldobler and Wilson, 1990; Gobin and Ito, 2000; Khila and Abouheif, 2008). Thus, to further understand the role of non-reproductive worker egg

production in *P. californicus* can allow for richer comparisons into how the reproductive infrastructure is exploited to evolve and sustain eusocial societies.

Although much remains to be clarified, our findings can be interpreted to support the view that the mechanisms underlying the worker division of labor may have been derived from regulatory networks of reproductive development. The RGPH suggests that the co-option of such networks may be a common route from which insect societies evolved complex social behaviors (Amdam et al., 2004). The finding that JH in *P. californicus* correlates with the foraging behavior of sterile workers in a manner similar to that of reproductive, colony-founding queens is consistent with this hypothesis. The inference that presumably ovarian-produced ecdysteroids may facilitate nurse behavior also is in line with the model of social co-option of solitary reproductive mechanisms. While neither of these associations has been causally linked to behavior, the correlations described here provide important additional information for understanding relationships between reproductive physiology and complex social behavior. A more robust evaluation of the RGPH would be made possible by future development of protocols for endocrine and functional genetic manipulation of ants. The ability to push forward with these investigations becomes more feasible due to the increasing number of tools available for ant researchers (Smith et al., 2009). For example, the growing number of annotated ant genomes (Bonasio et al., 2010; Smith et al., 2011; Suen et al., 2011, Wurm et al., 2011), including a closely related *Pogonomyrmex* species (Smith et al., 2011), opens up more ant systems to studies of the molecular genetics of behavioral regulation.

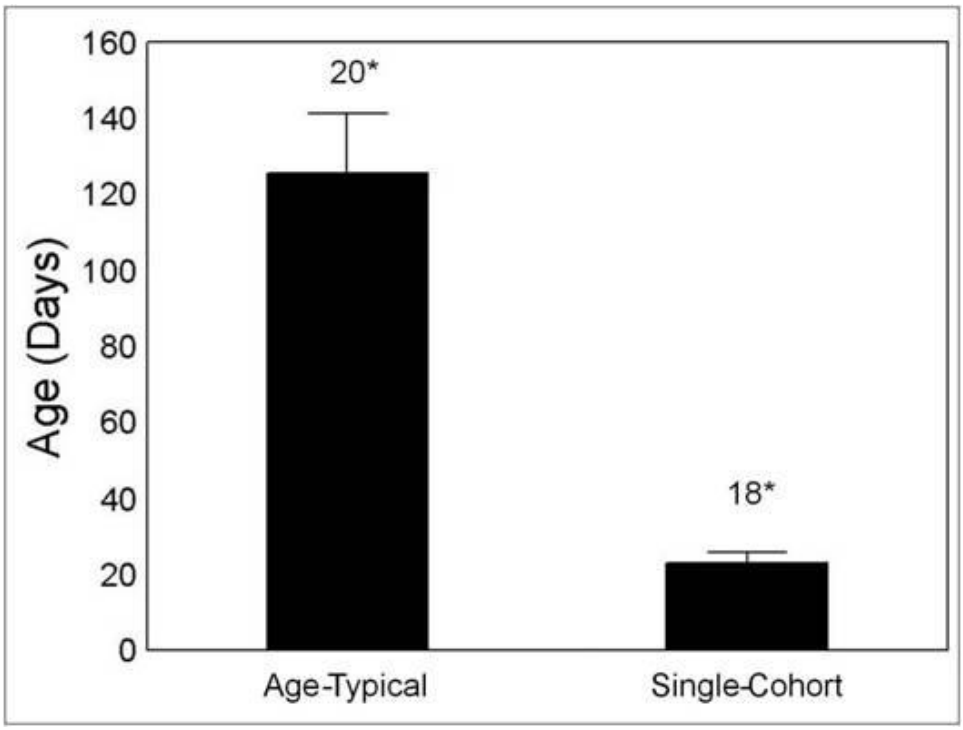

Figure 3.1: Age (days) of foraging confirmation of age-typical and single-cohort colonies. Single-cohort workers initiated foraging significantly earlier, denoted by asterisks (MWU test, $p<0.05$). Sample sizes are indicated.

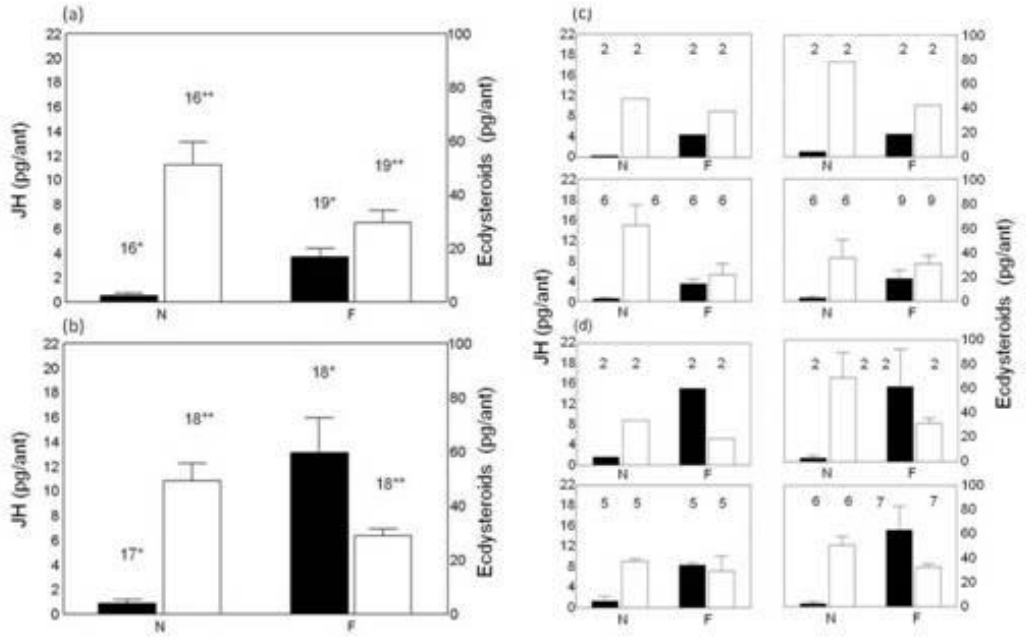

Figure 3.2: Mean +/- SE content (pg/ant) of juvenile hormone (JH; solid bars) and ecdysteroids (open bars) of (a) age-typical workers transitioning naturally from nest (N) to foraging (F) stages, and (b) single-cohort workers performing nest (N) or foraging (F) tasks in *P. californicus* colonies. Asterisks denote significant differences between compared groups. (Age-typical ecdysteroids: Student's *t*-test, $p<0.05$; all others: MWU test, $p<0.05$) between groups. In addition, JH content of single-cohort foragers is significantly higher than JH content of age-typical foragers (MWU test, $p<0.05$). For both the age-typical (c) and single-cohort (d) colonies, each component colony has too low a sample size for significant statistical analyses, however, the general trend in each colony is the same as the overall, result (a and b, respectively). Sample sizes are indicated.

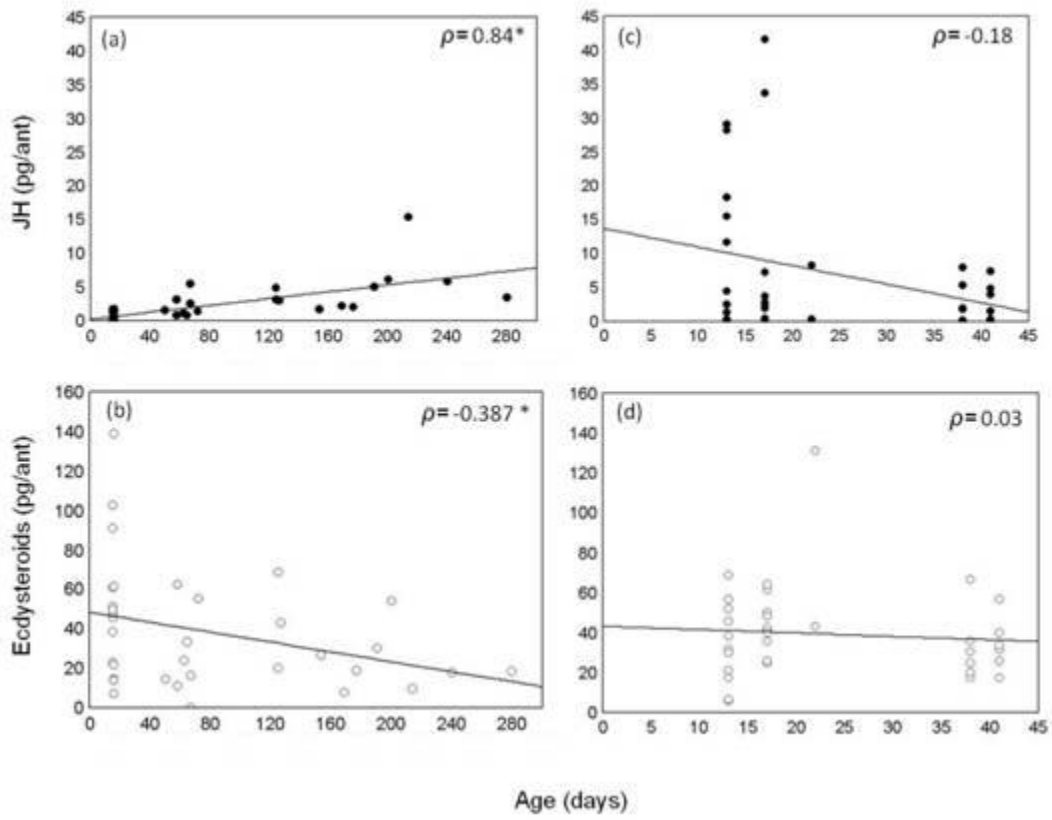

Figure 3.3: Distribution of hormone content in relation to age in age-typical and single-cohort *P. californicus* colonies. There is a significant positive association between JH (closed dots) content and age in age-typical colonies (a), but no such correlation exists for single-cohort colonies (b). Similarly, a significant negative correlation is observed between ecdysteroid (open dots) content and age in age-typical colonies (c), but not in single-cohort colonies (d). This relationship illustrates that the association between hormone content and behavior is not necessarily age associated, as the relationship disappears in single-cohort colonies. Asterisks denote significant correlations, and Spearman coefficients (ρ) are listed (Spearman Rank Correlation, $p<0.05$).

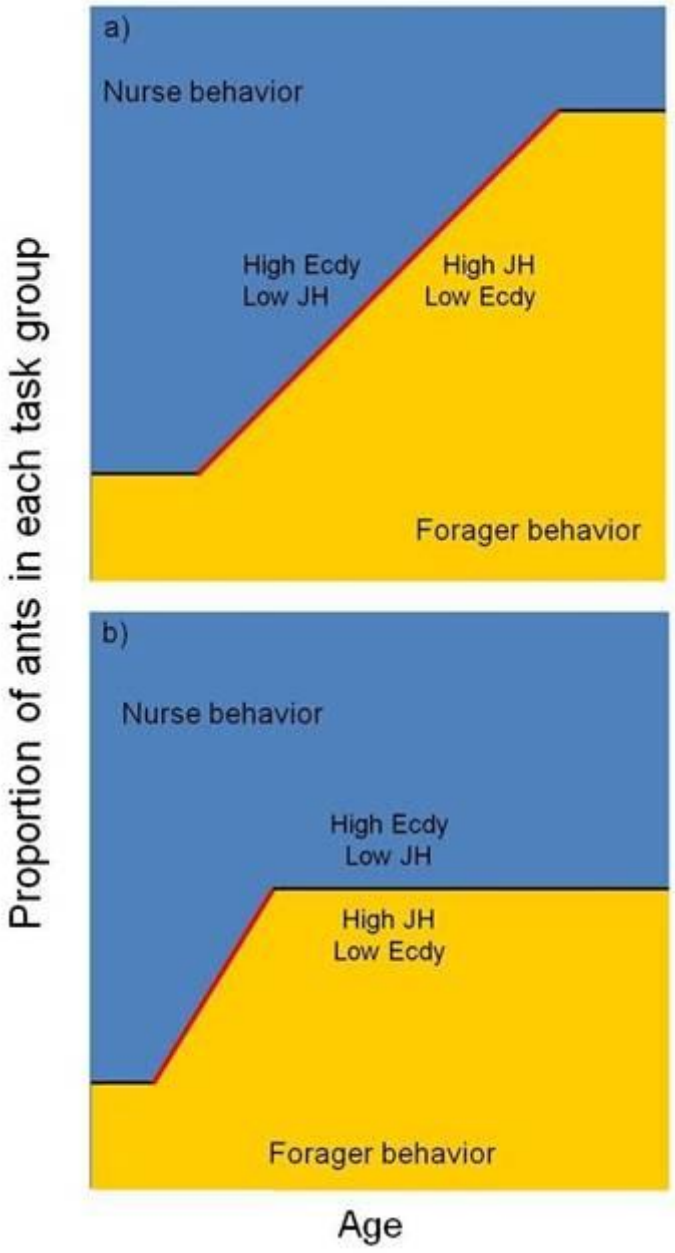

Figure 3.4: Hypothesized relationship between JH, ecdysteroids, age, and division of labor in *P. californicus* workers. Age is defined as the age of any given worker cohort, from adult emergence until death. That is, as a group of workers age (X-axis), the proportion performing different tasks changes (Y-axis). High JH content corresponds to foraging activities, and a high ecdysteroid content with nest tasks; the bisecting line represents the proportion of workers performing nurse (blue) or foraging (yellow), and demarks the different hormone levels. a) In age-typical

colonies, the vast majority of young workers has low JH levels, high ecdysteroid levels, and is inside the nest performing nursing tasks. As they age, more workers initiate foraging, which is a behavioral transition that is associated with high JH and low ecdysteroids. b) In single-cohort colonies, foraging onset begins at a much earlier age, and proceeds faster, until the colony has achieved the necessary balance between nurses and foragers. In both colony types, the onset of foraging coincides with increased JH and decreased ecdysteroid levels.

Chapter 4

DIVISION OF LABOR IS ASSOCIATED WITH AGE-INDEPENDENT CHANGES IN OVARIAN ACTIVITY IN *POGONOMYRMEX CALIFORNICUS* HARVESTER ANTS

ABSTRACT

An age-independent division of labor can develop in both the reproductive (queen) and non-reproductive (worker) castes of *Pogonomyrmex californicus* harvester ants, and individuals develop biases for in-nest activities or external foraging. These behavioral biases correlate with hormones that normally pace reproductive physiology and ovarian development. Additionally, ant ovaries normally atrophy in foragers compared to nest-biased workers (nurses). However, it is not clear whether these ovarian changes are in some way causal or due to changes in behavior or age, since foragers are typically older individuals. Here, we clarify this relationship in *P. californicus* queens and workers by comparing ovarian activity in same-aged ants that exhibit divergent behavioral biases. We found that foraging individuals had significantly reduced ovarian activity compared to their nest-biased counterparts, thereby linking changes in the ants' reproductive system to social task performance rather than to age. The general finding that ovarian physiology is associated with social insect behaviors is consistent with the hypothesis that the reproductive system of solitary ancestors provided building blocks for the evolution of insect societies.

INTRODUCTION

In most eusocial insect species, the success of the colony hinges on the completion of a variety of tasks, including food acquisition, brood care, and nest maintenance. These tasks are completed by a cooperative division of labor within the predominantly sterile worker caste. This division in task performance is age-based, with younger workers performing mostly in-nest tasks, like brood care, and older workers foraging for food or defending the nest (Hölldobler and Wilson, 2009). Since the ability of workers to switch between different tasks is an important component of the widespread ecological success of eusocial insects (Wilson, 1971), investigating the underlying physiology behind behavior may help us understand both how insect societies are regulated and how they evolved.

The proximate basis of temporal polyethism has been most heavily investigated in the honey bee, *Apis mellifera* (Ben-Shahar et al., 2002; Amdam et al., 2004, 2006; Rueppell et al., 2004; Hunt et al., 2007; Wang et al., 2010). Despite the fact that honey bee workers do not normally reproduce, behavioral transitions are affected by regulators that are normally associated with reproduction, including hormones (Robinson et al., 1987; Sullivan et al., 2000), yolk proteins (Amdam et al. 2004, Ihle et al., 2010), and even the whole ovary (Wang et al., 2010; reviewed by Amdam and Page, 2010). Thus, it has been hypothesized that these worker behaviors evolved from reproductive traits exhibited by a solitary ancestor. The reproductive ground plan hypothesis (RGPH) (West-Eberhard, 1987, 1996; Amdam et al., 2004; Amdam and Page, 2010) argues that, instead of evolving a new regulatory infrastructure, regulators

of development and behavior were co-opted from the solitary ancestral state, decoupled from reproduction, and then utilized to control social behavior in functionally sterile individuals.

Some of the major components likely co-opted in this transition are the endocrine regulators of reproductive processes. Juvenile hormone (JH) and ecdysteroids are the primary hormonal drivers of reproduction in most insects. JH is a key factor in the regulation of ovarian development, oogenesis, and reproductive behavior in many insects, including *Drosophila melanogaster* (Flatt et al., 2005) and *Aedes aegypti* (Knowlden, 1997). Ecdysteroids are produced by the insect ovary (Hagedorn et al., 1975; Lafont et al., 2005), and are involved in regulating the reproductive cycle, sometimes through interactions with JH (Raikhel et al, 2005; Bernardi et al., 2009). There is mounting evidence that these hormones also play an important role in regulating social behaviors. For example, elevated JH levels can signal foraging onset in honey bees (Robinson, 1987; Sullivan et al., 2000), and, in the fire ant, *Solenopsis invicta,* JH mediates the effect of queen primer pheromones that inhibit reproductive behaviors and development in nestmates (Brent and Vargo, 2003). Similarly, ecdysteroid levels are linked to dominance status in both bumble bees (Bloch et al., 2000) and queenless ants (Brent et al., 2006), where they likely affect the behavioral and physiological changes associated with the fluid reproductive roles in these species.

In addition to these systemic hormones, the ovary itself can be an important regulator of insect behavior. In mosquitoes, ovarian factors help control

host-seeking behaviors (Klowden, 1997), while in *Drosophila*, the ovaries modulate sensitivity to stimuli that influence behavior (Flatt et al., 2008). Similarly, in honey bees, worker ovarian development is robustly linked to age of foraging onset (Page et al., 1995; Amdam et al., 2006; Rueppell et al., 2008; Wang et al., 2009; Amdam and Page, 2010), and has a causal role in behavioral development (Wang et al., 2010). The ovaries may influence worker behavior through differential production of ecdysteroids (Amdam et al., 2010) and expression of genes coding for hormone receptors and sensory perception (Velarde et al., 2009; Wang et al., 2009, 2010, 2012).

In *Pogonomyrmex californicus* harvester ants, behavioral biases occur in both queens and workers. Individuals showing a bias towards foraging behavior exhibit elevated levels of JH relative to those that stay within the nest (Dolezal et al., 2009; 2012). Foraging-biased workers also have decreased levels of ecdysteroids (Dolezal et al., 2012). However, the hormonal association appears to be more correlative than directly causal, suggesting that other factors may be involved. While a direct link between behavior and ovarian activity has not been addressed in the ants, it has long been known that ovarian morphology differs drastically between workers in different task groups in many species. The ovaries of young, nurse workers are typically well-developed and active, while those of old foragers are heavily atrophied. However, it remains unclear whether these differences are due to the large age differences normally found between nurses and foragers in most colonies, or if these differences are linked to behavior *per se* (Hölldobler and Wilson, 1990). Based on the putative relationship between

reproductive physiology, reproductive hormones and behavioral preference in *P. californicus*, we hypothesize that ovarian activity is linked to non-reproductive behavioral preferences, independent of age. Since ovarian atrophy is associated with age-based foraging activity in ants (Hölldobler and Wilson, 1990), and *P. californicus* workers show decreasing levels of the ovarian-produced ecdysteroids after foraging initiation, we predict that decreased ovarian activity is linked to a preference for foraging, even when foraging is precociously stimulated via colony manipulation.

METHODS

P. californicus worker observation and collection

P. californicus single cohort colonies were formed and observed as described in Dolezal et al. (2012). *P. californicus* colonies were partially excavated from field sites in Maricopa County, Arizona in early November of 2010, and the light-colored callow workers were collected. Cuticle pigmentation was used as a marker for worker age, and the lightest colored, and thus youngest, workers were collected. Workers were brought back to the laboratory, and approximately 200 were added to each of four experimental colonies. Host colonies were 1.5-2.5 years of age, and had approximately 300 workers each. After two days, all of the original workers were removed, leaving only the queen, eggs, larvae, and 200 young, same-aged workers. Colonies were then observed 3-7 days per week, 2-3 times per day, for 5-10 minutes per observation. Foraging individuals were marked on the abdomen with small dots from a Sharpie® paint

pen. Once a worker had been observed foraging on three occasions, she and an in-nest worker from the same colony were collected together. These collections continued for the duration of the experiment, and all workers were collected between the ages of 23 and 33 days. The experiment was ended at this time, as the populations of all colonies had dropped below approximately 25 workers.

To verify that any patterns found in single cohort colonies reflected natural phenotypes, workers from unmanipulated field colonies were also collected. Age of these workers was unknown, but *P. californicus* workers exhibit age polyethism and typically transition to foraging as they age (Dolezal et al., 2012), so foragers were presumably older than nest workers. Foragers were collected at bait traps near mature nests, and in-nest workers were collected from the brood chambers of 4 nearby colonies.

***P. californicus* queen collection and observation**

P. californicus queens were collected immediately after mating flights in San Diego County, CA, USA in July 2008. Queens were collected from a population where queens readily form multiqueen associations. After collection, they were housed and observed as described in Dolezal et al. (2009). They were marked on the abdomen and thorax with one of two colors of enamel paint (Testor's: Rockford, IL, USA) one queen of each color was then introduced into a soil-filled nest jar. The soil was watered in small quantities when it became observably dry, and the colonies were fed a restricted quantity of Kentucky blue grass seeds. Associations were observed for 15 minute intervals 4 times per day for 15 days to identify behavioral biases. Queens were recorded as foraging when

observed outside of the nest entrance searching for or handling seeds. Associations were classified as having a division of labor if 10 or more foraging events were observed and more than 80% of those events were performed by one queen. The queen performing the majority of the foraging was categorized as foraging-biased, while the one performing the minority of foraging was categorized as nest-biased.

Ovarian Dissection, Fixation, and Staining

A total of 36 *P. californicus* queens and 44 workers were collected after observations, kept on ice, and dissected in PTW (1 X PBS; 0.05% Tween-20) using a dissection microscope. The entire ovary of each individual was fixed in a solution of dimethylsulfoxide (DMSO, 20 µl), 4% paraformaldehyde (200 µl) and heptane (600 µl) for 20 minutes. After fixation, ovaries were washed three times in PBT (1 X PBS; 0.3% Triton). For long-term storage, ovaries were then washed in increasingly concentrated methanol:PTW solutions (30:70, 50:50, 70:30), culminating in pure methanol. After completing sample collection, ovaries were rehydrated with 3 washes of decreasingly concentrated methanol:PTW solutions (70:30, 50:50, 30:70) culminating in pure PTW (Khila and Abouheif, 2008). Samples were then incubated in 1:1000 dilution of the DNA stain DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA) for 10 minutes, washed three times in PTW to remove excess stain, and mounted on microscope slides using Vectashield (Vector Labs, Burlingame, CA, USA). The stained and mounted samples were imaged using a Leica SP2 multiphoton scanning laser

microscope (Leica Microsystems, Buffalo, IL, USA) and a Nikon Fluphot inverted fluorescent microscope (Nikon Instruments, Melville, NY, USA).

Ovarian activity scoring

The insect ovary is made up of multiple tube-like filaments, called ovarioles, where egg development occurs. As the eggs mature, they increase in size and move towards the anterior of the animal. After the oocytes reach a certain size and maturity, the eggs uptake large quantities of yolk proteins and thus become vitellogenic (reviewed in Raikhel and Dhadialla, 1992). Therefore, we classified developing oocytes into one of several types (described below) based on their number, vitellogenic status, and location in each ovariole in queens and workers. The number of ovarioles comprising each ovary was also noted.

In workers, vitellogenic oocytes were identified by their large size and opacity, since vitellogenic oocytes are opaque and pre-vitellogenic oocytes are translucent (Raikhel and Dhadialla, 1992). In queens, vitellogenic oocytes were further classified based on their size and the status of their accompanying nurse cells. Large, vitellogenic (V) oocytes with completely degraded nurse cells were categorized as "V3". Smaller, vitellogenic oocytes with nurse cell clusters of approximately the same size were categorized as "V2". The smallest vitellogenic oocytes, with nurse cell clusters larger than the oocyte, were categorized at "V1". Pre-vitellogenic (PV) oocytes were categorized sequentially; the most posterior pre-vitellogenic oocyte was categorized as "PV1", and each subsequent (newer) oocyte was labeled "PV2", "PV3", etc (Fig. 4.1). This approach provides a metric for ovarian activity, as it shows how many eggs the ovary is producing and the

relative ages of each egg, with V3 being the oldest, and PV3 being the youngest. Workers, however, produce many fewer eggs than queens, so this more specific technique was abandoned in favor of simplified categories. In workers, oocytes were categorized simply as vitellogenic (V) or pre-vitellogenic (PV).

Statistical analyses

The data for all oocyte counts from *P. californicus* queens showed a general lack of normality and did not pass the assumption of homogeneity of variances (Levene's test; $P<0.05$). The oocyte counts for the workers also failed to meet parametric assumptions of normality. This required the non-parametric Mann-Whitney *U*-tests to compare behavioral groups. A Spearman rank test was used to determine if correlations existed between worker age and oocyte number. An alpha value of 0.05 was used for significance in all tests. Analyses were performed using Statistica 7.0 (Statsoft, Tulsa, OK, USA).

RESULTS

Ovarian activity in *P. californicus* workers

In workers, there were no significant differences between the numbers of ovarioles in nest-biased (non-foraging) and foraging-biased individuals from single cohort colonies (Mann-Whitney U Test; $N_n=20$, $N_f=24$; $p>0.05$; $Mean_{nest}=7.85$, $Mean_{forager}=7.58$). However, nest-biased workers had a significantly higher number of total oocytes ($N_n=20$, $N_f=24$; $p<0.001$); with the numbers of both pre-vitellogenic ($N_n=20$, $N_f=24$; $p<0.0001$) and vitellogenic ($N_n=20$, $N_f=24$; $p<0.0001$; Fig. 4.1F-G, Fig. 4.2) oocytes elevated. In fact, ovaries

of foragers were highly atrophied, and showed both a lack of oocytes and high number of apoptotic nuclei (Fig. 4.1 G). In addition, there was no correlation between the worker age and total oocyte number (Spearman rank correlation: N_n=20, N_f=24; ρ= -0.138; $p>0.05$, Fig. 4.3).

Workers from field colonies showed a similar pattern, with significantly more pre-vitellogenic (Mann-Whitney U Test; N_n=20, N_f=21; $p<0.0001$), vitellogenic (N_n=20, N_f=21; $p<0.0001$), and total (N_n=20, N_f=21; $p<0.0001$) oocytes in nest workers. In fact, no developing oocytes were found in any foraging workers. While this pattern is the same as that found in single cohort colonies, nest-biased single cohort workers had significantly more total oocytes than nest-biased workers from field colonies (N_{sc}=20, N_{field}=20; $p<0.0001$, Fig. 4.4).

Ovarian activity in co-founding *P. californicus* queens

Similarly to workers, there were no significant differences between the number of ovarioles in nest-biased (non-foraging) and foraging-biased queens (Mann-Whitney U Test; N_n=18, N_f=18; p=0.56; $Mean_{nest}$=29.6, $Mean_{forager}$=29.27). However, as we found in workers, nest-biased queens had more total oocytes in their ovaries than did foraging-biased queens (N_n=18, N_f=18; $p<0.001$).

Further analysis of the types of categorized oocytes in queen ovaries shows what types of oocytes drive these differences in queens. There were no significant differences in the numbers of the largest, best developed oocytes types: V3 (N_n=18, N_f=18; p=0.29) or V2 (N_n=18, N_f=18; p=0.18). However, nest-biased

queens had significantly more small V1 oocytes (N_n=18, N_f=18; p<0.001), as well as PV1 (N_n=18, N_f=18; p<0.001), PV2 (N_n=18, N_f=18; p<0.001), and PV3 (N_n=18, N_f=18; p<0.001; Fig. 4.1 B-E, Fig. 4.5) pre-vitellogenic oocytes.

DISCUSSION

Our results show that ovarian activity, denoted by the number and developmental stage of their oocytes, is highly correlated with task performance and is independent of age and reproductive caste in *Pogonomyrmex californicus* ants. In single cohort colonies, the ovary of nest-biased workers was well-developed and had many oocytes (Fig. 4.1F), while the ovary of foraging-biased workers was atrophied and supported few or no oocytes (Fig. 4.1G, 4.2). Workers collected from field colonies showed the same pattern, verifying that these differences are not an artifact of laboratory manipulation. This pattern is in agreement with the well-known relationship between ovarian status and foraging in many ant species, where young nurses have more developed ovaries than old foragers (reviewed by Hölldobler and Wilson, 1990). In our study, however, the use of single cohort colonies removed age as a significant factor (Fig. 4.3). Additional evidence that chronological age is not the driving force behind the ovarian atrophy observed in foragers is that all the workers sampled were relatively young, approximately 30 days post-emergence, compared to their potential lifespan in the laboratory (>300 days; Dolezal et al., 2012).

It is notable that, while workers collected in the field showed the same pattern of elevated ovarian activity in nest workers compared to foragers, nest

workers from the field had, on average, fewer total oocytes than those from single cohort colonies (Fig. 4.4). This discrepancy may be due to differences in sampling technique. Collection is much more precise in laboratory colonies; nest workers were sampled as they tended brood in observable laboratory nests. In field colonies, however, nests were excavated, and nest workers were collected from inside brood chambers; in the disorder of excavation, workers in the transition from nest to foraging tasks may have also been collected. It is also possible that the ants collected from the field were much older that than the single cohort ants, and that age may still cause some impairment of ovarian function regardless of behavioral phenotype. Lastly, field colonies are much larger, and likely have many more resource demands on the workers compared to the laboratory colonies that were fed *ad libitum*. Thus, field workers may have fewer resources to devote to oocyte production.

Part of the resource demands on *P. californicus* workers, is that, like many other ant species, the young nurses produce non-viable eggs to feed to developing brood (Wilson and Eisner, 1957; Hölldobler and Wilson, 1990; Gobin et al., 1998). In contrast, old foragers, who have ceased nurse tasks, show ovarian atrophy (Hölldobler and Wilson, 1990) and no longer produce nutritive eggs. This association between oocyte production and behavior appears similar to the 'social-exploitation' of vitellogenin by honey bee workers (Amdam and Omholt, 2003). While reproductively active individuals normally use the protein to produce eggs (Raikhel and Dhadialla, 1992), honey bee nurses have repurposed the protein to supply the hypopharyngeal gland with nutrients necessary to

produce food secretions for feeding brood (Crailsheim and Stolberg, 1989; Amdam et al., 2003; Seehus et al., 2007). The ovaries of *P. californicus* workers likely operate in a very similar manner to accomplish resource-sharing.

The same relationship between task performance and oocyte quantity found in workers was also observed in behaviorally-biased founding queens (Fig. 4.5). However, unlike workers, the ovary of foraging-biased queens is not atrophied and has some oocytes. This may be because, unlike workers, queen foraging behavior does not represent a terminal life stage. After the emergence of the first workers, queens cease foraging and then remain inside the nest as viable, reproductive queens for the remainder of their lives (Johnson et al., 2004; personal observation). While the ovaries of foraging queens do not atrophy, they do appear to become less active (Figs. 4.1E and 4.5) than those of non-foraging queens. The differences in total oocyte number between nest- and foraging-biased queens are driven by differences in the earlier stages of egg development, implying that, while both groups have some oocytes in their ovary, foraging-biased queens are producing significantly fewer new oocytes. Nest-biased queens have many oocytes at all levels of development: from large, vitellogenic to very small, pre-vitellogenic oocytes (Fig. 4.1 B-D). However, foraging-biased queens appear to have significantly slower oocyte production, as they have very few, young, pre-vitellogenic oocytes (Fig. 4.1E). The only oocyte quantities that are not different between nest- and foraging-biased queens are the most highly developed (V1 and V2). It is most likely that no differences exist between these

oocyte types, as these represent the oldest oocytes, which probably began developing before behavioral biases emerged.

The differences in apparent oocyte production between the cooperative queens beg the question of whether these differences result in some degree of reproductive skew; that is, whether the nest-biased queen is gaining greater reproductive advantage by staying in the nest and laying more eggs. While nest-biased queens do appear to produce more eggs, any skew between the queens is unlikely during this stage of colony development. The first workers are produced from eggs laid by both queens (Johnson et al., 2004), and then, most, if not all, of the subsequent eggs laid prior to worker emergence are used as a nutritional source for feeding this first group of developing larvae (personal observation). This behavior is not particularly surprising, as founding queens in other species produce large numbers of non-viable nutritive eggs (Gobin and Ito, 2000). Therefore, any egg production during this time is probably used only for food sharing. Furthermore, even if the eggs were viable, they would certainly be raised into workers; since these are sterile, they would represent no reproductive benefit. As in most ant species, reproductive gains are only made after the colony matures and produces new queens and males (Hölldobler and Wilson, 1990). In the case of *P. californicus*, this occurs long after the queens have ceased participating in foraging and brood care. Therefore, while it is possible that reproductive skew may occur between the queens at some point in their life cycle, there is no evidence that their behavioral role during founding affects this.

We also found that nest-biased queens (Fig. 4.5) had fewer total oocytes than workers (Fig. 4.2) despite the fact that queens have over three times more ovarioles. This is likely because queens are nutritionally-limited during this phase of their lives, and are not able to produce eggs at their highest rate. However, nest-biased workers are likely not as nutritionally limited, as they emerge into a colony buffered by stored food resources. Furthermore, queens and workers face very different demands for their nutritive eggs. Founding queens need to nourish a limited number of brood (Johnson, 2004), whereas workers must supply eggs to feed more brood, other workers, and the queen (Hölldobler and Wilson, 1990; Gobin et al., 1998). Therefore, despite the fact that workers have smaller ovaries than queens, they probably face a higher demand to produce nutritive oocytes than do founding queens.

In conclusion, our finding that ovarian activity is linked to age-independent behavioral changes in *P. californicus* workers and queens suggests that the ovary may play a role in the regulation of division of labor in this species. This finding is consistent with the RGPH, which argues that the regulatory infrastructure used by solitary ancestors to control reproductive behaviors may have been co-opted for use as the building blocks for regulating social behaviors, and that non-reproductive social behaviors are therefore regulated by reproductive factors. Despite this supporting evidence, additional work will be necessary to clarify the relationship between ovarian activity and behavioral development, and to identify other possible regulatory roles of this reproductive organ.

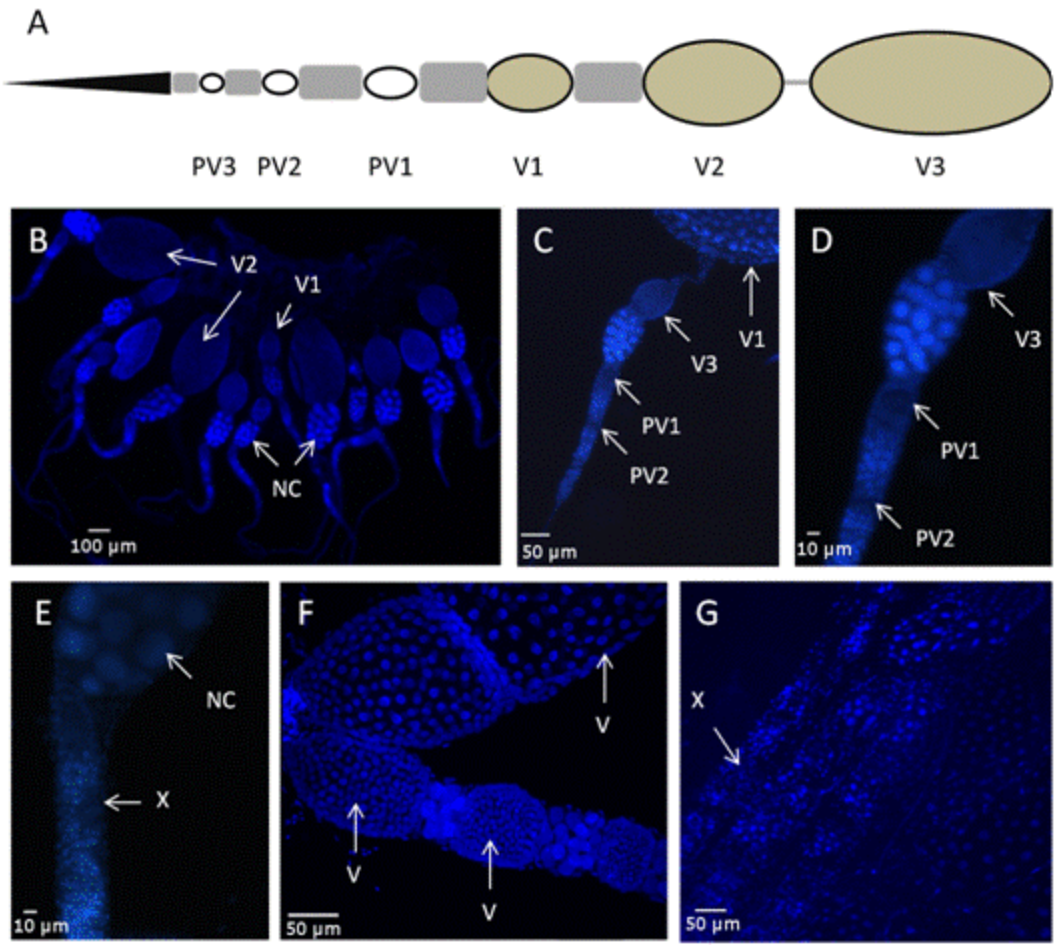

Figure 4.1: Scoring regime and examples ovaries of *P. californicus* queens and workers. A) Diagram of a single ovariole, showing the categorization regime for queen oocytes. The black triangle on the left represents the germarium, where new oocytes are produced; the right side is the posterior end of the ovariole. Vitellogenic oocytes (V) are shown in tan, and are numbered with increasing size (V1, V2, V3) relative to their accessory nurse cells (grey boxes). Pre-vitellogenic oocytes (PV1, PV2,PV3) are shown in white, and were counted sequentially. B-G) Oocytes and ovarioles with DAPI-stained DNA. Representative oocytes

categories are indicated. Nurse cells (NC) and sections of the ovariole where no oocytes are produced (X) are also labeled. B) Collection of connected nest-biased queen ovarioles. C) Single nest-biased queen ovariole. D) Enhanced magnification of ovariole in C, better showing early stage (PV) oocytes. E) Ovariole of a foraging-biased queen. The ovariole is still healthy and intact, but lacks any early-stage (PV) oocytes. F) Ovariole of a nest-biased worker, with many well-developed oocytes. G) Several ovarioles of a foraging-biased worker, with large quantities of apoptotic nuclei and no oocytes (X). Scale bars are shown.

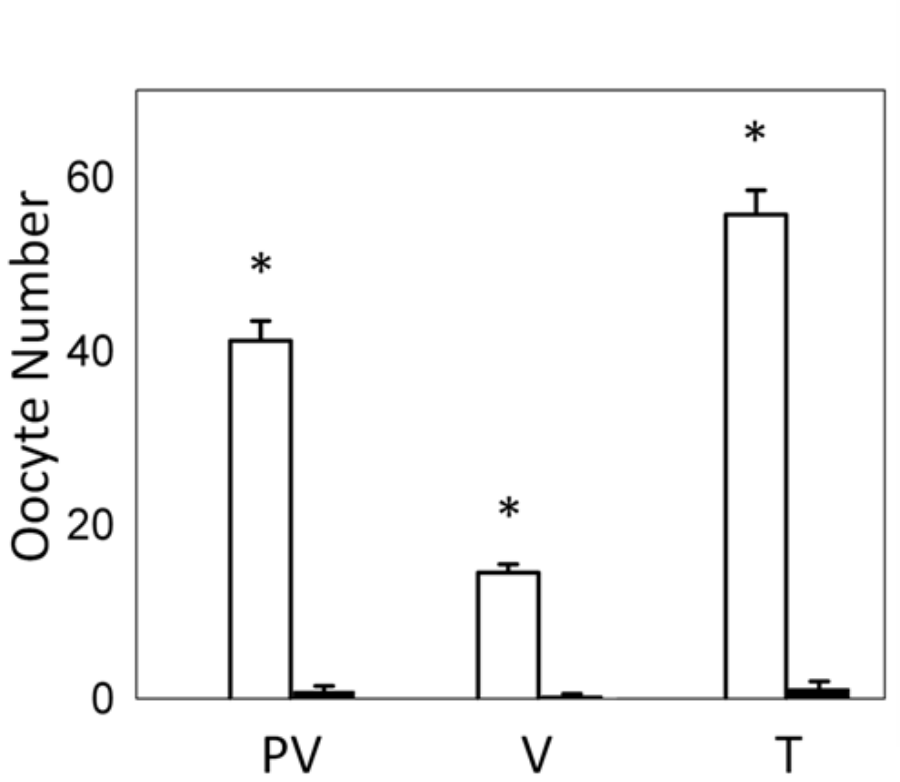

Figure 4.2: Oocyte numbers found in foraging- and nest-biased *P. californicus* workers. Mean +/- SE pre-vitellogenic (PV), vitellogenic (V), and total (T) oocytes found in nest-biased (white bars) and foraging-biased (black bars) single cohort workers. Asterisks denote significant differences ($p<0.05$).

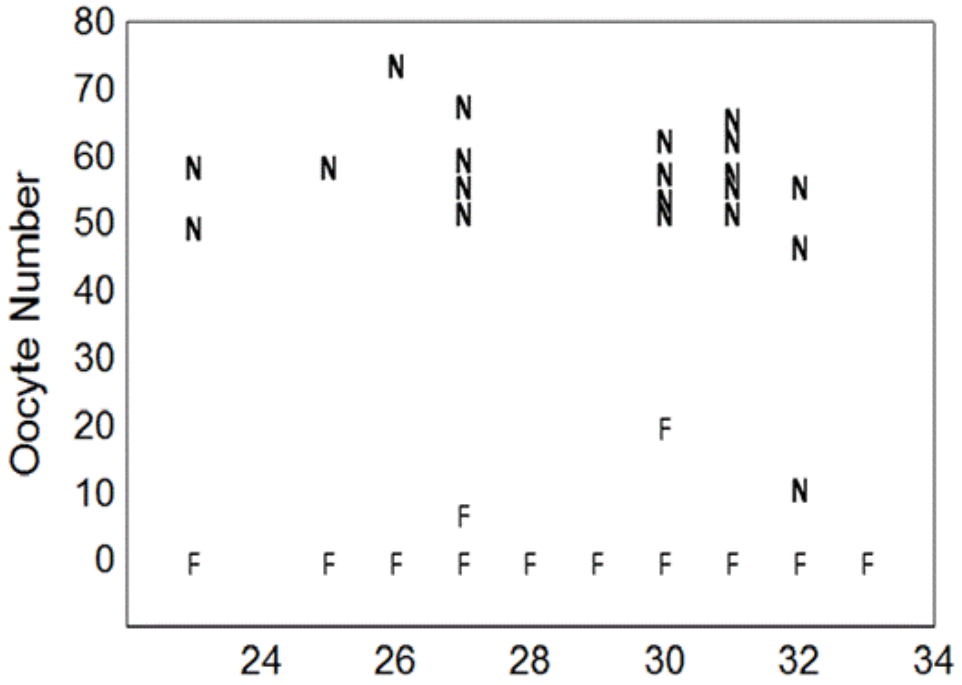

Figure 4.3: Oocyte number as a function of age in single-cohort *P. californicus* workers that were nest- (N) or foraging-biased (F).

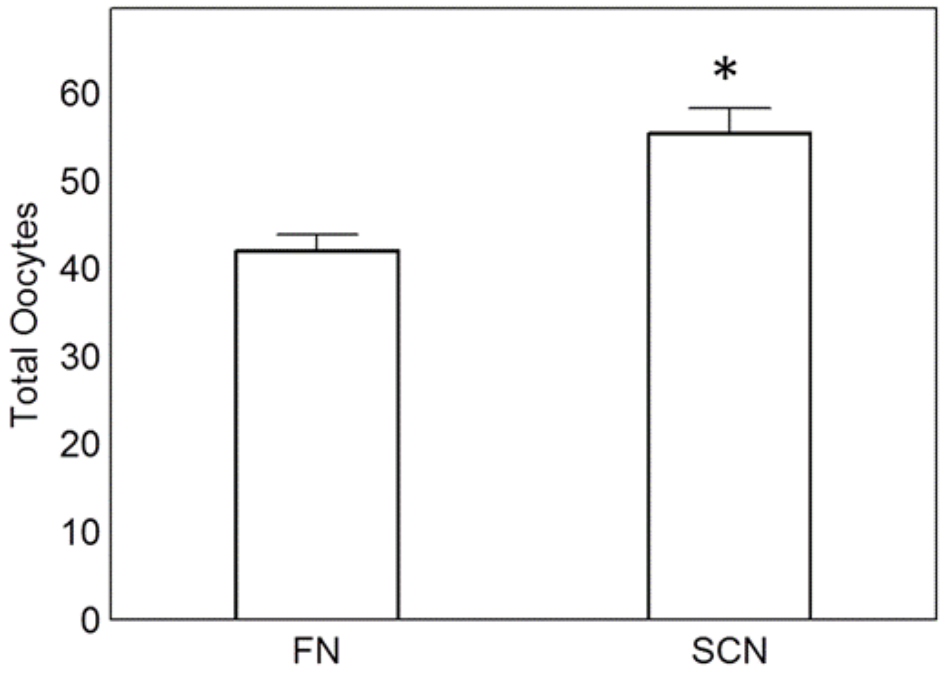

Figure 4.4: Oocyte numbers of field collected and single-cohort nest-biased workers. Mean +/- SE total oocytes found in field collected nest workers (FN) and single cohort nest workers (SCN). The groups differed significantly ($P<0.05$).

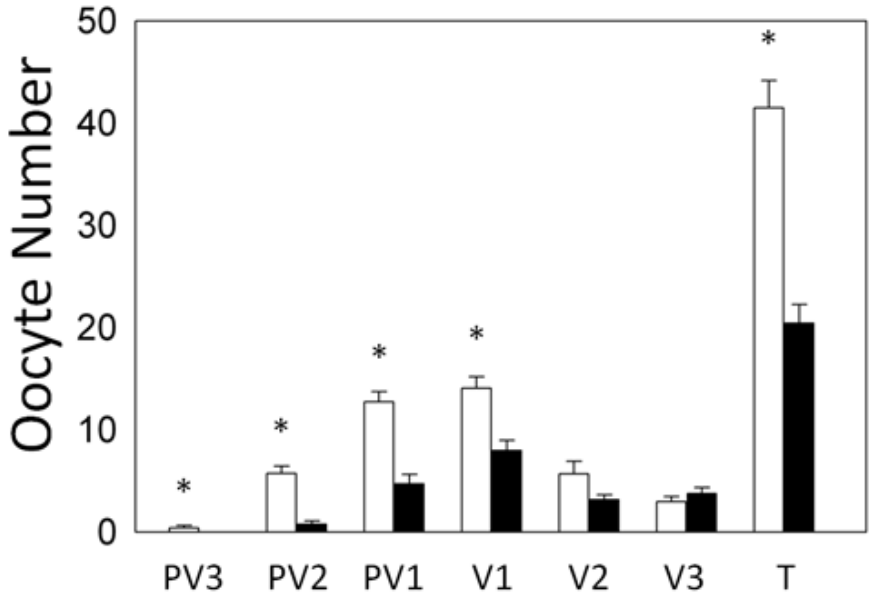

Figure 4.5: Oocyte numbers found in foraging- and nest-biased *P. californicus* founding queens. Mean +/- SE pre-vitellogenic 3, 2, 1 (PV3, PV2, PV1), vitellogenic 1, 2, 3 (V1, V2, V3), and total oocytes (T) found in nest-biased (white bars) and foraging-biased (black bars) *P. californicus* founding queens. Asterisks denote significant differences between the number of oocytes in nest-biased and foraging-biased queens ($p<0.05$).

Chapter 5

POGONOMYRMEX CALIFORNICUS QUEENS REMAIN BEHAVIORALLY PLASTIC AFTER COLONY FOUNDING

ABSTRACT

Advanced eusocial societies are typically defined by castes with a fixed range of behaviors. In some species, behaviors can change as individuals transition between life stages, usually in a fixed pattern. In *Pognomyrmex californicus* ants, queens in their colony founding stage spend their time foraging and caring for their brood. They exhibit some plasticity, in that cofounding queens can develop a division of labor in which one queen performs all the foraging tasks, and the other remains in the nest caring for brood. However, after the first workers emerge, both queens cease these tasks, and dedicate themselves solely to egg-laying. Whether or not the queens retain some behavioral plasticity at this "permanent" egg-laying stage and can return to earlier behaviors was previously unknown. Here, we disrupt the nests of established queen associations in which foraging has already ceased, effectively re-establishing the founding stage. We find that queens are capable of responding to changes in the social environment by resetting their behavioral development and returning to founding behaviors. Furthermore, we find that juvenile hormone (JH) content is elevated in foraging-biased queens compared to nest-biased queens, fitting the same pattern found in normal founding queens. We also observe that JH content is highly elevated in these reset foragers compared to previously reported levels in normal founding

queens. These results support a role for JH in regulating the behavioral development of queens, and suggest that behavioral castes may, in part, be maintained by changes to endocrine activational thresholds.

INTRODUCTION

Eusocial insect societies are defined by a dichotomy between reproductive and sterile castes. The existence of these castes allows individuals to specialize on subsets of tasks, helping to optimize colony efficiency. Within the sterile worker caste, which performs the majority of non-reproductive behaviors, individuals progress on a trajectory of behavioral development in which task performance changes with age (Hölldobler and Wilson, 2009). Changes in behavior usually follow this pattern, but some degree of plasticity is maintained throughout much of their behavioral development, as selective advantage is likely gained by the ability to respond to changes in the social environment (Nelson, 1927; Hölldobler and Wilson, 1990). While behavioral plasticity is relatively well-studied in workers, this type of behavioral plasticity could also be important in the reproductive queen caste, which likely encounters high levels of environmental variation during colony founding.

In most of the "advanced" ant subfamilies, a new colony is founded by a queen that uses her internal tissue resources to fuel the production of a first cohort of workers; during this time, she lays eggs and cares for her brood, but does not leave the nest. External resources are not brought into the nest until her workers emerge and begin foraging (Johnson, 2002; Hölldobler and Wilson, 1990).

However, in semi-claustral ants, like *Pogonomyrmex californicus*, founding queens lack the internal resources necessary to rear their first group of workers and must forage for provisions to successfully found a colony (Johnson, 2002; Hahn et al., 2004). In some populations of *P. californicus*, unrelated queens will cooperatively found nests. Often, queens in such groups will take equal responsibility for both brood care and foraging, but sometimes a strong division of labor develops between the founding queens. One queen may become biased towards leaving the nest on foraging trips while the other preferentially stays inside the nest, presumably caring for brood (Cahan and Fewell, 2004; Johnson, 2004; Dolezal et al., 2009). After the first group of workers emerges, queens enter a new behavioral stage, dedicate their resources solely to producing eggs, and never leave the nest again (Johnson, 2002; personal observation).

The emergence of workers, and their subsequent takeover of foraging and brood care tasks, has been hypothesized to play a role in ending queen foraging, possibly by increasing the threshold stimulus necessary to induce queens to leave the nest (Dolezal et al., 2009). In these colonies, unnecessary queen foraging is likely under strong negative selection, since mitigating risk to the queen is important for colony survival (Hölldobler and Wilson, 2009). However, there is probably a selective advantage for queens able to respond to environmental changes and reverse the normal trajectory of queen development if necessary. If the first clutch of workers is lost or greatly diminished, it would be beneficial for queens to 'reset' to an earlier behavioral stage and exhibit colony founding behaviors again. If this is the case, mechanisms would evolve to prevent the

queen from performing risky foraging trips after workers have taken over these tasks, but also to allow for a level of behavioral plasticity that lets queens respond to the loss of worker help.

To build a mechanism for the regulation of queen behavioral development, natural selection could have acted on proximate mechanisms already present as behavioral regulators. One possible regulator that could have been used to affect these queen phenotypes is juvenile hormone (JH), a systemic hormone that has effects on reproductive behavioral cycling in many insects (Raikhel et al., 2005), including ants (Sommer et al., 1993; Brent and Vargo, 2003; Brent et al., 2006). In addition, foraging bias, in both solitary queens and multiqueen associations of *P. californicus*, is associated with elevated JH levels (Dolezal et al., 2009), suggesting that JH signaling plays an important role in the development of foraging biases. Similar patterns have been observed in honey bees (Giray et al., 1999; Sullivan et al., 2000). However, in associations where no biases emerge (i.e., both queens forage equally), JH levels remain low, suggesting that elevated JH titers are not required for foraging behaviors to occur *per se*, but may instead be involved in reinforcing foraging behavior (Dolezal et al., 2009). If queens can respond to worker loss by resetting to the behaviors at colony founding, and JH plays an important role in regulating this plasticity, JH levels should differ in a pattern similar to that found in normal founding queens. Specifically, if reset queens are able to reinitiate colony founding and develop a subsequent division of labor, we expect JH levels to be elevated in foraging-biased queens, but remain low in nest-biased and unbiased queens.

Here, we show that queen behavior remains flexible even after a switch to a new life history stage. After worker emergence and cessation of queen foraging in multiqueen associations, removal of the workers and destruction of the nest resulted in a reinitiation of colony founding behaviors, showing that queens maintain a degree of behavioral plasticity that allows them to respond to changes in their social environment. In addition, these queens responded similarly to normal founding queens, with some associations forming a division of labor between nest-biased and foraging-biased individuals that was correlated with differences in JH titer. These data support the role of JH as a behavioral regulator in *P. californicus*.

METHODS

Behavioral observations and colony reset

P. californicus founding queens were collected during mating flights in July, 2010, in San Diego County, CA, USA. Queens were brought into the laboratory at Arizona State University, Tempe, Arizona, USA (natural light cycle, ~25º C) 1-2 days post-collection. Queens were marked on the abdomen and thorax with one of two colors of enamel paint (Testor's: Rockford, IL, USA); one queen of each color was then introduced into a soil-filled nest jar. From 520 collected queens, 260 associations were formed. The soil was watered in small quantities when it became observably dry, and the colonies were fed with restricted quantities of Kentucky blue grass seeds. Associations were observed for 15 min. intervals, four times per day, for 15 days to identify behavioral biases

towards nest tasks or foraging (Dolezal et al., 2009). Subsequently, colonies were maintained (fed and watered) and observed several times per week until worker emergence.

After approximately 80 days, a small number of workers were observed foraging in the 17 surviving nests. In addition, queens had not been observed outside for at least 15 days. At this point, the incipient colonies were disrupted: workers and brood were removed, and tunnels were destroyed. Queens were then introduced to new soil-filled jars with the same partner queens – "resetting" them to a founding stage with no nest or brood. They were then observed as above to identify whether a behaviorally biased association emerged. After 5 foraging bouts, queen behavior was identified as biased if 80% of the bouts were conducted by a single queen. If each queen performed 40-60% of the foraging observations, the association was identified as nonbiased.

JH measurements

Queen sampling, JH extraction, purification, and analysis were performed as described in Dolezal et al. (2009). Using samples from pooled whole body extracts of 5 queens, JH methoxyhydrin derivatives were created and quantified using an Agilent 6890 Series GC (Hewlett Packard, Palo Alto, CA, U.S.A.) equipped with a 30 m x 0.25 mm Carbowax Econo-Cap GC column (Alltech, Fresno, CA, U.S.A.) coupled to an Agilent 5973N inert mass selective detector/detection software (MSD/DS).

Statistics

Foraging-biased, nest-biased, and non-biased queen JH content was compared using a Kruskal-Wallis ANOVA; post-hoc analysis was conducted using the Mann-Whitney U test. To allow for comparisons of previous findings, JH content was normalized into fold differences; the mean JH content for nest-biased queens was equated to 1, and the mean content of foragers was then scaled to this value. These fold differences were then compared to the fold differences found in a previous study (Dolezal et al., 2009).

RESULTS

In the time between colony founding and worker emergence, colony mortality was very high, with only 17 associations surviving. Of these associations, 12 exhibited distinct behavioral biases, and 5 associations showed no bias. None of these associations were clearly biased before colony reset.

JH levels were significantly different between the groups (Kruskal-Wallis ANOVA, $p<0.001$), with JH elevated in foraging-biased compared to both nest-biased (Mann-Whitney U test, $N_{forager}=12$, $N_{nest}=12$, $p<0.001$) and unbiased (Mann-Whitney U test, $N_{forager}=12$, $N_{unbiased}=10$, $p<0.001$; Fig. 5.1) queens. The JH content of foraging-biased queens was 21.8 fold higher than their nest-biased counterparts. There were no significant differences in JH content between nest-biased and unbiased queens (Mann-Whitney U test, $N_{nest}=12$, $N_{unbiased}=12$, $p>0.05$, Fig. 5.1).

DISCUSSION

Removal of the workers and destruction of the nest was successful in 'resetting' *P. californicus* queens from the established stage back to the founding stage. For at least some time after worker emergence, queens are able to respond to changes in colony environment and reinitiate founding behaviors. This type of regressive behavioral plasticity is well-described in the worker caste of many ant species, but has not been previously described in queens. In general, ant workers change their task performance with age, switching from nest tasks to foraging as they get older. However, changes in the social environment can force workers to deviate from this pattern (reviewed in Hölldobler and Wilson, 1990, 2009). In *P. californicus* workers, for example, colonies with made up exclusively of young, same-aged individuals still develop a division of labor, even though many workers initiate foraging much earlier than they would under normal colony circumstances (Dolezal et al., 2012). This level of plasticity gives workers the ability to change their behaviors in response to the changing needs of the colony, in a dynamic environment (Hölldobler and Wilson, 2009). The ability to respond flexibly to changes in colony caste composition likely has a similar selective benefit for queens. The small number of workers produced in the initial clutch (Johnson, 2004) puts the colony at risk for losing a significant portion of the labor force to environmental hazards. Queens may not be able to afford to lose their capacity to contribute towards securing colony resources, although their ability to revert may diminish as they continue to age and become increasingly reliant on the help of their offspring.

Consistent with our previous findings that JH content is correlated with the division of labor in both founding queens (Dolezal et al., 2009) and workers (Dolezal et al., 2012), a foraging bias was found to be linked to elevated JH. However, nonbiased queens also foraged, even in the absence of this hormonal stimulus, indicating that high JH levels are not required for foraging behaviors to occur. A similar association is found in honey bees, where elevated JH levels are not required for foraging onset (Huang and Robinson, 1995; Sullivan et al., 2000), but may reinforce behavior to increase foraging propensity (Amdam and Omholt, 2003)..

Comparison of the reset queens with previously described normal founding queens (Dolezal et al., 2009) showed substantial fold differences in JH content between foraging- and nest-biased queens. In normal founding associations, foraging queens exhibited mean JH levels 3.37 fold higher than nest-biased queens (Dolezal et al., 2009); in reset associations, however, forager mean JH content was 21.8 fold higher than that of nest-biased queens. This finding may suggest a mechanism by which JH could regulate the transition of queens from the behaviors associated with a colony's founding stage to those of the established stage. One possibility is that changes in JH sensitivity occur after worker emergence, possibly due to changes in JH receptor levels. JH sensitivity can differ based on genotypic differences in honey bees (Giray et al., 1999). Furthermore, fire ant queens go through dramatic changes in how JH effects them during different life stages (Brent and Vargo, 2003), showing that the role of JH can differ within the life of a single individual.

If elevated JH levels are important in biasing *P. californicus* queens towards foraging, then it is plausible that changes in JH sensitivity could have been selected upon as part of a mechanism that prevents queens from leaving the nest after worker emergence. One means by which changes in JH sensitivity may be achieved is the downregulation of a JH receptor, such as the candidate JH receptor *ultraspiracle* (Riddiford, 2008). In honey bee workers, inducing such a reduction can, under some circumstances, result in dramatically increased JH levels during the transition from nursing to foraging behavior compared to unmanipulated workers (Y. Wang, unpublished data). This is similar to the disproportionate increase we observed in reset foraging queens. As queens develop from founding to a stage where workers are available to forage, JH receptor levels in queens, and thus JH sensitivity, could decrease, reducing the queens' susceptibility to a JH stimulus that could cause foraging behavior. This change would protect queens during the established stage, when enhanced rates of egg production may be driven by elevated JH titers (Raikhel et al., 2005), from leaving the nest to forage. When worker removal occurs, acting as a stimulus for queens to reinitiate founding behaviors, JH sensitivity would still be low, necessitating an increase in JH that greatly exceeds the level needed during the original founding phase to induce a strong bias towards foraging.

In summary, *P. californicus* queens appear to exhibit a system of behavioral development not unlike social insect workers. Their behavioral ontogeny follows a trajectory that starts with colony founding, followed by foraging and brood care, and terminating in a behavioral stage where they

dedicate their resources solely to egg-laying. However, like workers, the queens can respond to changes in their environment, returning to earlier task stages when necessary. The ability to react to a dynamic environment is likely adaptive for queens, since the loss of the first clutch of workers is always a possibility. Furthermore, we show that JH content is related to the behavioral differences observed in queens after colony reset, reinforcing the argument that this hormone is involved in the development of foraging biases. While it remains unclear whether JH plays a direct causal role in queen behavioral development, our results suggest that changes in JH sensitivity may have a modulatory role.

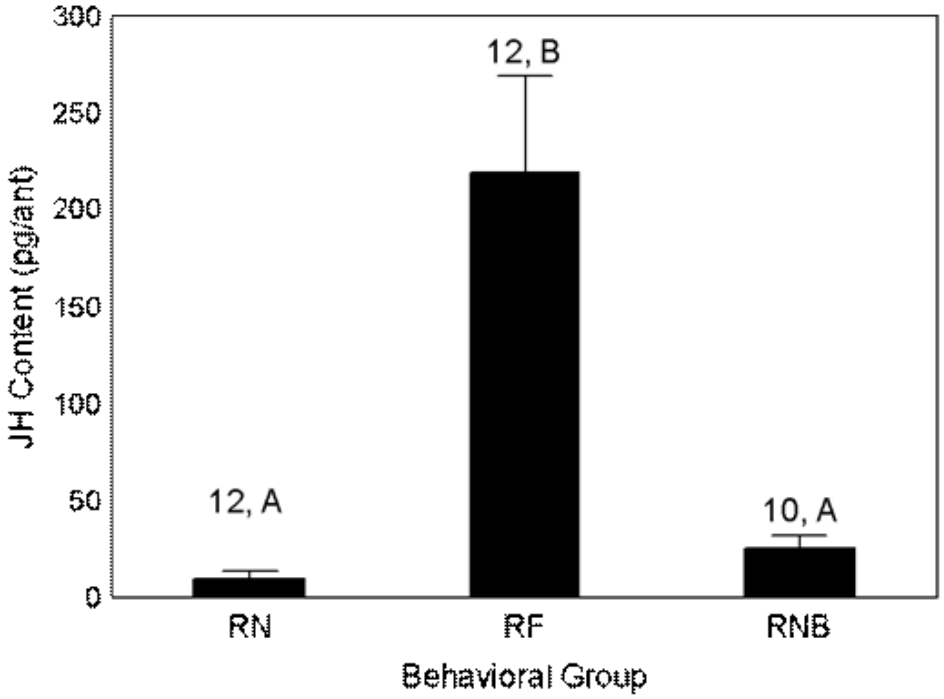

Figure 5.1: JH content of *P. californicus* queens after colony reset. Mean +/- SE JH content (pg/ant) of cofounding *P. californicus* queens from different behavioral groups after being reset to the nest-founding stage: nest-biased (RN), foraging-biased (RF) and nonbiased (RNB). Letters denote significant (Mann-Whitney U test, $p<0.05$) differences between the groups. Sample sizes are indicated.

Chapter 6

EFFECTS OF RAPAMYCIN TREATMENT ON TASK PREFERENCE IN *POGONOMYRMEX CALIFORNICUS*

ABSTRACT

Nutrient-sensing pathways like Target of Rapamycin (TOR) have an array of important effects on eukaryotic life history regulation. In eusocial insects, many life history traits are regulated by social interactions between colony members. This suggests that changes in TOR signaling could be involved in the regulation of social phenotypes, including the behavioral division of labor. Since one effect of TOR signaling is on reproductive physiology, we focus on a system where individuals have an active reproductive system and exhibit discrete division of labor: associations of *Pogonomyrmex californicus* queens that can develop a division of labor between nest- and foraging-bias during colony founding. When queens are treated with the pharmacological TOR inhibitor rapamycin before colony founding, they develop a foraging bias significantly more often than expected, indicating that the TOR pathway may be involved in the development of behavioral biases in these queens. This result supports the hypothesis that nutrient-sensing may be important in regulating the division of labor among social insects.

INTRODUCTION

Some nutrient-sensing pathways, like Target of Rapamycin (TOR) signaling, are conserved throughout eukaryotes and contribute to essential patterns of life histories, including growth, development and reproduction (Oldham and Hafen, 2003). In most social insect species, many of these processes are under some level of social control; that is, they are regulated by interactions between colony members (Hölldobler and Wilson, 2009). This suggests that pathways like TOR signaling could be adapted to sculpt social phenotypes, including behaviors. One of the major behavioral features in social species is some form of division of labor. This is most notable in the eusocial insects, including honey bees and ants. In these groups, behavior differs among the members of the worker caste, which allows groups of individuals to increase efficiency *via* task specialization (e.g.: larval care vs. foraging), thereby enhancing their ecological success. However, the regulatory mechanisms underlying this division of labor are not fully understood (Wilson, 1971; Hölldobler and Wilson, 2009).

TOR signaling regulates many important physiological effects in insects, including reproductive activity. In the German cockroach, downregulation of TOR signaling results in decreased production of the gonadotropic juvenile hormone (JH), leading to downregulation of yolk protein synthesis and inactivation of the ovary (Maestro et al., 2009), and TOR signaling is a key component of yolk protein synthesis is mosquitoes (Hansen et al., 2004). In fruit flies, TOR inhibition leads to egg destruction within the adult ovary (Thomson

and Johnson, 2010). Both JH and ovarian physiology also play important roles in social insect behavior, with effects on foraging onset and collection preferences (Hölldobler and Wilson, 2009; Amdam and Page, 2010). While the role of TOR has been studied in regard to honey bee division of labor, the results are inconclusive so far (Ament et al., 2008). If TOR signaling predominantly affects adult ovarian activity, as it appears to in cockroach and fruit fly adults, it may be difficult to ascertain its function in honey bee workers which have comparatively undeveloped ovaries. While differences in reproductive physiology do affect honey bee behavioral preferences, these are predominantly based on differences between individual bees' physiology at adult emergence (e.g., ovariole number, a static physiological component) (Amdam and Page, 2010). As adults, changes in reproductive activity are small (Winston, 1987), which may make TOR-mediated behavioral changes difficult to observe.

Therefore, it is desirable to study the role of TOR in modulating social behavior using a species that exhibits both reproductive activity and behavioral division of labor in the same individual, unlike in honey bees in which these are functions are performed separately by queens and workers, respectively (Winston, 1987). One such species is *Pogonomyrmex californicus*, which exhibits behavioral division of labor in the sterile worker caste as well as in reproductive founding queens (Johnson, 2002; 2004). Unlike honey bees and many ant species, newly-mated *P. californicus* queens must both forage for provisions and care for larvae prior to the emergence of their first brood. Furthermore, in some populations, queens can found colonies cooperatively (Johnson, 2002;, 2004;

Dolezal et al., 2009), and within these multiqueen groups, behavioral biases can emerge where one queen performs nest tasks and the other forages (Dolezal et al., 2009). These behavioral partitioning parallels that observed in workers (Hölldobler and Wilson, 2009; Dolezal et al., 2009; 2012), and thus provides an opportunity to compare behavioral physiology in both reproductives (queens) and non-reproductives (workers) within the same species. In both castes, the transition from nest tasks to foraging appears to be associated with reproductive hormones and ovarian physiology (Dolezal et al., 2009; 2012; unpublished data), suggesting a shared underlying regulatory network adapted fro multiple purposes.

Because of these unique colony and caste dynamics, these queens represent a rare opportunity to study the relationship between division of labor and TOR signaling in a context where such relationships should be more readily defined and differentiated. If TOR signaling affects task performance, then artificial downregulation of this pathway will result in predictable behavioral changes. Since blocking the TOR pathway prevents oocyte maturation in cockroaches (Maestro et al., 2009) and *Drosophila* (Thomson and Johnson, 2010), and lower oocyte production is associated with foraging bias in queens (see Chapter 5), we predict that perturbation of this pathway will make queens more likely to specialize on foraging.

Here, we use rapamycin/FK506 pharmacology to examine this relationship. Rapamycin (RAP) and FK506 are structural analogs that compete for the same binding protein; however, only the RAP-protein complex blocks the TOR pathway. Therefore, RAP action can be antagonized by the presence of

FK506 as a competitive inhibitor (Hansen et al., 2004). Our use of RAP/FK506 shows that RAP treatment strongly biases queens towards both developing a division of labor and becoming a foraging specialist. This suggests that the TOR pathway may be an important regulator of queen division of labor, possibly *via* interactions with the reproductive system and brain.

METHODS

Queen collections

P. californicus founding queens were collected in San Diego County, CA, USA in July 2010 and brought to the laboratory (constant 28° C, natural photoperiod). They were then housed in glass test tubes filled with 2mL of water that was stopped up with a clean cotton ball. The queens were enclosed inside the tubes with another cotton ball at the end of the chamber. One of the five treatments described below was dissolved in the water. Queens were housed with access to this water for 3 days, after which they were marked using a paint pen (Sharpie: Oak Brook, IL, USA), with colors coded to their treatment.

Pharmacology

RAP and FK506 (L.C. Laboratories, Woburn, MA, USA) were both dissolved in 95% ethanol and then diluted in water to 2% ethanol vehicle. This resulted in a final RAP concentration of 200µg/ml. To ensure effective competitive inhibition, FK506 final concentration was 1000µg/ml. Queens were treated with one of five treatments, presented as 2ml of solution inside their test

tube chamber: untreated water control, 2% ethanol sham, FK506, FK506+RAP (competitive inhibitor control of RAP), or RAP.

Queen pairings and observations

Next, queens were paired such that each queen that had received water (untreated control) was co-housed with a queen that received one of the four pharmacological treatments. Pairs (Sham; N=57, FK506; N=65, FK506+RAP; n=67, RAP; N=77) were introduced into soil-filled glass jars, where they could be easily observed for foraging biases (Dolezal et al., 2009). Data collection was performed blind to prevent observer bias; i.e., the observer was unaware of what treatments the color markings represented. The soil substrate was watered as needed, and the queen associations were fed a restricted quantity of Kentucky blue grass seeds. Queens were observed for 15 minute intervals four times per day for 15 days to identify behavioral bias; foraging was recorded when queens were observed outside of the nest entrance searching for or handling seeds. Associations were classified as having a division of labor if 10 or more foraging events were observed and more than 80% of those events were performed by one queen. The queen performing the majority of the foraging tasks was categorized as foraging-biased, while the one performing the minority was categorized as nest-biased.

RESULTS

In *P. californicus* queen associations where one queen was treated with FK506 (N=65) or FK506+RAP (N=70), a division of labor occurred as often as

expected, using the sham control (N=67) as an expected value (Chi-squared test , $p>0.05$). In associations where the treated queen received RAP (N=77), a division of labor occurred significantly more often that would be expected (Chi-squared test, $p<0.05$; Fig. 6.1). When associations did exhibit a division of labor, the incidences of foraging bias in queens treated with sham (N=16), FK506 (N=15), or FK506+RAP (N=13) did not differ from a 50/50 expected distribution (Chi-squared test, $p>0.05$). However, RAP (N=35) treated queens became foragers significantly more often than expected (Chi-squared test, $p<0.05$; Fig. 6.2)

DISCUSSION

The TOR pathway has an array of effects on growth, development, and reproduction (Oldham and Hafen, 2003), but its role in adult social insect behavior is poorly understood (Ament et al., 2008). Our findings show that exposure to RAP, a pharmacological agent that inhibits TOR signaling, results in *P. californicus* queens developing a foraging bias more often than expected. While we did not quantify *TOR* expression, the strong bias caused by RAP but not by RAP mixed with a competitive inhibitor (FK506), suggests that RAP affected the TOR pathway as it does in other insect species (Hansen et al., 2004; Patel et al., 2007). This finding also suggests that the TOR pathway may play an important role in the development of behavioral biases in these queens.

In adult *Drosophila*, perturbation of the TOR pathway causes mid-stage oocytes to be destroyed (Thomson and Johnson, 2010), and decreased ovarian activity is also linked to foraging bias in *P. californicus* queens (Dolezal,

unpublished data). Since ovarian activity affects a host of systemic factors in insects (Klowden, 1997; Raikhel, et al., 2005; Flatt et al., 2008), a relationship between TOR signaling and reproductive physiology could contribute to the effect of RAP treatment on *P. californicus* queen behavior. If a decrease in ovarian activity is involved in biasing queens towards foraging, it is possible that RAP treatment could negatively affect ovarian activity, changing signals produced there, and lead to the high incidences of foraging bias observed in this study.

These behavioral changes could also be linked to differences in how individual queens sense and respond to resource scarcity. In *Drosophila,* adults respond to starvation by downregulating ovarian activity (Pritchett et al., 2009) and entering a stage of hyperactivity where they are more active in searching for food (Lee and Park, 2004). Perturbation of the TOR pathway causes starvation-like responses in *Drosophila* (Oldham and Hafen, 2003; Pritchett and McCall, 2012) and larval honey bees (Patel et al., 2007; Kamakura, 2011; Mutti et al., 2011) even in the presence of high quality food, implying that TOR is important in the ability to correctly sense the nutritional environment. Therefore, if queen foraging bias is stimulated by their sensation of nutritional resource scarcity, then RAP treatment could prevent them from sensing nutritional signals, mimicking starvation, and stimulating the development of a foraging bias. When *P. californicus* queens first establish a nest, the internal resource used in producing the initial clutch of eggs may delete the queens reserves to a threshold level that triggers the cessation of oogeneis and promotes foraging. In an association of foundresses, one queen may deplete her reserves sooner or have a different

activational threshold causing her to forager sooner. By doing so, the forager brings food in, preventing the other queen from sensing resource scarcity and keeping her nest-biased, leading to the division of labor we observe.

These types of changes could also be linked to TOR effects on the brain. In mammals, TOR may regulate brain control of energy balance (Cota, 2009), and there is also evidence for nutrient-sensing effects on the brain in *Drosophila* (Jacinto and Hall, 2003). Nutrient-sensing can also act *via* the brain in mosquitoes to regulate reproductive physiology (Gulia-Nuss et al., 2011). Both ants and bees show quantifiable changes in brain anatomy that correlate with complex behavior (Whitfield et al., 2003; Gronenberg et al., 1996;1999; Zube and Rössler, 2008), though causal connections with TOR have not been investigated. Thereby, sensation of the nutritional environment could signal changes in the brain *via* the TOR pathway, likely through interactions with other components in the ovary and fat body, resulting in a cascade of effects that can lead to *P. californicus* queen behavioral biases.

It is also important to note that the *P. californicus* queens in our study were not receiving pharmacological treatments throughout the behavioral observations, but only in the 3 days prior to colony foundation. In rats, the half-life of RAP after administration to the organism is approximately 24 hours (Crowe et al., 1999), so it is unlikely that significant levels of RAP were persisting inside founding queens for long after the cessation of treatment (15 days). Therefore, early RAP treatment is sufficient to cause a foraging bias to

develop, pushing queens into a foraging-biased behavioral trajectory that continues long after the RAP stimulus has diminished or ceased entirely.

In conclusion, treatment of *P. californicus* queens with RAP has clear behavioral effects, causing queens to develop a foraging bias significantly more often than would normally be expected. While this suggests that TOR signaling may be an important factor in the behavioral development of these ants, it is unclear how changes in this pathway interact with other physiological systems involved in the division of labor. Further research can investigate how perturbation of the TOR pathway affects these systems, and how these effects compare with the patterns found in queens that develop behavioral biases under control conditions, allowing for a better understanding of how nutrient-sensing could play a role in social organization and behavior.

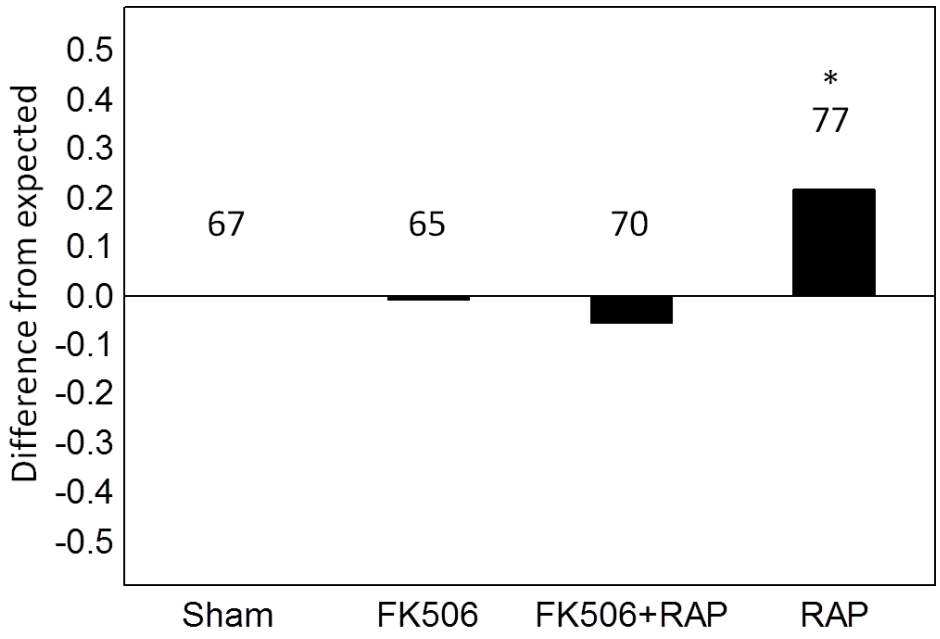

Figure 6.1: Incidence of division of labor in pharmacologically treated *P. californicus* founding queens. Asterisks denote significant differences from an expected distribution (Chi-squared test, $p<0.05$) and sample sizes are indicated. Associations with a sham-treated individual are treated as the expected proportion of associations in which a division of labor developed, and bars show proportional difference from this expected proportion; positive values indicate a division of labor is more common, negative values less common. Within paired associations, RAP-treated queens were involved in a division of labor more often than sham treated queens.

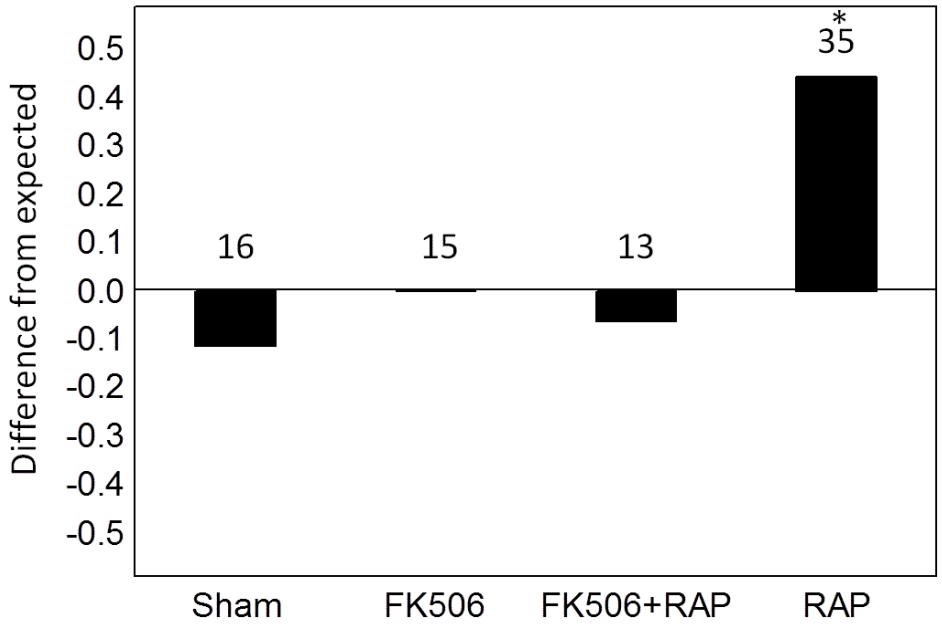

Figure 6.2: Incidence of foraging biases in pharmacologically treated *P. californicus* founding queens. Asterisks denote significant differences from an expected distribution (Chi-squared test, $p<0.05$) and sample sizes are indicated. The expected proportion of foraging bias was 50/50: either queen equally likely to become a forager. Bars show proportional difference from this expected proportion; positive values indicate a division of labor is more common, negative values less common. Within colonies with a clear division of labor, RAP treated queens become foraging-biased more often than expected from a 50/50 behavioral distribution.

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