

Articles

Endocrine physiology of the division of labour in *Pogonomyrmex californicus* founding queens

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

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A major factor leading to the ecological success of social insects is the evolution of a system of division of labour (Wilson 1971). Although many studies have explored this aspect of social organization, it is unclear how complex labour 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 labour 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 affectors of social behaviour (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 & Vargo 1997; Page & Amdam 2007), but one idea receiving increasing support is that complex social phenotypes emerged through co-option of gene

networks and endocrine signalling 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 behaviour are ecdysteroids and juvenile hormones (JH). In adult females, ecdysteroids are primarily produced in the ovary (Lafont et al. 2005). Changes in circulating ecdysteroid titres 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 labour. In the primitively eusocial bumblebee, *Bombus terrestris*, differences in ecdysteroid titre appear to be linked to reproductive and social status (Bloch et al. 2000b). A similar positive correlation of reproductive ranking with ecdysteroid titre is also found in the queenless ant *Streblognathus peetersi* (Brent et al. 2006). However, this correlation does not hold for all social insects. For example, in adult honeybees, ecdysteroid titres 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 behaviour and reproduction in the solitary *Drosophila melanogaster* (Flatt et al. 2005) and *Aedes aegypti* (Klowden 1997), as well as in some social

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insects. JH influences ovarian physiology in queens and guarding behaviour in workers of a primitive eusocial wasp (*Polistes canadensis*) (Giray et al. 2005). It affects queen maturation and reproduction in *Solenopsis invicta* (Brent & Vargo 2003), is correlated with reproductive status in the queenless ant species *Diacamma* (Sommer et al. 1993) and *Streblognathus peetersi* (Brent et al. 2006), and JH titre 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 & Hartfelder 1998), and it is correlated with the onset of foraging behaviour 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 behavioural processes pertaining to division of labour. To better understand how this social complexity might evolve, it is necessary to take a comparative approach, examining the proximate mechanisms regulating behaviour 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 behaviours 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 semiclastral 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 (nonforaging) to field-biased (foraging) behaviour (Johnson 2002), which mimics the behavioural 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 multiqueen associations (pleometrosis) (Johnson 2004; R. Overson, unpublished data). Under pleometrotic conditions, behavioural biases can emerge between queens, so that one primarily performs nest tasks and the other forages. This variation in the behaviour of these founding queens may be produced by differences in endocrine activity.

Here, we study changes in titres of ecdysteroids and JH coinciding with (1) the behavioural progression of single-founding *P. californicus* queens and (2) the behavioural biases shown by cofounding *P. californicus* queens. If the partitioning of labour within a nest results from the exploitation of these endocrine networks, we predicted that in-nest tasks and foraging behaviours 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 labour between cofoundresses.

METHODS

Single-founding (Haplometrotic) Queen Collection and Observation

We collected *P. californicus* founding queens during and directly after their yearly mating flights in July 2006 in San Diego County, California, U.S.A. Queens were collected from two behaviourally and geographically discrete populations in which new queens founded colonies either by themselves (haplometrosis) or with one or more cofounders (pleometrosis). All were kept under laboratory

conditions for the duration of the experiment (constant 28 °C, natural photoperiod). Haplometrotic queens were kept in plastic nestboxes 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 nestbox, 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 (R. Overson, unpublished data) indicated that 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 behaviour was monitored daily for 15 days following oviposition.

Cofounding (Pleometrotic) Queens

Although pleometrotic queens are willing to cofound new nests, each is still fully capable of individual colony founding, showing the same polyethism observed in haplometrotic queens. To determine whether the behavioural biases that can occur between cofounding queens are associated with endocrine changes similar to those associated with the behavioural 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. We used 390 queens 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 behaviour of paired queens for 15 min intervals, four times per day for 15 days. During each observation, we noted when a queen was outside of the nest arena foraging for seeds. Once there were at least 10 observed foraging events, the queen pairings were categorized as being either behaviourally biased or nonbiased. 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. Nonbiased associations, of which there were 14, were those in which each queen performed approximately 50% of the foraging. Any pairing that failed to meet our strict criteria for categorization as biased or nonbiased was not used for hormonal analysis.

Sampling and Hormone Assay

Because of limited availability, only one single-founding queen was used for each hormone titration. Cofounding queens were available in greater number; therefore, two individuals from the same behavioural 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, 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 & Vargo 2003). The methanol layer was lyophilized, resuspended in 250 µl of methanol and stored at –80 °C until analysis. Duplicate 10 µl aliquots of the methanol section of each sample

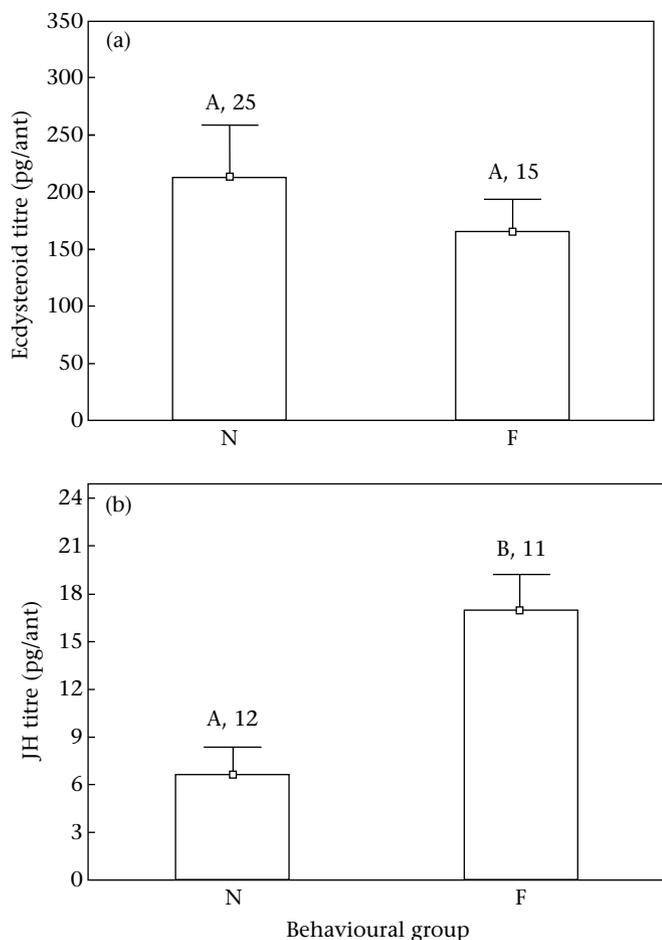


Figure 1. Mean \pm SE titre (pg/ant) of (a) ecdysteroids and (b) juvenile hormone (JH) of single-founding (haplometrotic) queens during nest-biased (nonforaging) (N) and field-biased (foraging) (F) stages. Letters denote significant differences between the groups. Sample sizes are indicated.

were incubated overnight with 100 μ l of (3H)-20-hydroxyecdysone stock (1 mg/ml, NEN) in borate buffer, and 100 μ l of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH) at 4 °C 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 & Gilbert 1986). To minimize intra- and interassay variability, new standard competition curves were generated for each set of samples run, using 20-hydroxyecdysone stock (Sigma, St Louis, MO, U.S.A.) in quantities of 15.6–2000 pg, a range that was well within the detection limits. After 18 h, 20 μ l of cleaned protein A solution (Pansorbin; CalBiochem, San Diego, CA, U.S.A.) 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 μ l 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 titre JH using the gas chromatography/mass spectrometry (GC-MS) method of Bergot et al. (1981) as modified by Shu et al. (1997) and Brent & Vargo (2003). The homogenized samples were eluted through aluminium oxide columns with hexane, 10% ethyl ether–hexane and 30% ethyl ether–hexane. The sample was then suspended and derivatized in

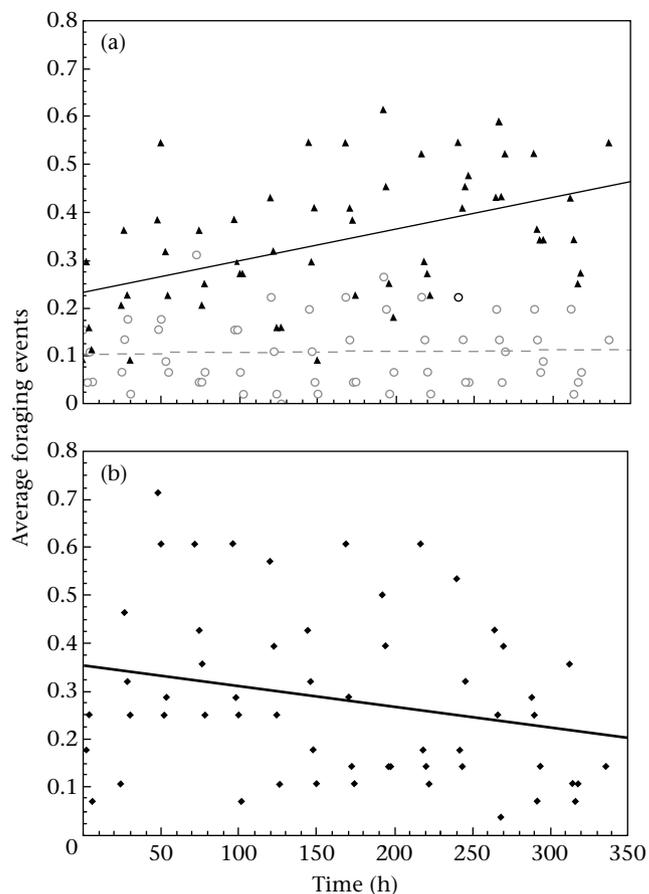


Figure 2. Average foraging events over time for (a) field-biased (solid black trendline, black triangles) and nest-biased (dotted trendline, open circles) queens and (b) queens with no observed behavioural bias.

a methyl-d alcohol and trifluoroacetic acid solution. The derivatized sample was resuspended in hexane and again eluted through aluminium oxide columns; 30% ethyl ether was used to remove nonderivatized 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 analysed using an HP 6890 Series GC (Hewlett Packard, Palo Alto, CA, U.S.A.) equipped with a 30 m \times 0.25 mm Carbowax Econo-Cap GC column (Alltech, Fresno, CA, U.S.A.) coupled to an HP 5973N inert mass selective detector/detection software (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 analysed 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 pg.

Statistical Analysis

Because of a general lack of a normally distributed data, we used Mann–Whitney *U* tests to test for ecdysteroid and JH titre differences between the single-founding nest-biased and field-biased queens and between cofounding nest-biased and field-biased queens. Significance values were adjusted by Dunn's methods to

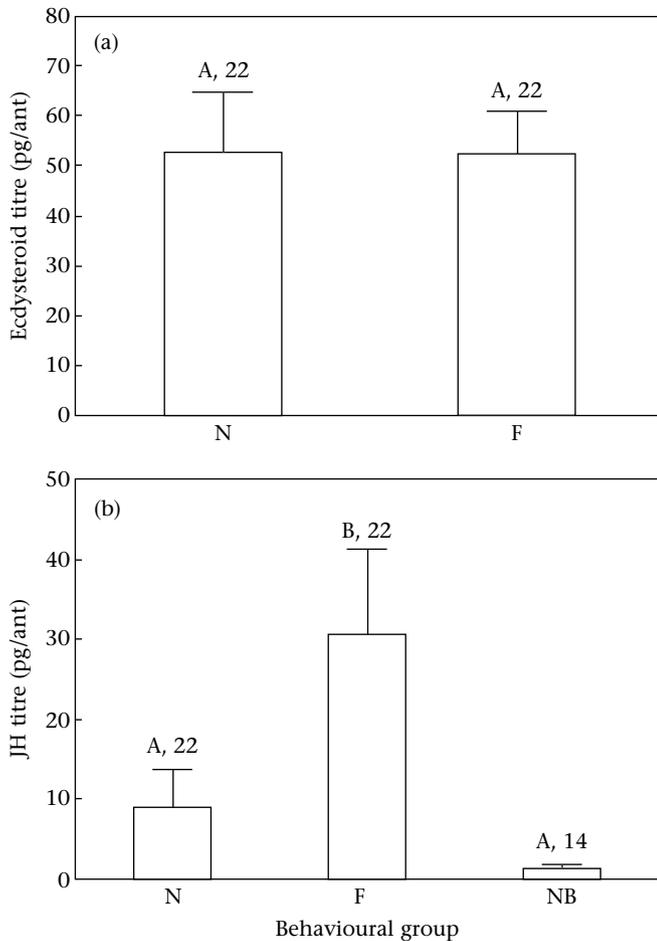


Figure 3. Mean \pm SE titres (pg/ant) of (a) ecdysteroids and (b) juvenile hormone (JH) in cofounding (pleometrotic) queens from different behavioural groups: nest-biased (nonforaging) (N), field-biased (foraging) (F) and nonbiased (NB). Letters denote significant differences between the groups. Sample sizes are indicated.

compensate for the multiple comparisons between the three behavioural groups of queens from pleometrotic associations. All analyses were performed using SigmaPlot version 11.0 (SYSTAT Software, Inc., San Jose, CA, U.S.A.).

RESULTS

Single-founding (*Haplometrotic*) Queens

There were no differences in ecdysteroid titre between queens in the nest-biased (nonforaging) and field-biased (foraging) stages (Mann–Whitney U test: $U = 185$, $N_1 = 25$, $N_2 = 15$, $P = 0.94$; Fig. 1a). This finding is similar to results from the honeybee, where ecdysteroid titres remain constant in queens and in workers going through the behavioural 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 titres. The concentration of JH was three times higher in the field-biased queens than in nest-biased queens (Mann–Whitney U test: $U = 19$, $N_1 = 11$, $N_2 = 12$, $P = 0.0038$; Fig. 1b). These results led us to predict that cofounding queens that partitioned labour between in-nest tasks and foraging would have similar ecdysteroid titres but different JH levels, with JH being elevated in the foraging queens.

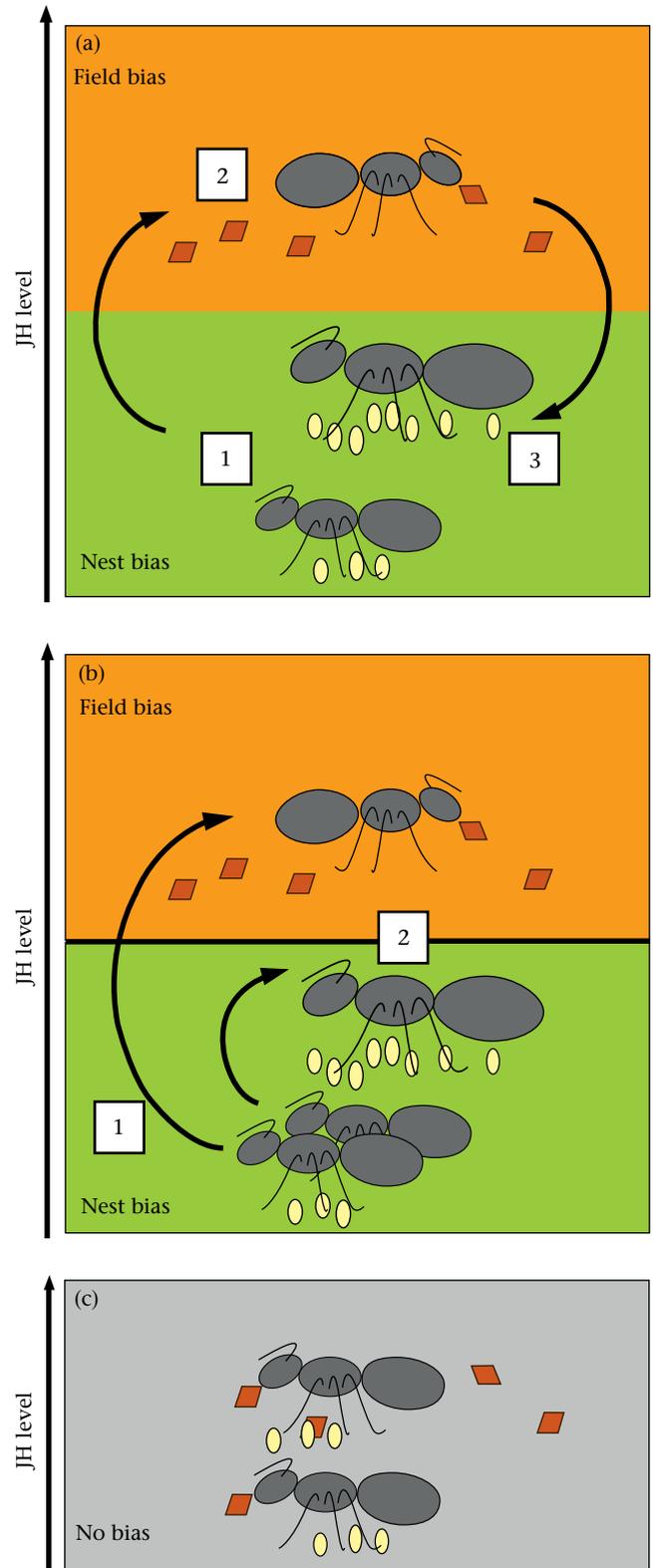


Figure 4. The hypothesized relationship between juvenile hormone (JH) and the behavioural bias of *P. californicus* queens. An increased JH titre does not cause the onset of foraging behaviour, but biases behaviour towards foraging/outside-nest activities. (a) Single-founding queen: the newly mated queen (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

Cofounding (Pleometrotic) Queens

Our observations of behaviour showed that among cofounding 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. 2a). As predicted from the results for haplometrotically founding queens, nest- and field-biased pleometrotic queens did not have divergent ecdysteroid titres (Mann–Whitney U test: $U = 381$, $N_1 = N_2 = 22$, $P = 0.85$; Fig. 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. 3b), which was comparable to the mean titre 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 cofounding queens were of similar age, we can discount the possibility that the link between JH titre and behaviour emerged simply because these traits co-occur independently as a consequence of increasing chronological age.

Queens from cofounding associations where no behavioural bias occurred showed decreasing foraging activity over time (Fig. 2b), but the frequency of activity remained within the same general range as that observed for the field-biased queens (Fig. 2a). Furthermore, despite foraging more frequently at a higher rate than nest-biased queens, a post hoc test showed that the queens in associations that did not partition labour ($N = 14$) had JH titres comparable to those 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. 3b). These results suggest that a high JH titre may bias *P. californicus* behaviour towards tasks in the field, but it is not required for the onset of foraging behaviour.

DISCUSSION

We found that the ecdysteroid titre was not significantly correlated with foraging behaviour 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). *Pogonomyrmex 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 found a robust association between JH titre and behavioural biases in founding queens. Nest bias was linked to a low JH level, and field-bias was linked to a high JH level. This association between hormone expression and behavioural bias was independent of age, as it occurred during the sequential behavioural development of single foundresses (Fig. 4a) as well as during the concurrent division of labour of similarly aged cofounding queens (Fig. 4b). Yet, when behaviourally biased queens were compared to cofounding queens that did not show bias, it became clear that JH itself did not cause foraging behaviour. Queens without behavioural biases showed little within-group variation in JH, and thus, there was no discernable difference between individuals in terms of observed

behaviour or endocrine status (Fig. 4c). We conclude that JH is probably one component of a regulatory system that can establish a behavioural bias in queens of *P. californicus*, but this hormone is not required for the performance of foraging.

A very similar conclusion has been reached for the regulation of foraging behaviour in honeybee workers. Foraging workers have elevated JH titres (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 honeybees, and possibly in *P. californicus*, is the expression of vitellogenin, a yolk precursor protein. As vitellogenin titres decline in maturing honeybee workers (Engels & Fahrenhorst 1974), both foraging behaviour and JH synthesis increase (Guidugli et al. 2005; Nelson et al. 2007). JH may reinforce the forager behavioural 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 & Omholt 2003). *Pogonomyrmex californicus* queens did not begin foraging until after their first clutch of eggs had been produced, which would normally coincide with a decline in vitellogenin production. They also ceased foraging around the time they began producing a second clutch of oocytes. This result suggests that the *P. californicus* queens may rely on the same reproductively linked double repressor mechanism as *A. mellifera* workers to regulate foraging behaviour.

The results also suggest that it is possible to develop divergent behavioural 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 may be the common route by which insect species have evolved greater social complexity (Amdam et al. 2004). If future research shows that the behaviour 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.

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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.

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