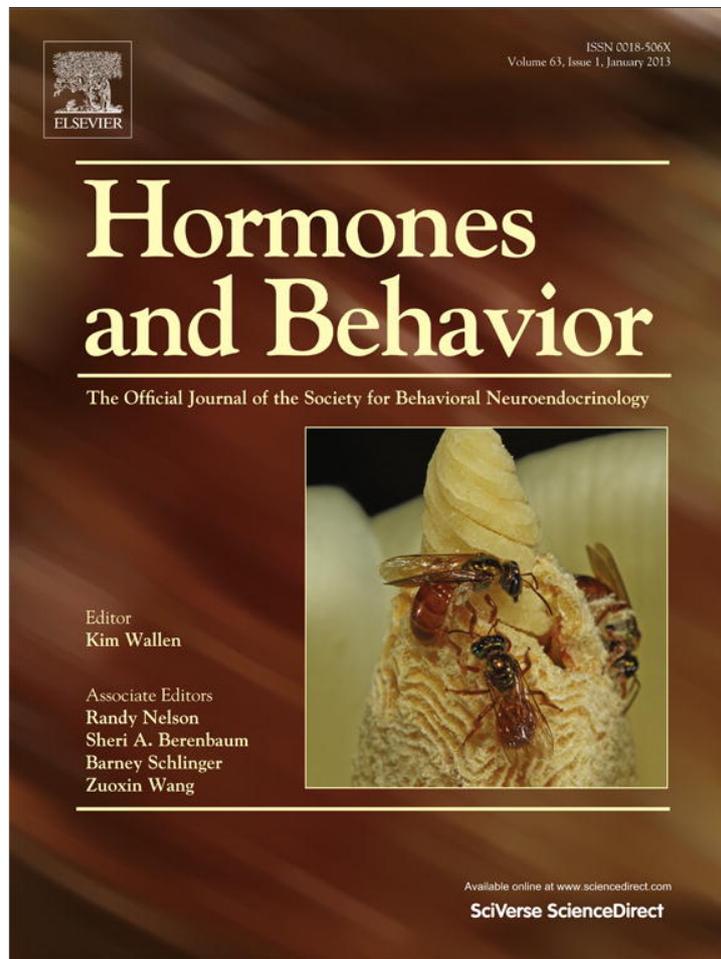


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Juvenile hormone levels reflect social opportunities in the facultatively eusocial sweat bee *Megalopta genalis* (Hymenoptera: Halictidae)

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ABSTRACT

The evolution of eusociality is hypothesized to have involved de-coupling parental care from reproduction mediated by changes in endocrine regulation. While data for obligately eusocial insects are consistent with this hypothesis, we lack information from species representative of the transition from solitary reproduction to eusociality. Here we report the first evidence for a link between endocrine processes and social behavior in a facultatively eusocial bee, *Megalopta genalis* (Halictidae). Using females that varied in social, reproductive, and ecological context, we measured juvenile hormone (JH), a major regulator of colony caste dynamics in other eusocial species. JH was low at adult emergence, but elevated after 10 days in all nesting females. Females reared in cages with ad lib nutrition, however, did not elevate JH levels after 10 days. All reproductive females had significantly more JH than all age-matched non-reproductive females, suggesting a gonadotropic function. Among females in established nests, JH was higher in queens than workers and solitary reproductives, suggesting a role for JH in social dominance. A lack of significant differences in JH between solitary reproductives and non-reproductive workers suggests that JH content reflects more than reproductive status. Our data support the hypothesis that endocrine modifications are involved in the evolutionary decoupling of reproductive and somatic effort in social insects. These are the first measurements of JH in a solitary-nesting hymenopteran, and the first to compare eusocial and solitary nesting individuals of the same species.

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Introduction

An important source of evolutionary novelty may be the rearrangement of existing regulatory and physiological processes (Gerhart and Kirschner, 1997; West-Eberhard, 2003). The evolution of eusocial insects (species with reproductive queens and sterile workers) from solitary ancestors provides an excellent example of this repurposing (West Eberhard, 1987). While solitary insects both reproduce and forage, eusociality requires de-coupling reproduction (the tasks of the queen phenotype) from parental care (the tasks of the worker phenotype). Juvenile hormone (JH) is one of the primary regulators of insect ovarian development and activity (Flatt et al., 2005; Hartfelder, 2000). In many social insects, JH also influences dominance status and division of labor (reviewed in Bloch et al., 2009; Hartfelder, 2000). Thus, West-Eberhard (1996, 2003)

hypothesized that JH contributes to regulating the switch between queen-like behaviors (reproduction) and worker-like behaviors (foraging) in solitary bees and wasps. Increasing or suppressing JH could lead to more consistently queen- or worker-like behavior, respectively, functioning as a mechanism for de-coupling reproduction from parental care, and thus promoting division of labor.

Studies of primitively eusocial insects (species without morphologically distinct behavioral castes), including the bumble bee *Bombus terrestris*, the paper wasps *Polistes dominulus*, *P. canadensis*, and *P. metricus*, and the halictid bee *Lasioglossum zephyrum*, generally demonstrate a role for JH in regulating division of labor. In these species, JH correlates with ovarian development and reproduction: queens have large ovaries and high JH titers, while workers have small, non-reproductive ovaries and low JH titers (Bell, 1973; Bloch et al., 2000; Giray et al., 2005; Röseler and Röseler, 1978; Röseler et al., 1980; Tibbetts and Izzo, 2009; Tibbetts and Sheehan, 2012; Tibbetts et al., 2011a). JH titers are also influenced by social context, nesting opportunity, and nutrition. In both *B. terrestris* and *P. dominulus*, worker JH titers increased following experimental removal of the queen, suggesting that queen dominance suppresses JH levels in nestmates (Bloch et al., 2000; Tibbetts and Huang, 2010). *L. zephyrum* females do not enlarge their

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ovaries without access to nesting substrate (Bell, 1973), and the effects of increased JH are greater in better-fed *P. dominulus* individuals (Tibbetts and Izzo, 2009). Together these findings generally support the hypothesis that the increased dominance and reproduction of queens is enabled by higher JH titers, and that queens suppress worker JH titers through aggressive interactions and/or control of nutrition. The role of JH in the evolutionary origins of queen and worker phenotypes is unknown, however, because JH has never been measured in a solitary-nesting bee or wasp to determine its influence on behavioral phenotype. Thus, it is impossible to evaluate how solitary regulatory mechanisms may have been modified to regulate social phenotypes without comparison to a solitary phenotype.

To fill this gap, we measured JH in a facultatively eusocial sweat bee, *Megalopta genalis* (Halictidae). *M. genalis* is not phylogenetically basal to *Bombus*, *Polistes*, or *Lasioglossum* (which represent independent origins of primitive eusociality in three different hymenopteran families). Rather, its significance is that a facultatively eusocial species permits direct comparison of solitary and social phenotypes in the same species, providing insights into factors that facilitated the evolutionary transition from solitary to social (Michener, 1985; Schwarz et al., 2007; Wcislo, 1997). We examined the role of JH in reproduction and dominance among females from both solitary nests (reproductive female without workers) and eusocial nests [reproductive queen with sterile worker(s)]. Based on the hypothesized link between reproduction and ovary development in solitary bees, we expected queens and solitary reproductive females to have higher JH than newly emerged adults with undeveloped ovaries. Additionally, we expected non-reproductive workers to have lower JH levels than their queens. We further compared these groups with females kept in the lab with adequate nutrition but without access to a nest, and with new nest foundresses, to measure the effect of ontogenetic, social, and ecological context on JH. If access to nutrition limits *M. genalis* JH levels independent of nesting context, then well-fed females in isolation should have high JH levels. If queen dominance limits JH levels, then females given access to nesting substrate without the presence of a queen should have elevated JH.

Methods

Natural history précis

M. genalis nests are initiated by a single female (Wcislo et al., 2004). These foundresses either become social queens or solitary reproductives. Eusocial nests form when a daughter remains at her natal nest as a subordinate non-reproductive worker that begins foraging 6–10 days after emergence. Workers and queens are readily distinguished through observation; the former do nearly all the foraging and the latter rarely leave the nest (Wcislo and Gonzalez, 2006). In a solitary nest, each offspring disperses approximately 6–10 days after eclosion (Kapheim, 2010; Kapheim et al., 2012) (ESM 1).

Observation nests and rearing conditions

All fieldwork was conducted on Barro Colorado Island (BCI), Panama (ESM 1). We collected bees from natural nests, and reared them under ambient conditions. When females emerged as adults, they were put into either cages or observation nests made of balsa wood between two sheets of plexiglass (ESM 2). Observation nests with newly emerged females were placed in the forest and monitored. Cage-reared bees were placed in circular 8 cm high \times 7 cm diameter plastic containers and fed ad lib a honey:water:soy-protein (45:45:10 by volume) powder mixture (following Ref. Kapheim et al., 2012).

Overview of behavioral groups

In total, we measured JH content in seven groups of *M. genalis* (Table 1). 1) *Workers* were collected from social observation nests

when 10 days old. We confirmed workers' status by marking nestmates and filming foraging flights. Only workers filmed returning from foraging trips were included in the study. 2) *Queens* were collected at the same time as the workers in their nests. 3) *Solitary reproductives* were collected from observation nests 10 days after their first offspring emerged, in order to match the treatment of the queens. These nests were not social because all offspring had dispersed. Average adult age at collection was 65.8 ± 5.8 SD days for queens and 65.8 ± 5.4 days for solitary females. 4) *Newly emerged bees* were collected the day after adult emergence. 5) *Ten-day old cage bees* were kept in social isolation for 10 days post-emergence with ad lib food, but no opportunity for nesting. 6) *Ten-day old observation nest foundresses* are those females we placed as singletons into empty observation nests when they emerged. We allowed them to initiate nesting, and then collected them when they were 10 days old. These females would have become solitary reproductives or social queens if left uncollected. 7) *Naturally dispersing foundresses* were females that had left their natal nests and initiated their own nests in sticks that had been regularly monitored for new nesting. They were collected within 4 days of nest initiation. The exact age of these bees is unknown, but they are likely ~10 days old, based on typical ages of females that disperse (Kapheim, 2010; Kapheim et al., 2012).

Reproduction assessment

At the time of collection, we noted the number and stage of each brood cell present in each nest for comparisons between queens and solitary reproductives (summed across pairs used in hormonal analysis – see below) with a Mann–Whitney U-test. All 10 day old observation nest foundresses and natural dispersers began nest construction [defined as building an entrance collar (Kapheim et al., 2012)], but had not laid an egg. We calculated a “nesting index” to quantify nest reproductive stage based on the following characteristics, summed for each pair used in hormonal analysis: nest collar only = 0, cell construction = 1, complete empty cell = 2, some pollen provisions = 3. We used this nesting index to compare reproductive stage between pairs of queens and solitary reproductives used for hormonal analysis with a 2×2 Fisher's exact test, based on whether or not either individual of a pair had a partially provisioned cell in their nest at the time of collection. We also used this nesting index to assess the relationship of JH and reproductive development among pairs of new nest foundresses with a Spearman's rank correlation.

JH extraction and analysis

Adult bees were collected between 26 February and 8 May 2010, at similar times of day, into individual glass vials containing 1 ml 50% acetonitrile, and stored at -80 °C until analysis. Whole-body extracts were used for JH extraction, and gas chromatography/mass spectrometry was used to determine JH content (following Brent and Vargo, 2003; Brent and Dolezal, 2009; Dolezal et al., 2009, 2012; Penick et al., 2011) (ESM 3). 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 MS SIM mode, monitoring at m/z 76 and 225 to ensure specificity for the d3-methoxyhydrin derivative of JHIII. Total abundance was quantified against a standard curve of derivatized JH III. We paired two randomly selected bees within each treatment group to extract enough JH for measurement. Sample sizes and values reported below refer to these pairs. We tested for differences between all groups with a Kruskal–Wallis test, followed by Conover post-hoc comparisons.

Results

There was significant variation in JH content among treatment groups (Kruskal–Wallis $\chi^2_6 = 40.21$, $p < 0.001$; Fig. 1). Queens had more JH than solitary reproductives (Conover post-hoc test; $p < 0.05$)

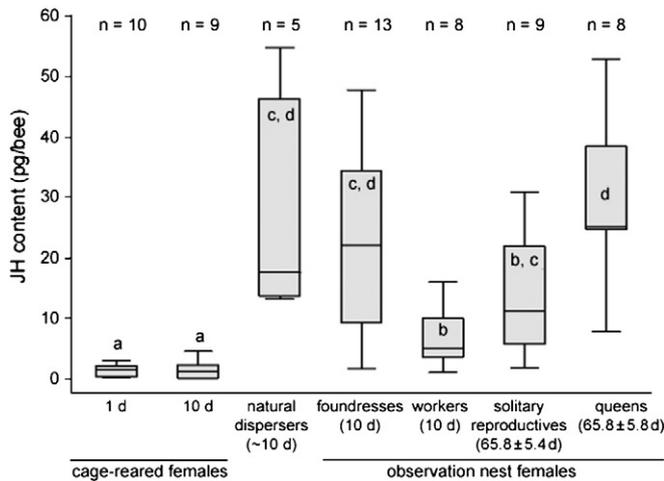


Fig. 1. Relationships of social status and rearing conditions with JH content. For each behavioral group boxes represent the interquartile range, with a line at the median value; whiskers reach the maximum and minimum values. Sample sizes are given above each box, and ages are indicated below each label (error estimates are standard deviations). Behavioral groups with different letters in the box were significantly different from other groups, while those with the same letter were not significantly different (Kruskal–Wallis test followed by a Conover post hoc test, $\alpha = 0.05$).

and workers ($p < 0.001$), but not the natural dispersers or observation nest foundresses. These latter comparisons may be confounded by greater age of the queens. JH content of solitary reproductives and workers did not significantly differ. Queens and solitary reproductives did not differ in the number of brood cells present at collection (mean \pm standard deviation summed by pairs = 5.29 ± 1.80 and 6.22 ± 2.33 , respectively; $Z = -0.27$, $n = 16$ pairs, $p = 0.79$), and both were equally likely to have been provisioning at least one brood cell at the time of collection (Fisher's exact test, $n = 16$ pairs, $p = 0.30$). The brood cell data indicate that the queens and solitary females sampled were in similar stages of the reproductive cycle.

The 10 day cage-reared bees showed no increase in JH content relative to the newly emerged bees. Among observation nest foundresses and natural dispersers, JH was positively correlated with nesting index (Spearman's $\rho = 0.58$, $n = 17$ pairs, $p = 0.015$). JH in these two groups did not significantly differ, but both showed significant increases relative to newly emerged bees (Conover post-hoc test; both $p < 0.001$). Workers had more JH than newly emerged bees ($p < 0.01$) and caged bees ($p < 0.01$), but less than observation nest foundresses ($p < 0.01$) and natural dispersers ($p < 0.001$).

Discussion

While JH has been repeatedly implicated in the evolution of insect eusociality (reviewed in Bloch et al., 2009; Hartfelder, 2000; Robinson and Vargo, 1997), this is the first study examining JH function in a facultatively eusocial species. Facultatively eusocial species may be more representative of the transition from solitary reproduction to eusociality than the obligately eusocial species used in previous studies because some females express solitary behavior. We tested the hypothesis that the expression of reproductive division of labor is associated with changes in levels of JH. Our data are consistent with this hypothesis, and provide additional support that ecological and social context modifies the link between JH and reproductive behavior (references in Introduction). Our data also suggest a potential role for developmental variation in shaping this association.

Consistent with a context-dependent interpretation, JH content in *M. genalis* was generally associated with reproductive opportunity. Individuals with access to nesting sites had more JH than similarly aged and well-fed cage-reared bees, and queens had more JH than non-reproductive workers. Workers can enlarge their ovaries and

reproduce when queens are removed or die (Kapheim, 2010; Smith et al., 2009), so JH levels may also respond to changes in social context, although this remains to be tested with removal studies. Moreover, JH content appeared to rise as *M. genalis* females approach egg-laying: JH was higher in foundresses than in newly eclosed adults, and was positively correlated with progress in establishing and provisioning a nest as measured by the nesting index. Likewise, paper wasp (*P. dominulus*) foundresses with more established nests had higher JH levels than those just beginning nest initiation (Tibbetts et al., 2011b).

JH levels did not significantly differ between solitary reproductives and non-reproductive workers, despite the differences in their reproductive activity and likely differences in ovary size, based on previous studies (Kapheim et al., 2012; Smith et al., 2008, 2009). This may simply reflect lack of statistical power: median JH level of solitary reproductives was higher than that of the workers, and our sample sizes were relatively low and within-group variability was high. The age-matched reproductive counterparts to the workers, the observation nest and natural nest foundresses, did have significantly higher JH levels, but had not yet laid eggs. In previous studies, these two foundress groups also had larger ovaries than workers (Kapheim et al., 2012).

Our data are consistent with an expanded role for JH beyond that of a gonadotrope. Social queens had higher JH levels than solitary reproductives, despite equal reproductive activity. This may result from queens increasing JH in order to maintain social dominance (Bloch et al., 2000; Röseler et al., 1984; Tibbetts and Huang, 2010). If true, this might explain both the higher JH levels of queens than solitary reproductives, despite equal reproductive activity, and the lack of a difference between solitary reproductives and workers, despite unequal reproductive activity. These JH patterns are mirrored by those involving ovary size: social queens on average had larger ovaries than both their subordinate females as well as solitary reproductives (Kapheim et al., 2012; Smith et al., 2009).

An alternative interpretation of the context-dependent JH levels is that JH levels are a predictor, rather than a consequence, of sociality, under the hypothesis that JH levels may predispose a foundress toward becoming a queen or remaining solitary. Our results revealed a great deal of variation in JH content among observation nest foundresses and natural dispersers, such that these groups were not significantly different from either queens or solitary reproductives. Some variation may result from developmental biases, such that those queens that would have

Table 1

Summary of study comparisons, interpretations, and group differences in JH. See Introduction and Results for details.

Paired comparison	Effect tested	JH difference?
Workers vs. observation nest foundresses	Queen social influence on reproductive opportunity	Yes
Queens vs. solitary reproductives	Social dominance	Yes
Newly emerged bees vs. observation nest foundresses and natural dispersers	Ontogenetic rise in JH with nesting opportunity	Yes
Newly emerged bees vs. workers	Ontogenetic rise in JH with queen presence	Yes
Newly emerged vs. cage bees	Ontogenetic rise in JH with nutrition but without queen or nesting opportunity	No
Workers vs. queens	Division of labor	Yes, but confounded by age
Workers vs. solitary reproductives	Non-reproductive vs. reproductive behaviors	No, but confounded by age
Observation nest foundresses and natural dispersers vs. queens and solitary reproductives	Initiating vs. maintaining reproduction	Equivocal

higher JH levels are better able to recruit workers (Kapheim, 2010; Kapheim et al., in review). Previous studies support this hypothesis by demonstrating that whether a foundress establishes a social or solitary nest may be the result of how well the bee was provisioned as a larva and the adult body size she achieved (Kapheim, 2010; Kapheim et al., 2012). Likewise, predisposition to a worker phenotype may result from inadequate larval nutrition, which would limit JH biosynthesis early in adulthood (Kapheim et al., 2011). The correlation between nesting stage and JH level further suggests that bees with elevated JH may initiate nesting sooner, which has been documented for a paper wasp, *Ropalidia marginata* (Agrahari and Gadagkar, 2003).

Our results provide the first empirical data to assess the role of JH in reproduction and social dominance in a species with facultatively eusocial behavior, enabling comparisons among socially dominant (queens) and subordinate (workers) bees, and those having a solitary life-style. These comparisons provide support for prior hypotheses that JH plays a significant role in decoupling reproductive and somatic behavior at a transitional cusp between solitary and social behavior, which is central to the evolution of eusociality. Manipulative studies of developmental conditions, coupled with detailed observations of aggressive interactions, can build on our study to test the relative role of pre- and post-imaginal influences on JH suppression among workers. Collectively such data will provide a full understanding of the mechanistic bases for decoupling the queen and worker phenotype, and more generally, the endocrine bases for maternal strategies of facultatively eusocial insects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yhbeh.2012.08.012>.

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24 2004). Both males and females are produced throughout the year, although male
25 offspring are more common in late February and early March than during other times of
26 the year (Kapheim, 2010; Wcislo et al., 2004).

27 **ESM 2.** *Rearing and observation nest details.*

28 Semi-natural observation nests were constructed by sandwiching a 1 cm x 9 cm x
29 33 cm piece of ultra-light density balsa wood (*Ochroma pyramidale*) between two pieces
30 of clear acrylic sheeting. A 9 cm long shallow groove was cut from one end of the wood
31 to serve as an entrance and tunnel. The acrylic sheeting was covered with opaque fabric
32 or plastic, secured with binder clips, and hung under a plastic roof in the BCI forest (ESM
33 Figure 1).

34 Each observation nest was seeded with a single newly emerged female, which we
35 collected from natural nests at the larval or pupal stage and reared in tissue culture trays
36 in ambient conditions until the day after they eclosed. Upon introducing adult females to
37 observation nests, we plugged the nest entrance with a piece of cotton for a few hours to
38 prevent them from immediately flying away. After an initial adjustment period, the
39 females were allowed to leave and enter the nest freely. Behavior in these nests appeared
40 similar to the behavior of bees in natural and modified natural nests. Because the tunnel
41 in an observation nest was pre-excavated, the first sign of nest construction of females in
42 observation nests was to construct the constricted circular entrance collar typical of
43 *Megalopta* nests (ESM Figure 1). Observation nest foundresses then began constructing
44 cells just like those in new natural nests.

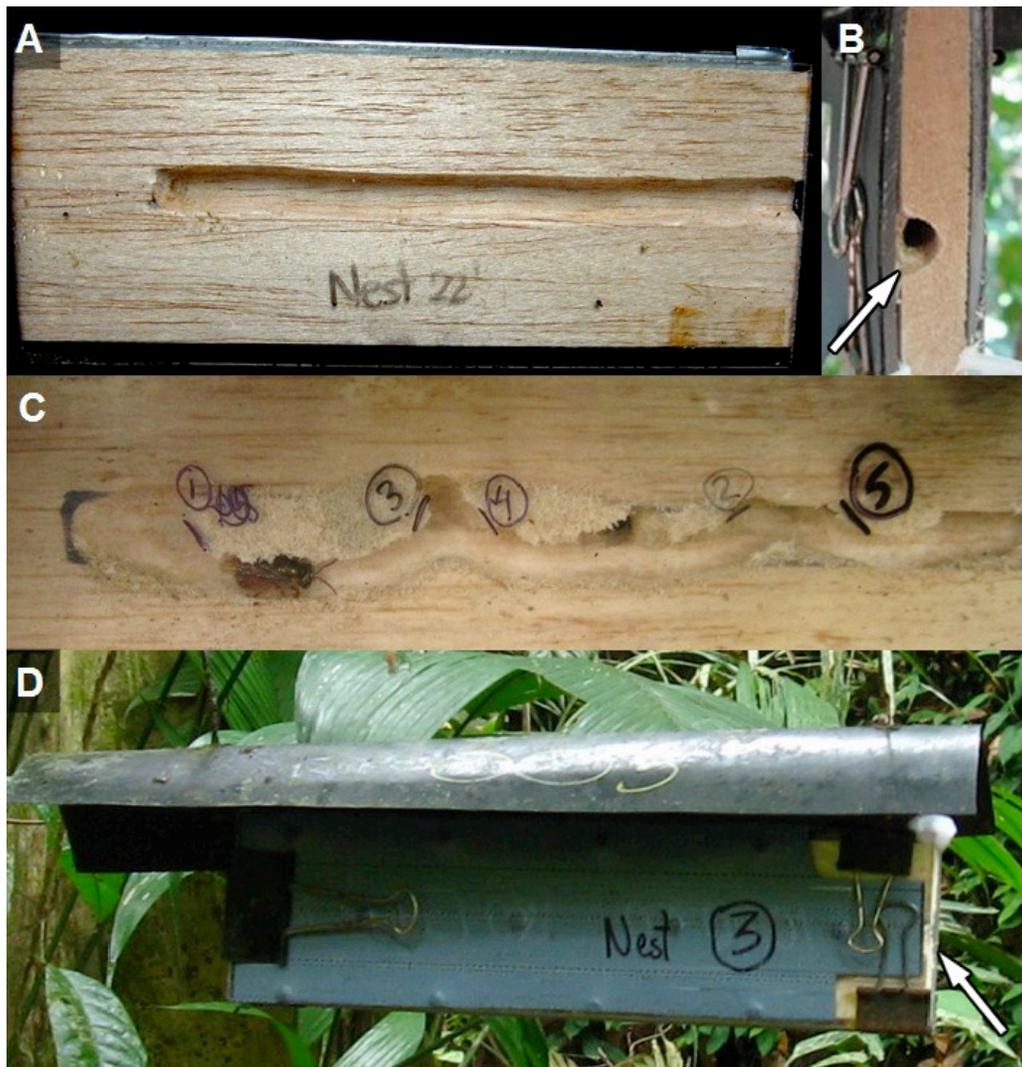
45 All foundresses were marked with a dot of white paint before their offspring
46 emerged. We censused observation nests every 4 days to record newly constructed cells,

47 newly closed cells, and newly emerged offspring. We could predict offspring emergence
48 dates based on the date each cell was closed, and censused daily around this time in order
49 to know precise emergence dates. Newly emerged females were marked with a pattern of
50 white dots distinct from that of the foundress. Foraging behavior was recorded under
51 infrared light with a Sony miniDV camcorder positioned near the entrance of each nest
52 during the short windows of time when *M. genalis* forage: approximately 90 minutes
53 before sunrise and 60 minutes after sunset (Kelber et al., 2006).

54 **ESM 3.** *Gas chromatography/mass spectrometry methods.*

55 JH was quantified using the gas chromatography/mass spectrometry (GC–MS)
56 method of Bergot et al. (Bergot et al., 1981) as modified in Brent & Vargo (Brent and
57 Vargo, 2003). For each sample, a pair of females was pulverized in hexane using a glass
58 tissue grinder. The homogenized samples were eluted through aluminum oxide columns
59 with hexane, 10% ethyl ether–hexane, then 30% ethyl ether–hexane. After drying in a
60 vacuum centrifuge, the sample was then suspended and derivatized in a methyl-d alcohol
61 and trifluoroacetic acid solution. The derivatized sample was dried, then resuspended in
62 hexane and again eluted through aluminum oxide columns; 30% ethyl ether-hexane was
63 used to remove nonderivatized components and 50% ethyl-acetate–hexane was used to
64 collect the JH derivative. After vacuum drying, the purified sample was resuspended in
65 hexane, then analyzed using an Agilent 6890 Series gas chromatograph (Agilent
66 Technologies, Santa Clara, CA, U.S.A.) equipped with a 30 m X 0.25 mm Carbowax
67 Econo-Cap GC column (Alltech, Fresno, CA, U.S.A.) and coupled to an Agilent 5973N
68 inert mass selective detector. JH isoform was confirmed by first running test samples in
69 SCAN mode for known signatures of JH 0, JH I, JH II, JH III and JH III ethyl; JH III was

70 confirmed as the primary endogenous form in this species. Subsequent samples were
71 analyzed using the MSD running in SIM mode. Helium was used as a carrier gas. The JH
72 III derivative was monitored at m/z 76 and 225 to ensure specificity; total abundance was
73 quantified against a standard curve of JH III and an internal standard of farnesol (Sigma-
74 Aldrich, St. Louis, MO, U.S.A.). The detection limit of the assay is approximately 1 pg.
75



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77 **ESM Figure 1** *M. genalis* observation nest design. (a) Each nest was constructed
78 with a standardized piece of balsa wood. (b) New foundresses build an entrance
79 collar, and this is easily recognizable as a sign of new nest activity (see arrow). (c)
80 The interior of an observation nest after the foundress has constructed cells. The

81 location of the entrance to each cell is marked with a black line drawn on the outside
82 of the acrylic sheeting. The foundress is visible at lower left. (d) A nest hanging in the
83 forest on BCI, under a roof and covered with opaque plastic. The entrance is at right
84 (see arrow).

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