Insect science

Insect Science (2016) 00, 1–6, DOI 10.1111/1744-7917.12411

ORIGINAL ARTICLE

Methoprene does not affect juvenile hormone titers in honey bee (*Apis mellifera*) workers

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Abstract Methoprene, a juvenile hormone (JH) analog, is a widely used insecticide that also accelerates behavioral development in honey bees (*Apis mellifera*). JH regulates the transition from nursing to foraging in adult worker bees, and treatment with JH or methoprene have both been shown to induce precocious foraging. To determine how methoprene changes honey bee behavior, we compared JH titers of methoprene-treated and untreated bees. Behavioral observations confirmed that methoprene treatment significantly increased the number of precocious foragers in 3 out of 4 colonies. In only 1 out of 4 colonies, however, was there a significant difference in JH titers between the methoprene-treated and control bees. Further, in all 4 colonies, there was no significant differences in JH titers between precocious and normal-aged foragers. These results suggest that methoprene did not directly affect the endogenous JH secreted by *corpora allata*. Because methoprene caused early foraging without changing workers' JH titers, we conclude that methoprene most likely acts directly on the JH receptors as a substitute for JH.

Key words behavioral development; juvenile hormone analog; juvenile hormone; methoprene; precocious foraging

Introduction

Honey bee (*Apis mellifera* L.) workers show division of labor and change their tasks as they age. In colonies with a stable age demography, workers perform cell cleaning and brood rearing during the first 2 weeks, then nectar processing and comb building in the third week, and finally to foraging when they are approximately 21 d old (Seeley, 1982). This progression of tasks is correlated with levels of juvenile hormone (JH). JH titers in blood and rates of JH biosynthesis both increase with age; they are low in bees that perform in-hive tasks and higher in foragers (Rutz *et al.*, 1976; Fluri *et al.*, 1982; Robinson *et al.*, 1987, 1989; Huang *et al.*, 1991, 1994; Huang & Robinson, 1995). Applying JH, JH analog, or JH mimic to bees causes workers to forage earlier (Jaycox *et al.*, 1974; Jaycox, 1976; Robinson, 1985, 1987; Robinson & Ratnieks, 1987; Robinson *et al.*, 1989; Sasagawa *et al.*, 1989). These results suggest that JH is not only correlated with behavioral development in honey bees, but plays a key role in modulating the pace of behavioral transition in workers.

One study showed that workers with their *corpora allata* (CA, the sole source of endogenous JH) removed still commenced foraging (Sullivan *et al.*, 2000), suggesting that JH may not be the sole regulator of behavioral development (Page & Peng, 2001). Another study, however, demonstrated that a suite of genes that characterize foragers can be turned on directly by applying a JH analogue methoprene (Whitfield *et al.*, 2006). Taking together, these results suggest that though JH plays a major

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role in the nurse to forager transition, redundant pathways and mechanisms might exist.

JH analogs and JH mimics represent a class of insecticides that were designed specifically to disrupt endocrine-regulated processes unique to insects. Methoprene, the isopropyl ester of 11-methoxyacid, is structurally similar to JH and is classified as a JH analog (Klowden, 2002). Methoprene has been used as a growth regulator to control insect pests (Amos & Williams, 1977). In honey bees, however, it has been used extensively as a substitute for JH in causing precocious behavioral development (e.g., Jaycox *et al.*, 1974; Jaycox, 1976; Robinson, 1985, 1987; Robinson & Ratnieks, 1987; Robinson *et al.*, 1989). It exerts no effects on foraging preference in honey bee foragers (Deng & Waddington, 1997) and is considered both more potent and more stable than the naturally occurring JH-III (Henrick *et al.*, 1976).

Because the functions of methoprene and JH have been so similar, it is possible that the two work through the same physiological pathways. JH and methoprene have been shown to activate common pathways to regulate gene expression in *Drosophila melanogaster* (Noreiga *et al.*, 1997; Ashok *et al.*, 1998; Restifo & Wilson, 1998). Although this may also be true for honey bees (Whitfield *et al.*, 2006), it is still unclear how methoprene causes accelerated behavioral development in honey bees. There are 3 alternative hypotheses. One is that methoprene acts as a substitute or agonist for JH, thus directly causing behavioral development in honey bees (Agonist hypothesis). If this is true, lower endogenous JH titers would be required to reach the foraging "threshold" in methoprene-treated bees. Because most measurement assays will only detect JH in hemolymph, not methoprene, this hypothesis predicts lower JH titers in methoprene-treated foragers. Methoprene could also make bees more sensitive to endogenous JH, so that methoprene-treated bees will forage earlier even if their JH titers are lower or comparable to that of control bees (Sensitivity hypothesis). Third, methoprene could cause bees to increase endogenous amounts of JH, either by direct induction or by suppression of JH initially which causes bees to overcompensate with a higher JH titer later (Elevated JH hypothesis). The first 2 hypotheses will both predict lower JH titers in foraging methoprene-treated bees; thus JH titers cannot distinguish these 2 alternatives. The third hypothesis is consistent with the long delay (about 2 weeks) between methoprene treatment and foraging behavior (Robinson, 1985, 1987). In this study, we aimed to distinguish between these 3 hypotheses, by comparing JH titers at different ages, including JH titers at the onset of foraging, between bees that were treated versus not treated with methoprene.

Materials and methods

Focal bees

Experiments were conducted during the summer of 2007 at the Michigan State University Bee Biology Building, East Lansing (42º40′44″ N, 84º28′38″ W), Michigan, USA. Bees were maintained according to standard beekeeping practices. Frames of near-emerging brood were brushed of adult bees and incubated (34 °C, RH 50%) overnight. Newly emerged bees (<24 h) were placed in a plastic pan with a thin layer of vegetable oil smeared on the rim to prevent escape, then each marked with a spot of paint (Testor's PLA, Testor, Vernon Hills, IL, USA) on their dorsal side of thoraces. There were 125 bees in the treated group and 125 in the control. Bees in the treated group were each topically treated on the abdomen with 200 μg methoprene (Sigma-Aldrich, USA) dissolved in 2 μL of acetone, while the control group was treated with 2 μL acetone alone. This dose has consistently been shown to induce precocious foraging in honey bees (Robinson, 1985, 1987). Both groups of bees were then returned to their natal colonies. The experiment was replicated using 4 different colonies, each containing 25 000–30 000 workers and 1 laying queen.

Hemolymph sampling

Hemolymph was taken for JH titer measurement on the 4th day and then once every 3–4 d thereafter until both groups started foraging. Bees were collected with forceps into a ziplock bag, buried into ice and bled according to the standard procedure: a small hole was pricked with a bent insect pin between segments in the intersegmental membrane. The hemolymph was collected in a capillary tube (Drummond Wiretool 1–5 μL), then measured to the nearest 0.5 mm with a ruler (later converted to μL) and mixed with 500 μL acetonitrile (HPLC grade, EM Science, Gardena, CA, USA) in a 12 mm × 125 mm culture tube with a Teflon-lined lid. These samples were then stored at –20 °C until analysis. Ten bees were sampled but 8 bees per group per colony were bled each time. We were careful to collect hemolymph no more than an hour after bees were collected from the hives, to ensure that stress did not alter the natural JH titers (Lin *et al.*, 2004). All sampling was conducted between 900 and 1300 h, so that diurnal changes in JH titer (Elekonich *et al.*, 2001) would not be a factor affecting the results.

JH titer measurement

JH III, the only JH isoform found in honey bees (Hagenguth & Rembold, 1978), was extracted from

the hemolymph and measured with a chiral-specific radioimmunoassay. JH was extracted from the mixture of hemolymph and acetonitrile after adding 1 mL 0.9% NaCl and mixing with 1 mL hexane (×2). Tubes with the hexane phase were dried to concentrate the JH using a Savant solvent drying system (SS21, Thermo-Fischer, Waltham, MA, USA). Then 100 μL methanol was added and vortexed vigorously, 2 aliquots (20 μL each as one of the duplicate) were taken out and dried. A 200 μL mixture of JH antibody (diluted 1:14 000 times) and radio-labeled JH (~10 000 DPM) was added to each tube and incubated for 2 h at room temperature for the radiolabeled and sample JH to compete for binding sites on the antibodies. The reaction was slowed down by incubating the tubes in ice water for 10 min, and then stopped by adding 500 μL dextran-coated-activated charcoal to absorb the free JH that was not bound to the antibodies. The tubes were centrifuged at 2000 × *g* for 3 min and the supernatant decanted into individual scintillation vials. Scintillation cocktail (2.5 mL Ultima Gold, PerkinElmer, Waltham, MA, USA) was added to each vial and radioactivity in each sample was determined by a Scintillation Counter (Packard Tri-Carb 2100 TR, PerkimElmer, Waltham, MA, USA). Kaleidagraph (Synergy Software, Reading, CA, USA) was used to perform a non-linear regression on the standard curve data to obtain 5 parameters that were used to convert DPM into JH amounts (Huang & Robinson, 1996). Each sample was measured in duplicate and any sample with high variation (coefficient of variation > 8%) was redetermined the next day using the remaining methanol aliquots.

Behavioral observations

Behavioral observations were made to verify that methoprene indeed accelerated behavioral development in workers (days 12–20). Observation was done by placing a wire mesh over the hive entrance and collecting paint-marked returning foragers with an insect vacuum. Foragers were defined as bees carrying pollen in the pollen baskets or bees with extended abdomens. Foragers were frozen and not returned to their colonies. We collected the very first foraging bees in both the treated and control groups and bled them to compare their JH titers ($n = 8$ per treatment).

Statistical analyses

Means and standard errors (SE) are reported throughout this paper. Differences in behavioral development were analyzed with survival analysis (treating those started foraging as census = 1 and those that did not start foraging as census = 0). JH titers were analyzed with analysis of variance (ANOVA), with age and methoprene treatment as independent variables and JH titer as the dependent variable. JH titers were transferred (log (JH+1)) to satisfy homoscedasticity requirement of ANOVA. Both were done by using R 3.3.0 (R Core Team, 2016).

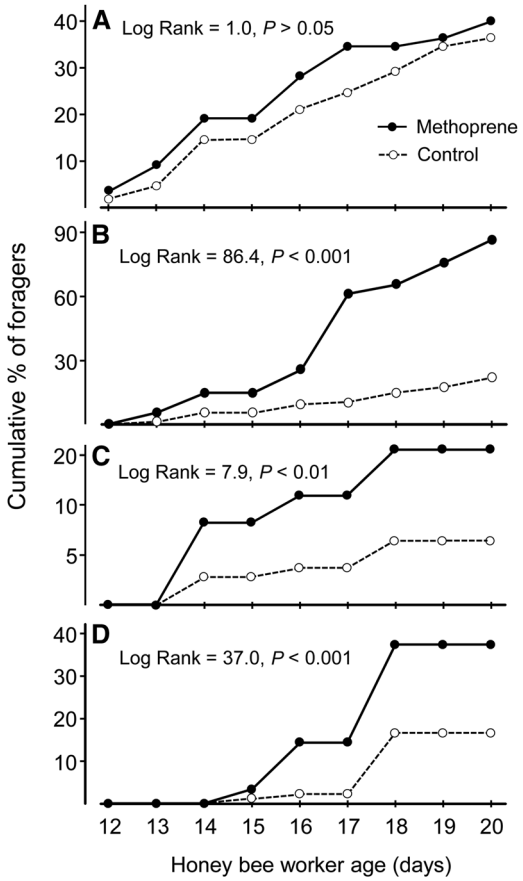

Fig. 1 Number of precocious foragers in methoprene-treated (solid) and control (empty) bees in 4 colonies. Kaplan–Meier survival analysis showed that methoprene-treated bees foraged significantly earlier than the control bees ($P < 0.01$), except in Colony A, which showed no significant difference between the 2 groups of bees ($P > 0.05$).

Results

Behavioral development

The behavioral data collected showed that methoprene had a significant impact on foraging of treated bees in 3 out of 4 colonies (Fig. 1). There were significantly more foragers in the methoprene-treated group than that of the control 12–20 d postemergence, with the exception of Colony A (Fig. 1 and statistics therein). This confirms

that the methoprene treatment was effective in inducing precocious foraging in our treated group.

JH titers in control and methoprene treatment colonies

When all 4 colonies were analyzed together, there were significant differences in JH titers among the 4 colonies ($F_{3,252} = 7.56$, $P < 0.0001$), so each colony was analyzed separately using two-way ANOVAs, with worker age and treatment (methoprene and control) as factors. Each colony showed significant differences in JH titers due to age ($F_{3,59} = 7.43$, 4.87, 43.71, and 39.79 for Colony A, B, C, and D, respectively, $P < 0.01$ for all). However, only Colony B showed a significant difference ($F_{1,59} = 8.74$), with all other colonies with no significant differences, in JH titers between the methoprene-treated bees and the control ($F_{1,59} = 1.27$, 0.22, and 0.49 for Colony A, C, and D respectively, $P > 0.2$ for all 3 colonies). *Post hoc* comparison showed that in Colony B, day 11 was the only day causing this difference ($P < 0.05$, Fisher's Least Square Differences).

In all 4 colonies, there were no significant differences between JH titers of the earliest methroprene-treated versus untreated foragers (all 4 *t*-tests, $P > 0.05$, Fig. 2).

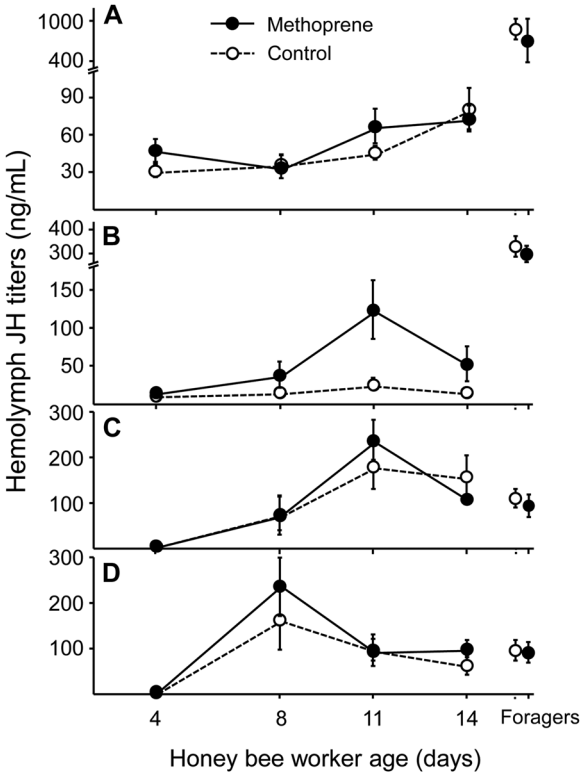

Fig. 2 Juvenile hormone (JH) titers (mean ± SE) of various aged bees and known foragers in methoprene-treated (solid) and control (empty) bees in 4 colonies. Only Colony B showed a significant effect of methoprene treatment on JH titers, and this difference was largely due to difference on 1 single day, day 11. $N = 8$ bees per data point except foragers had 10–12 bees per data point. Statistical analysis done after log (JH+1) transformation but untransformed data are used here.

Discussion

This study examined the effects of methoprene on behavioral development and JH titers in worker honey bees. Behavioral data from 3 out of 4 colonies verified that methoprene did cause precocious behavioral development in adult workers. However, the acceleration is not as strong as observed in previous studies (Robinson, 1987), perhaps due to frequent rain during our experiments. The smaller effect of behavioral acceleration might have prevented our seeing a JH difference between the 2 groups of bees, even if there were differences. This is because we sampled bees for JH at ages 4, 8, 11 and 14 and there were very few bees foraging even in the methoprene-treated group by age 14 (Fig. 1).

Nonetheless, we would have expected to see a consistent JH difference between the 2 groups in preforaging bees if methoprene was indeed modulating JH titers. Previous experiments with precocious foragers in single-cohort colonies have observed such accelerated increases in JH titers (Jassim *et al.*, 2000). In 3 out of 4 colonies of this study, JH titers remained the same across different ages between the 2 groups, yet methoprene-treated bees were foraging earlier than the control. Because the elevated JH hypothesis implies a consistent difference between the treated and untreated groups, with the methoprene-treated bees showing an accelerated JH increase, our results are not consistent with this hypothesis.

It is more difficult to distinguish between the agonist and sensitivity hypotheses. Foragers in both the methoprene-treated group (precocious) and the control group (normal aged) had similar JH titers (Fig. 2, last data point). This result is puzzling because it does not fit nicely with either the sensitivity or the agonist hypothesis. If sensitivity to JH is increased in methoprene-treated bees, we expect that methoprene-treated bees should be foraging with significantly lower JH titers. The agonist hypothesis also predicts that JH titers should be lower in methoprene-treated bees that are foraging earlier: if bees are responding to both internal JH and the external methoprene, they should require lower endogenous JH to make the nurse to forager transition.

Previous studies on the effects of methoprene lead us to believe that the agonist hypothesis is the most plausible explanation of our results. Methoprene has been shown to eliminate the delay in foraging caused by an allatectomy (Sullivan *et al.*, 2000), the removal of the corpora allata glands that secrete JH. In both typical and single-cohort colonies, foraging onset was delayed in allatectomized workers but normal in allatectomized workers treated with methoprene. These results imply that methoprene can affect workers independently of its endogenous JH secretions. That conclusion would be less consistent with the sensitivity hypothesis, which indicates that methoprene's action is dependent on the presence of endogenous JH secretions.

In conclusion, our data can only clearly reject the elevated JH hypothesis because there were no consistent differences in JH titers between the methoprene-treated and control bees. Most importantly, first foraging bees in both groups showed indistinguishable JH titers in all 4 colonies. Of the 2 hypotheses that are consistent with our data, we find the agonist hypothesis to be the most plausible given our comprehensive understanding of methoprene's effects.

Methoprene-treated workers show other changes in behavior. One recent study (Chang *et al.*, 2015) showed that besides accelerating behavioral development, methoprene-treated bees also showed reduced foraging span, total time spent foraging and the number of completed foraging trips. It is not clear if these effects are specific to methoprene treatment, or perhaps due to the earlier foraging in younger bees. For example, it is possible that more naturally induced precocious foraging (such as in single cohort colonies, colonies composed with only newly emerged bees, Huang & Robinson, 1996), earlier foragers will show the same symptoms. Indeed, nonmethoprene induced precocious foragers from single cohort colonies, show similar changes in behaviors. Vance *et al.* (2009) found that precocious (7–14 d old) foragers show reduced maximal wingbeat frequency and maximal average angular velocity compared to normal-aged (15–28 d old) foragers. Further, actual foraging performance also is lower in nonmethoprene-induced precocious foragers: bees foraging early (younger than 14 d) spent less time outside the hive, completed fewer flights, and performed longer foraging trips than more normal foragers (foraging at or later than 14 d) (Perry *et al.*, 2015). These results suggest that precocious foragers, regardless whether induced by colony environment (Vance *et al.*, 2009; Perry *et al.*, 2015) or by methoprene (Chang *et al.*, 2015), perform at reduced capacity than normally aged foragers; these reductions are not due to methoprene but due to starting foraging when too young. Methoprene therefore can be used to induce earlier foraging without using the special colony demography manipulation for behavioral studies.

Acknowledgments

We thank the MSU HSHSP (High School Honors Science Program) for supporting SL, and Michigan Beekeepers Association and MSU Agricultural Experimental Station for supporting this research. We thank 1 anonymous reviewer for critical comments that improved this manuscript.

Disclosure

The authors have declared that they have no conflict of interest.

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Manuscript received September 12, 2016
Final version received October 5, 2016
Accepted October 18, 2016