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Evolution

Common endocrine and genetic mechanisms of behavioral development in male and worker honey bees and the evolution of division of labor

(*Apis mellifera*/behavior genetics/juvenile hormone/temporal polyethism)

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ABSTRACT **Temporal polyethism is a highly derived form of behavioral development displayed by social insects. Hormonal and genetic mechanisms regulating temporal polyethism in worker honey bees have been identified, but the evolution of these mechanisms is not well understood. We performed three experiments with male honey bees (drones) to investigate how mechanisms regulating temporal polyethism may have evolved because, relative to workers, drones display an intriguing combination of similarities and differences in behavioral development. We report that behavioral development in drones is regulated by mechanisms common to workers. In experiment 1, drones treated with the juvenile hormone (JH) analog methoprene started flying at significantly younger ages than did control drones, as is the case for workers. In experiment 2, there was an age-related increase in JH associated with the onset of drone flight, as in workers. In experiment 3, drones derived from workers with fast rates of behavioral development themselves started flying at younger ages than drones derived from workers with slower rates of behavioral development. These results suggest that endocrine and genetic mechanisms associated with temporal polyethism did not evolve strictly within the context of worker social behavior.**

Adult female honey bee workers display "temporal polyethism," a progressive change in the performance of tasks related to colony growth and development. This form of behavioral development is the basis of age-related division of labor, a central feature of insect societies (1, 2). There are several theories explaining the adaptive significance of temporal polyethism (3, 4), but the evolution of mechanisms regulating this highly derived behavioral trait has received less attention (5).

We used male honey bees (drones) to investigate how mechanisms regulating temporal polyethism may have evolved. Drones, virtually ignored except for in studies of mating biology (6), are useful for this purpose because they display an intriguing combination of similarities and differences in behavioral development relative to workers. Both drones and workers spend some period of time in the nest after adult emergence before they begin activities outside. Drones spend only a few days in the nest after adult emergence, beginning mating flights, on average, at 6–9 days of age (6–10). In contrast, workers spend over half of their adult life, 2–3 weeks, working in the nest before beginning to forage (6). Moreover, drones do not participate in colony division of labor. Their only known function is to mate with virgin queens; they take daily flights until they mate and/or die (6–10).

Hormonal and genetic mechanisms regulating temporal polyethism have been identified. Juvenile hormone (JH) is involved in the regulation of temporal polyethism (1). The JH blood titer increases with worker age; it is low in bees that work in the nest and increases with the onset of foraging (11). Treating bees with JH, JH mimic, or JH analog induces precocious foraging (12, 13). Variation in worker genotype also influences the expression of temporal polyethism (1, 14, 15). Differences in rates of behavioral development are due, in part, to genotypic differences among workers (15, 16).

We performed three experiments to determine whether behavioral development in drones is regulated by mechanisms common to workers. In experiment 1, we treated drones with a JH analog and predicted that they would exhibit precocious flight activity, as do workers. In experiment 2, JH blood titers were measured in drones at different ages and stages of behavioral development. We predicted an age-related increase in JH associated with the onset of flight, as in workers. In experiment 3, we tested for common genetic regulation of behavioral development in drones and workers. We predicted that drones derived from workers with fast rates of behavioral development will start flying at younger ages than drones derived from workers with slower rates of behavioral development.

MATERIALS AND METHODS

Experiment 1: Effect of JH Analog Treatment on Drone Behavioral Development. One-day-old adult drones, obtained by placing combs of pupae in a 33°C incubator, were each marked with a colored, numbered, tag on the thorax and treated topically on the abdomen with either 5 μl acetone (control) or 500 μg racemic methoprene dissolved in 5 μl acetone (treatment). Methoprene has demonstrated JH-like activity in all insect species tested, at the molecular (17), physiological, and behavioral (18, 19) levels. The efficacy of methoprene as a JH analog in honey bees is well established (11–13, 20, 21). We used a 500 μg dose, double the optimum dose used in previous studies with workers, because drone mass is ≈2 times greater (6). Untreated drones were not used as an additional control because a previous study showed that topical treatment of acetone had no effect on age at first foraging for workers (12). This allowed us to study the effects of methoprene on as many drones as possible; colonies usually only maintain a few hundred drones at any one time (6), so there is a limit to how many can be introduced for observation.

Treatment and control drones were introduced to a honey bee colony in a hive fitted with a Plexiglas covered observation ramp and a dead bee trap (22). Observations at the hive entrance was made for 2–3 h daily from 15:00 to 18:00 h, the time of maximum drone flight in our region. Observations

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Abbreviation: JH, juvenile hormone.

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began on day 2 and ended after ≈50% of the experimental drones started flying. We most likely observed the experimental drones' very first flights because there were none during the first several days of observation. This experiment was performed twice and observations were performed blind with respect to treatment in trial 2. The mortality of experimental drones was determined both by monitoring the dead bee trap daily and by opening the hive and censusing the colony every 2–3 days. Drones from unrelated source colonies were used in each trial.

This experiment was performed with "single-cohort colonies," each composed initially of 2000 adult worker bees 1–3 days old (11), because they accept a higher proportion of introduced marked drones than do larger colonies with more typical age structures (unpublished observations). Previous results of hormonal analyses of worker honey bee behavior from single-cohort colonies agree with those from more typical colonies (11, 15, 20). The consistency of results for drones from a single-cohort colony and a more typical colony was examined in experiment 2.

Experiment 2: Blood Titers of JH for Drones at Different Ages and Stages of Behavioral Development. Drones of known age were obtained by marking 1-day-old adult drones with a spot of paint on the thorax prior to introduction. Drones from the same source colony were introduced either to a single-cohort colony (as in experiment 1) or to a typical colony (with ≈20,000 bees of all ages). Observations began on day 1 and continued until day 15, when all remaining marked drones in each colony were sampled. Drones younger than 6 days of age had not yet flown and were collected inside the hive at the end of the drone flight period; 6-day-old drones were collected at the hive entrance after their first flight. Older drones also were collected returning to the hive; we waited a few days after the first drones were observed flying to increase the likelihood that these older drones had one or more days of flight experience. Unlike in experiment 1, our observations were not designed to reveal the ontogeny of flight for a group of drones but rather to sample individuals of known age and behavioral status.

Blood (0.86–17.7 μl per individual) was collected from a neck-membrane incision. JH was quantified from individual drones with a radioimmunoassay (RIA) (23) validated for adult worker honey bees (24). JH III is the only JH homolog found in honey bees, based on both gas chromatography/mass spectroscopy (25) and RIA (24, 26). Since the lipophilicity of JH can theoretically affect quantification (W. G. Goodman, personal communication), we determined whether lipids in drone blood influence RIA accuracy. Ten blood samples were split; one half of each sample was subjected to a C_{18} Sep-Pak procedure to remove lipids (24) and the other half was not. As is the case for workers (24), lipids did not affect RIA analyses of drone blood: JH titers in both sample halves were significantly correlated with each other ($r = 0.8$, $P < 0.01$) and the slope (1.07 ± 0.29) of the regression line was not significantly different from 1 ($P > 0.1$, t test, df = 8, $t = 0.086$).

To further compare the development of adult drones in a single-cohort colony relative to a more typical colony, we measured sperm concentration in some of the same individuals used for JH quantification. Semen was collected from 12- and 13-day-old drones (0.4–2.0 μl per individual), diluted in 10 ml water, and the spermatozoa counted with a Neubauer counting chamber (27).

Experiment 3: Genotypic Differences in Rates of Behavioral Development for Worker Honey Bees and Their Sons. We identified "source colonies" with workers that exhibited either fast or slow rates of adult behavioral development, as in previous studies (15, 16). Each source colony was headed by a queen instrumentally inseminated with semen from a single, different, drone. Genotypic differences in rates of behavioral development were determined by measuring the age at onset of foraging for workers in experimental colonies, each composed of a single cohort of 1-day-old individuals from two source colonies. Differences in rates of behavioral development were assessed by determining the proportion of workers from each source colony that became precocious foragers relative to their representation in the whole experimental colony. Precocious foragers were bees that started collecting nectar or pollen at 7–10 days of age, which is about 2 weeks earlier than in a typical colony (6).

After screening eight pairs of source colonies, we identified two pairs that showed the biggest differences in rates of worker behavioral development. Workers from these four source colonies were induced to lay (parthenogenetic, haploid) male eggs according to the following procedure. One queenless, broodless colony was established from each source colony with ≈5000 1–3 day old workers (28). These colonies were given ample food (honey and pollen in combs and a supplemental pollen and sugar syrup mixture) to help induce worker ovary development, and empty drone comb for workers to lay haploid eggs (29). The resulting 1-day-old adult drone progeny from each pair of source colonies were individually tagged and introduced into a common single-cohort colony, unrelated to either source colony. Observations on the onset of flight were as in experiment 1, and were performed blind with respect to genotype.

RESULTS

In both trials of experiment 1, methoprene-treated drones started flying at significantly younger ages than did acetone-treated drones (Fig. 1). The age at first flight for acetone-treated drones is well within the range reported for untreated drones (6–10). This suggests that acetone alone had no effect on drone behavioral ontogeny in this study, as is the case for workers (12). Differences in the number of drones flying from treatment and control groups cannot be attributed to differences in drone mortality. There were no significant differences in mortality in the days prior to the onset of flight; in trial 1, mortality was 10% for both treated and control drones and in trial 2, 15% and 8%, for treated and control drones, respectively ($G = 1.77$, df = 1, $P > 0.18$).

In experiment 2, drones had high JH titers at the time they began taking flights, in both the single-cohort colony and the more typical colony (Fig. 2). There were no significant colony differences for drone JH titers ($P > 0.82$, $F = 0.051$) or sperm concentration (mean ± SE; single-cohort colony, $3.96 \times 10^6 \pm 6.9 \times 10^5/\text{mm}^3$; typical colony, $4.43 \times 10^6 \pm 6.5 \times 10^5/\text{mm}^3$; $n = 7$ for each colony, $P > 0.56$, Mann–Whitney U test, $U = 20$, $U' = 29$). Based on these results, JH titer data from the two colonies were pooled for further analysis. JH titers of drones increased significantly on day 5 and peaked on day 6, coincident with the onset of flight. JH titers decreased after day 10 even though flight continued.

The behavioral screening for experiment 3 identified two pairs of source colonies with the biggest differences in rates of worker behavioral development. In both cases, one source colony's workers were significantly over-represented in the precocious forager sample relative to their abundance in the experimental colony they were tested in (Fig. 3). The workers from the over-represented source colony thus showed faster rates of behavioral development than did workers from the other source colony (referred to as "fast" and "slow" workers, respectively, in Fig. 3).

Sons of workers with faster rates of behavioral development themselves started flying at significantly younger ages than did sons of workers with slower rates of behavioral development (Fig. 4). As in experiment 1, differences in the number of drones flying cannot be attributed to differences in mortality between the two groups. In both trials, mortality was higher for the "faster-developing" drones, presumably due to the earlier onset of flight [trial 1, 26% and 14% for fast- and slow-

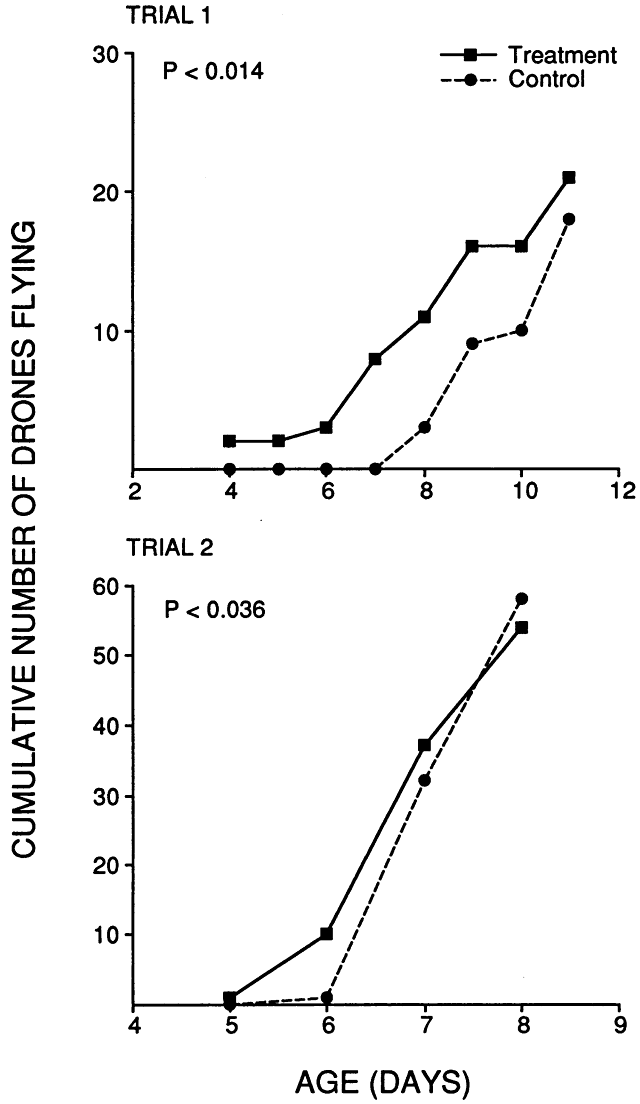

FIG. 1. Effect of the JH analog methoprene on rate of behavioral development in drone honey bees. P values based on results of Mann–Whitney *U* tests on age at first flight for the individuals in the treatment and control groups (trial 1, $z = 2.462$; trial 2, $z = 2.091$). In trial 1, 21 of 50 and 18 of 50 drones were observed flying in the treatment and control groups, respectively; in trial 2, 54 of 100 and 58 of 100, respectively.

developing drones, respectively ($G = 1.56$, df = 1, $P > 0.21$); trial 2, 21% and 10%, respectively ($G = 3.82$, df = 1, $P = 0.05$)].

DISCUSSION

These results demonstrate common endocrine and genetic mechanisms that regulate behavioral development in both drone and worker honey bees. Finding common regulatory mechanisms in different sexes of the same species is not unusual for solitary animals (e.g., ref. 30). But the existence of common regulatory mechanisms is striking for the highly eusocial honey bee because drones and workers are so different. Drones show evidence of intense selection for behavioral and morphological adaptations associated with mating (7) but have not been selected to participate in colony division of labor. In contrast, workers typically are more or less sterile and spend their entire life engaged in a series of tasks related to colony growth and development.

These results suggest that endocrine and genetic mechanisms associated with temporal polyethism did not evolve strictly within the context of worker social behavior. If this hypothesis is correct, the observed genetic variability in both drone and worker behavioral development may have been important in the evolution of delayed behavioral development in workers, which is an essential feature of temporal polyethism (5). Worker honey bees spend almost half of their adult life working in the nest before initiating foraging, while primitively social bees and solitary bees begin flight much sooner (7). The evolution of delayed behavioral development may have in-

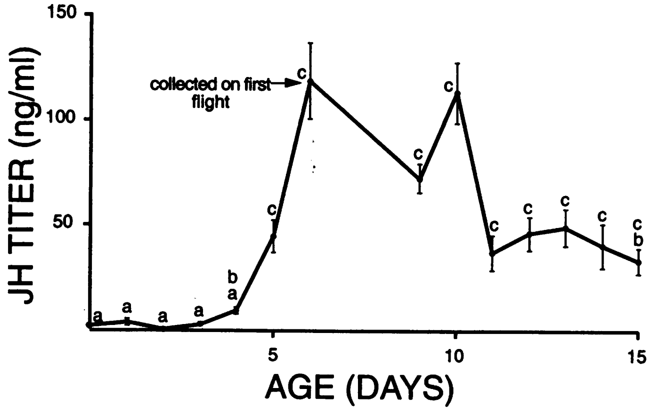

FIG. 2. JH blood titers for drone honey bees at different ages and stages of behavioral development. Data points (mean ± SE) with different letters are significantly different from one another ($P < 0.05$; Scheffé multiple-comparison test; $F = 73.8$, $P < 0.001$; log[1 + *x*] transformed data). For days 1–4, $n = 20$; for day 5, $n = 13$; for day 6, $n = 10$; and for days 9–15, $n = 12$ (except for day 12, $n = 11$).

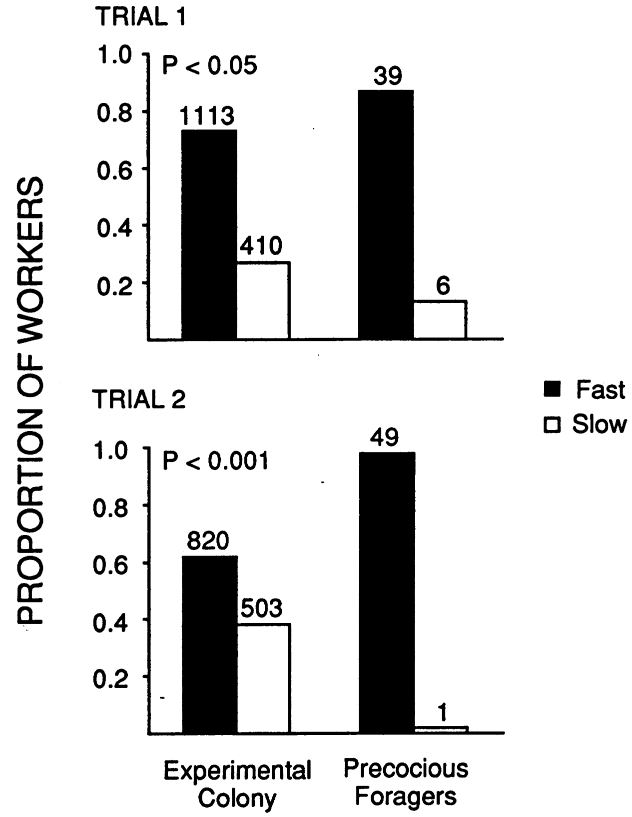

FIG. 3. Genotypic differences in rates of behavioral development for worker honey bees. Data show proportions of workers from each source colony in samples of precocious foragers and the whole colony. *P* values based on a comparison of these proportions by two-way *G*-tests (using actual frequencies above each bar; first pair, df = 1, G = 4.997; second pair, df = 1, $G = 40.085$).

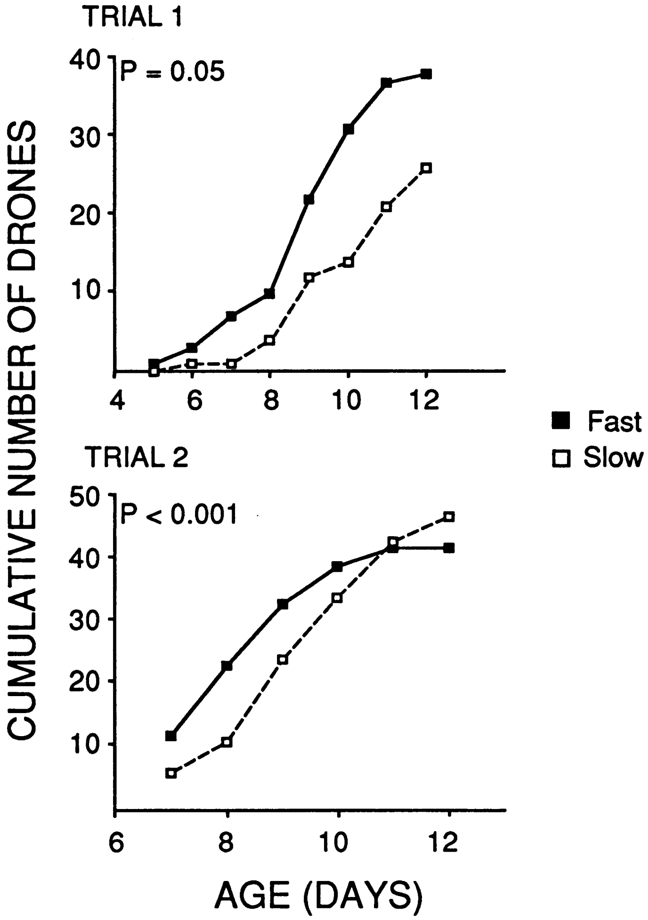

FIG. 4. Genotypic differences in rates of behavioral development for drone sons of fast and slow developing worker honey bees. *P* values based on results of Mann–Whitney *U* tests (trial 1, $z = 1.959$; trial 2, $z = 3.59$) on age at first flight for individuals in the two groups. In trial 1, 38 of 50 fast and 26 of 50 slow developing drones were observed flying; in trial 2, 42 of 100 fast and 47 of 100 slow developing drones were observed flying.

volved changes in JH-dependent mechanisms such as JH production and target tissue hormone sensitivity (31), or as-yet-undiscovered JH-independent mechanisms. The possibility that endocrine and genetic mechanisms regulating temporal polyethism actually preceded the evolution of eusociality could be evaluated by studying solitary bees, but interspecific comparative analyses of endocrine mechanisms can be complicated by "hormonal pleiotropy" (31). Genetic variability for rate of behavioral development in drones may also be related to variation in drone reproductive success, but this has not been studied.

Our results suggest that the regulation of behavioral development in drones differs from that of workers in at least two ways. Although JH titers in drones increased with age and remained high during the period when drones initiated flight, titers then decreased with age, and older drones flew even after their JH levels dropped. This pattern is very similar to recently reported age-related changes in rates of JH biosynthesis *in vitro* by the corpora allata of drones (32). In contrast, both JH titers and rates of biosynthesis in workers increase with age and then typically remain high in foragers during the summer (11, 24, 33). An intriguing exception to this was recently reported; workers foraging during the late winter or early spring were found to have low JH titers and rates of biosynthesis (34). These results suggest that higher JH titers are required for higher levels of flight activity; summer foragers take more flights per day than do drones or workers foraging in the late winter or early spring. The suggestion that higher JH titers are associated with increased flight activity also is consistent with findings from queen honey bees, which show less flight activity than do summer foragers. Queens have a JH profile that is more similar to that of drones than to workers; they have a JH peak early in adult life prior to the onset of flight activity and then fly when titers are low (35). However, it is not known whether behavioral development in queens also is regulated by JH, and experiments on this question would be complicated by the honey bee's monogynous society. This hypothesis can be further tested by treating drones and workers with different doses of JH analog, before or after the onset of flight, and measuring the ontogeny and intensity of flight.

The second difference between workers and drones observed in this study is that the rate of drone behavioral and reproductive maturation was similar in both a single-cohort colony and a typical colony, whereas it is well known that worker behavioral development is very sensitive to colony conditions, in particular colony age demography (15, 36, 37). While we did observe differences in drone behavioral ontogeny in experiments 1 and 3, they were probably related to differences in genotype, time of year, and other factors, but not to colony age demography, since both of these experiments were conducted with single-cohort colonies. Our results, though limited to individuals from just one single-cohort colony and one typical colony, suggest that drones are less sensitive than workers to changes in colony age demography.

This study, and two recent papers on drone learning (38, 39), highlight the utility of drones in intraspecific comparative analyses of mechanisms of complex behavior. They both demonstrate that mechanisms that may have significance in social evolution are expressed by both haploid and diploid genotypes. Given the emergence of the haploid zebra fish as a model in molecular neurobiology (40, 41), the haploid drone also may be useful in future molecular analyses of behavioral development.

This paper is dedicated to the memory of George C. Eickwort, whose unparalleled breadth and depth in social insect biology contributed so much to the study of the evolution of social behavior. We thank Z.-Y. Huang for assistance with hormone assays; J. C. Kuehn for expert help in maintaining colonies; D. W. Borst for JH antiserum; W. G. Goodman for helpful technical suggestions; S. A. Cameron, B. N. Danforth, D. W. Pfennig, R. E. Page, and D. Yanega for discussion; and S. N. Beshers, K. Crailsheim, S. A. Collins, S. M. Farris, L. M. Heuser, Z.-Y. Huang, J. C. Kuehn, D. Schulz, J. P. Sullivan, D. P. Toma, C. Wagener-Hulme, M. J. West-Eberhard, and M. L. Winston for reviewing the manuscript. We also thank C. D. Michener and two anonymous reviewers for suggestions that improved this paper. We appreciate Sandoz Corporation's generous gift of methoprene. This study was supported by grants from National Institute of Mental Health (MH 47374), U.S. Department of Agriculture (92-37302-7856 and 94-34302-0568), and a U.S. Department of Agriculture Hatch grant (ILLU-12-0302) to G.E.R.

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