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Juvenile hormone profiles of worker honey bees, *Apis mellifera*, during normal and accelerated behavioural development

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Abstract

Juvenile hormone III (JH) haemolymph titres were quantified in adult worker honey bees under colony conditions conducive to either typical or accelerated behavioural development. JH titres of bees under conditions of accelerated behavioural development were significantly higher than same-aged bees under more typical conditions, even before the onset of foraging. These results are consistent with previous findings indicating that JH plays a causal role in timing the onset of foraging behaviour in honey bees. We also detected a peak of JH in 2–3 day old adult bees, the significance of which is unknown.

Keywords: Apis mellifera; Division of labour; Juvenile hormone; Behavioural development; Radioimmunoassay

1. Introduction

Many social insect colonies are characterised by an age-related division of labour in which workers change jobs as they age. In honey bee colonies, young workers (1–3 weeks old) perform tasks inside the nest, such as brood care ("nursing"), comb building, and food processing; while older workers (>3 weeks old) carry out tasks outside the hive such as foraging and colony defence. Juvenile hormone (JH) has been shown to be involved in the regulation of honey bee age-related division of labour. Young workers specialising in tasks inside the nest have low haemolymph JH titres and rates of JH biosynthesis, whereas foragers and soldiers have high JH titres and biosynthesis rates (Rutz et al., 1976; Fluri et al., 1982; Robinson et al., 1987; Huang et al. 1991, 1994). Manipulating JH levels experimentally also affects the pace of behavioural development, suggesting that JH plays a causal role. For example, treating newly emerged adult workers with JH or JH analogue causes them to become foragers precociously (Jaycox, 1976; Robinson, 1985; Sasagawa et al., 1989), while removing the corpora allata delays the onset of foraging (Sullivan et al., 1996).

Honey bee age-related division of labour also shows plasticity. Behavioural development, from hive activities to foraging, can be accelerated, delayed, or even reversed in response to changing colony conditions (reviewed by Robinson, 1992). For example, in a "single-cohort colony", experimentally composed at the outset of only newly emerged bees, some workers become foragers at approximately 7 days of age (Nelson, 1927; Milojévic, 1940; Robinson et al., 1987), 2 weeks younger than under typical conditions. The JH titres of precocious foragers are as high as those of normal-aged foragers (Robinson et al., 1989), consistent with the idea that high JH accelerates behavioural development.

While it is known that JH titres increases with age in honey bees prior to the onset of foraging during normal behavioural development (Robinson et al., 1987; Huang et al., 1994), JH profiles of pre-foragers under conditions of accelerated behavioural development have not been determined. One purpose of this study was to compare JH titres of young, pre-foraging bees under conditions of typical and accelerated behavioural development. A

Abbreviations: JH=Juvenile hormone; RIA=radioimmunoassay; CA=corpora allata.

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more rapid rise of JH titres is predicted under conditions of accelerated behavioural development.

The second purpose of this study was to determine whether a peak of JH occurs early in adulthood. Data collected for another purpose by Kaatz et al. (1992, Fig. 1) from caged bees in the laboratory and small experimental colonies suggest the existence of such a peak. We were interested in determining whether this peak occurs consistently and under more natural conditions, and if so, whether variation in this peak is associated with variation in the rate of behavioural development.

2. Materials and methods

2.1. Focal bees

Bees were from colonies maintained according to standard techniques at the University of Illinois Bee Research Facility, Urbana, IL. They were typical of current North American populations of *Apis mellifera* in this area (a mix of predominantly European subspecies; Phillips, 1915; Pellet, 1938). One-day-old (0–14 h post-emergence) bees were obtained by incubating frames of honeycomb containing sealed brood in an incubator (34°C and 80% RH). They were either marked on the thoracic dorsal surface with a paint dot (Testor's PLA) or tagged with coloured number tags (Opalith, Germany) for individual identification. Paint-marked bees were used for JH analyses and tagged bees were used for behavioural analyses.

For each trial, the marked bees were divided into two groups. The first group, consisting of 100 tagged and 300 paint-marked bees, was introduced into a typical colony. The second group, consisting of 50 or 100 tagged and 200 paint-marked bees, was introduced into a single-cohort colony. Four trials were performed for this experiment. The focal bees were the offspring of queens (Colonies 42, 45 and 68) that were instrumentally inseminated with semen from a single, unrelated drone. Other bees from these queens were shown to have relatively fast rates of behavioral development (unpublished data, determined as in Giray and Robinson, 1994), an important consideration for the single-cohort colonies (see below).

2.2. Experimental colonies

Single-cohort colonies were established by placing the focal bees with a mated queen, two frames of pollen and honey, and 3000 1-day-old unmarked "background" bees in a small ("nucleus") hive. The unmarked bees came from a colony whose bees had relatively slow rates of behavioral development (Colonies 33, 75 and 59). We created single-cohort colonies in which the focal bees were expected to have faster rates of behavioural development than the background bees to increase the numbers of focal bees experiencing accelerated behavioural development. This was important because we wanted to measure JH titres in bees with accelerated behavioural development *before* they actually initiated foraging, but unfortunately we have no other reliable index of accelerated behavioural development in a single-cohort colony besides precocious foraging. Because only about 5–10% of the bees in a typical single-cohort colony become precocious foragers (Huang and Robinson, 1992; Giray and Robinson, 1994), sampling bees randomly from such a colony would not provide an accurate reflection of the JH titres in bees with accelerated behavioural development.

The genetic manipulation described above was employed in trials 1 and 2; it did result in a majority of the precocious foragers belonging to the focal cohorts, but many precocious foragers came from the background groups as well (data not shown), which suggests that many of the focal bees were not experiencing accelerated behavioural development. Therefore, in trials 3 and 4 we used both the genetic manipulation and a demographic manipulation to increase the numbers of focal bees that experience accelerated behavioural development in the single-cohort colony. In trials 3 and 4, background bees were not added to the colony at the time it was established, but rather 4 days later. Previous studies (Page et al., 1992) have shown that such age differences can strongly influence behavioural development in a single-cohort colony, with the older individuals much more likely to become precocious foragers.

Typical colonies (Colonies 65, 21, 13 and 34) had populations of ~40,000 adult workers and were unmanipulated except as noted above. Each colony pair (typical and single-cohort colony) were studied at the same time and located near each other in the same apiary to minimise environmental influences on foraging behaviour.

2.3. Behavioural observations

A wooden ramp with glass cover (41 cm×61 cm) was fitted to the hive entrance to facilitate behavioural observations of tagged focal bees (Winston and Katz, 1982). "Spot checks" for foraging by focal bees were performed by blocking the entrance for 10–30 min with an 8-mesh hardware cloth and looking for focal bees returning to the hive with signs of foraging (pollen loads in corbiculae on the legs or an abdomen distended with nectar or water). These spot checks began when focal bees were 3 days old in single-cohort colonies, and 5 days old in typical colonies, well before they were expected to forage. After focal bees were observed in this way, more detailed observations began, conducted for two 1-h periods, one in the morning and one in the afternoon. No observations were made during times of mass "orientation flights", when pre-foraging workers learn the

location of their hive (Winston, 1987). The identity of each tagged bee was recorded with a laptop computer, which automatically appended the time of exit or entrance. A bee was classified as a forager if it had visual signs of foraging (as above) or a round trip flight time of >5 min (Robinson, 1987). Observations were conducted daily, weather permitting, until 50% of the tagged focal bees that were introduced to the colony began foraging. The age of the workers at this point was called "F_{50}" and used as a measure for the rate of behavioral development. The mortality of tagged bees, if any, should be higher in the smaller single cohort colonies (ant predation, orientation loss, etc.), thus the difference in F_{50} is probably an underestimate of the differences in behavioural development between the two colony types.

2.4. Haemolymph samples

Starting on day 2 for each trial, paint-marked workers (N=10) were collected daily (10:00-13:00 h) from both the typical and single-cohort colony. Workers were randomly collected from within the hive using a modified portable vacuum. Sampled bees were immediately brought into the laboratory and anaesthetised on ice. Haemolymph was collected with a Drummond Wiretrol (1–5 μl) capillary tube and measured to the nearest 0.1 μl. The haemolymph was then transferred into 500 μl of acetonitrile in a 13 mm×100 mm glass culture tube with Teflon-lined cap and stored at −20°C until JH analysis.

Samples were collected daily until the F_{50} was reached in the single-cohort colony. After that point, we continued to collect samples from bees in the typical colony every 5 days until the F_{50} was reached in that colony. On the day on which the F_{50} was reached in each colony, we sampled in-hive bees and returning foragers (N=10 each). These samples were taken to provide an indication of homogeneity in behavioural development among the focal bees in a colony. Since the bees of both groups were the same age, we expected little or no differences in JH titres, if rates of behavioural development were similar among the bees in a focal cohort. As explained above, we were concerned about this issue for bees in single-cohort colonies.

2.5. Determination of juvenile hormone titres

Titres of JH III, the only form of JH found in honey bees (Hagenguth and Rembold, 1978), were measured for individual bees using a chiral-specific radioimmunoassay (Hunnicutt et al., 1989) specifically validated for adult worker honey bees (Huang et al., 1994). JH in haemolymph samples was extracted with hexane and the hexane was evaporated using a vacuum centrifuge (Savant). Methanol (50 μl) was added to each tube followed by vortexing. To lower the detection limit of this assay, we made the following modifications to previously published procedures (Huang et al., 1994; Huang and Robinson, 1995). We used larger aliquots of each sample (two aliquots, 20 μl each), which amounted to 40% of the sample in each aliquot, rather than 10–20% as in previous studies. The aliquots were transferred to 10 mm×75 mm glass tubes and dried, because the large amount of methanol could interfere with binding of JH with the antiserum. To each dried tube, a 200-μl aliquot of premixed antiserum (1:28 000) and 10 000 DPM of [10-^{3}H(N)]-JH (NEN, 629 Gbq/mmol) were added and then vortexed. These modifications doubled the detection limit. There is no evidence that JH was adsorbed to the tubes due to the extra drying step; standard curves made with and without this modification did not differ significantly (N=4 pairs of curves, P>0.70, ANOVA). In addition, the JH titres of foragers and 1-day-old bees determined in this study are consistent with those of previous studies (Huang et al., 1994; Huang and Robinson 1995, 1996).

Capillary tubes and other glassware that may contact JH were baked at 500°C for 3.5 h prior to use to minimise JH adsorption (Strambi et al., 1981). All solvents were HPLC grade, obtained from either EM Science, Fisher Scientific, or J.T. Baxter Chemical Co.

2.6. Effects of incubation and laboratory handling on JH titres

To mark bees, it is standard procedure to transfer frames of a brood from the hive to the incubator, remove newly emerged bees from the frames with a soft-bristled brush, and keep the bees in a pan in the laboratory before they are marked and then returned to the hive. Because one of our objectives was to determine whether a peak of JH occurs very early in adulthood, we were concerned about the possible effects of this procedure on JH titres. We therefore compared the JH titres of bees marked in this way to bees that received minimal handling (N=30 bees per group). These minimally handled bees were marked in the field by locating brood in the hive that contained newly emerging adults; the bees were marked with a brush without being picked up as soon as they emerged. Haemolymph sample were taken on 3 consecutive days, when the bees were 1, 2 and 3 days old. Bees marked in the standard way were returned to their colony and collected at the same time as the minimally handled bees.

2.7. Statistical analyses

Means and standard errors (SE) are reported throughout this paper. Differences in JH titres between typical colonies and single-cohort colonies were analysed with two-way ANOVA (age and colony type as independent variables; log or square root transformations to obtain normal distributions). Only data for the first 7–13 days

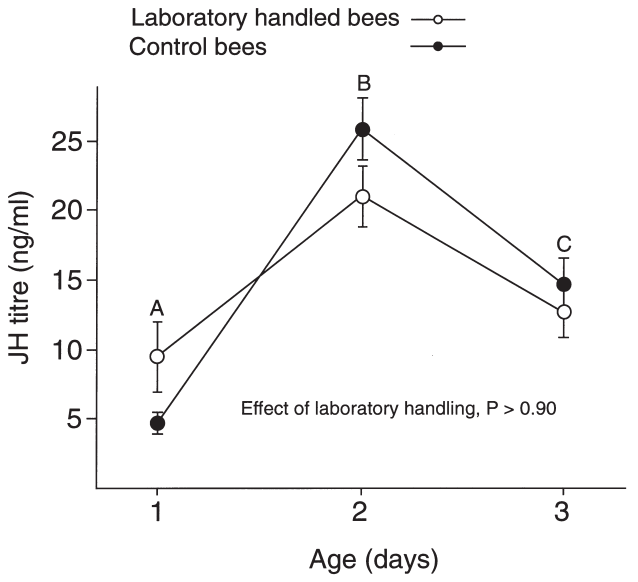

Fig. 1. Effect of laboratory handling on JH titres (mean±SE). N=10 bees per day. There was no difference ($P>0.9$) in JH titres due to handling; data points with different letters show significant differences due to age (at the 5% level using Tukey's HSD test, following ANOVA; $P<0.01$ for age effect).

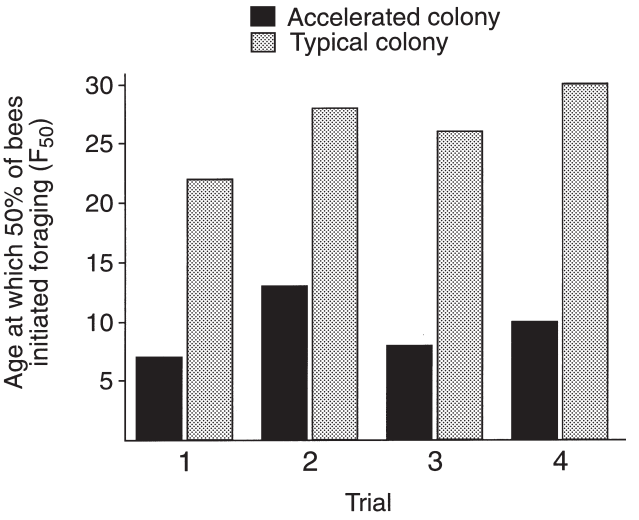

Fig. 2. Differences in rate of behavioural development for bees in typical or single-cohort colonies, based on the age (days) at which 50% of the focal bees began foraging (F_{50}). Difference: $P<0.001$, paired t-test.

were analysed, because data collection stopped earlier in single-cohort colonies. We also excluded data for 1-day-old bees and precocious foragers.

Evidence for a peak in JH in early adulthood was assessed for each colony with one-way ANOVA followed by Tukey's HSD, for days 1–4 (SAS Institute Inc., 1985).

3. Results

3.1. Effects of incubation and laboratory handling on JH titres

There was no significant difference between minimally handled bees and those handled with standard laboratory procedures, either in overall JH titres ($P>0.90$, F=0.01), or in age-related changes in JH titres (interaction between age and handling: $P>0.20$, F=1.52). There was a significant effect of age on JH titres in both groups ($P<0.001$, F=30.54), due to a peak of JH on day 2 (Fig. 1).

3.2. Behavioural development and JH titres in typical and single-cohort colonies

In four out of four trials, focal bees showed precocious behavioural development in single-cohort colonies relative to bees in typical colonies (Fig. 2). The F_{50} for bees in single-cohort colonies was 7–13 days, compared with >21 days for bees in typical colonies ($P<0.001$, t=12.17, paired t-test).

In four out of four trials, JH titres in pre-foraging workers varied significantly both with age ($P<0.01$) and colony type ($P<0.05$; ANOVA, Fig. 3A–D). The interaction between age and colony type was also significant ($P<0.001$) in all four trials. This indicates that worker JH titres varied with age in both colony types, but the patterns of age-dependent variation were different under the two conditions. The difference in JH titres between the two colony types was not caused solely by differences due to the presence of foragers in the single-cohort colony samples. Excluding data on F_{50}, there still were colony type differences in three out of four trials ($P>0.05$ for trial 4). This suggests that bees in the two colony types had different JH titres before the onset of foraging.

On the day when 50% of bees became foragers in each colony, we sampled in-hive bees and returning foragers (N=10). These samples were taken to provide an indication of homogeneity in behavioural development among the focal bees in a colony. Since the bees of both groups were the same age, we expected little or no differences in JH titres, if rates of behavioural development were similar among the bees in a focal cohort. In two out of three trials in typical colonies (in-hive sample not collected in trial 3), there were no significant differences between in-hive bees and foragers ($P>0.3$ and $P>0.4$ for trials 1 and 4, respectively, F-test; the difference is significant for trial 2: $P<0.01$). For single-cohort colonies, in-hive bees had significantly lower JH titres than foragers in three out of four trials ($P<0.05$ for trials 1, 2 and 4, $P>0.10$ for trial 3, F-test).

JH titres of precocious and normal foragers differed significantly in two out of four trials ($P<0.01$, trials 1 and 3), but were similar in trials 2 and 4 ($P>0.05$).

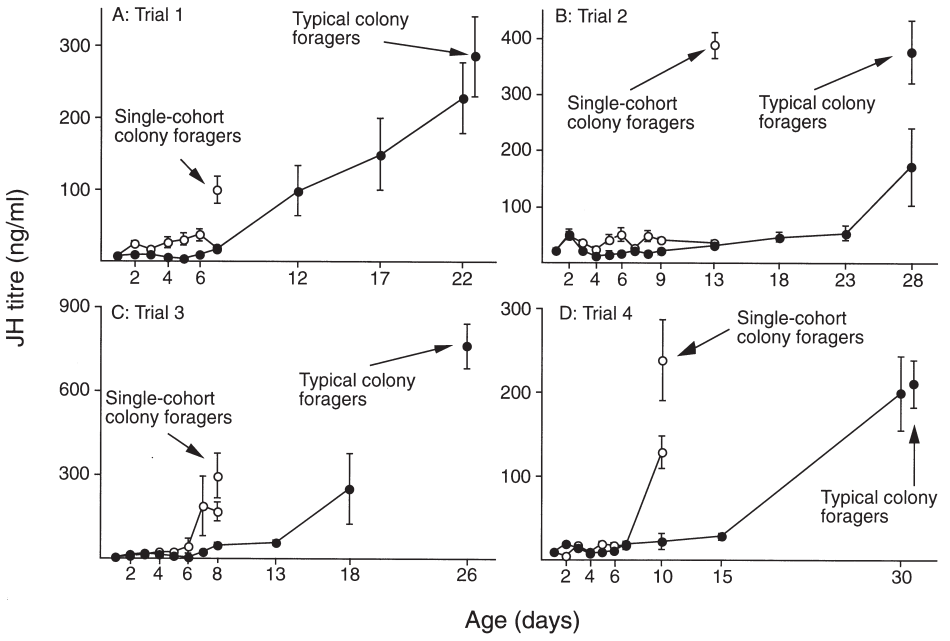

Fig. 3. Age-related changes in juvenile hormone titres (mean±SE) in typical (solid circles) and single-cohort colonies (open circles). Isolated points indicate bees were sampled as returning foragers on the same day the in-hive bees were sampled. *N*=10 bees per data point. Results of statistical analyses in text.

3.3. Early peak in JH titres

Visual inspection of the data in Figs. 1 and 4 suggests that a JH peak occurred in seven out of nine colonies on either day 2 or day 3. In seven out of nine colonies there was a significant ($P<0.05$) increase in JH titres on days 2 or 3 compared with day 1. Five out of nine colonies showed a significant drop in JH titres after day 2 but prior to day 5. Three out of nine colonies showed both a significant rise and then a significant drop in JH titres during the first 4 days. The difference in peak JH value between the two colony types was significant only in trial 1 (Fig. 4).

4. Discussion

This study compares age-related changes in the JH titres of honey bees that experience conditions conducive to either typical or accelerated behavioural development. Results from typical colonies agree with previously published results (Robinson et al., 1989 Huang et al. 1991, 1994). Precocious foragers from single-cohort colonies showed levels of JH as high as normal-aged foragers in two of four colonies, also as reported previously (Robinson et al., 1989). We show here for the first time that bees that apparently would have become precocious foragers (if they were not sampled for JH) also showed an increase in JH titres, before the onset of foraging. These results are consistent with the hypothesis that JH plays a causal role in determining the age at onset of foraging in honey bee colonies (Robinson and Vargo, 1997).

The increase in JH in single-cohort colonies was probably an underestimate because not all the bees we sampled were likely to have experienced accelerated behavioural development (see Section 2). This is especially the case in trials 1 and 2, where JH titre data indicate that there was considerable heterogeneity in the rate of behavioural development among the focal bees in the single-cohort colonies ($P<0.001$ for JH titre between in-hive bees and returning foragers). This was not as marked for trials 3 and 4 ($P>0.1$ and $P=0.02$, respectively), which indicates that the combination of genetic and demographic manipulations was more effective than genetic manipulation alone in promoting more homogeneous accelerated behavioural development. Comparisons of JH titres under conditions of typical and accelerated behavioural development would be improved if there were other behavioural indices of accelerated behavioural development in a single-cohort colony prior to precocious foraging. Task-related differences in location can be used as good indicators of behavioural development in typical colonies (Robinson, 1985), but it is not known whether this would work in single-cohort colonies, due to their small size.

There is a peak of JH around day 2 or 3 that is detectable in most colonies. The peak is evident in data collected for another purpose by Kaatz et al. (1992), and is clearly seen in this study, which was designed to look for it. Results of the “handling” experiment indicate that the peak is not an artifact of incubating and handling bees in the laboratory.

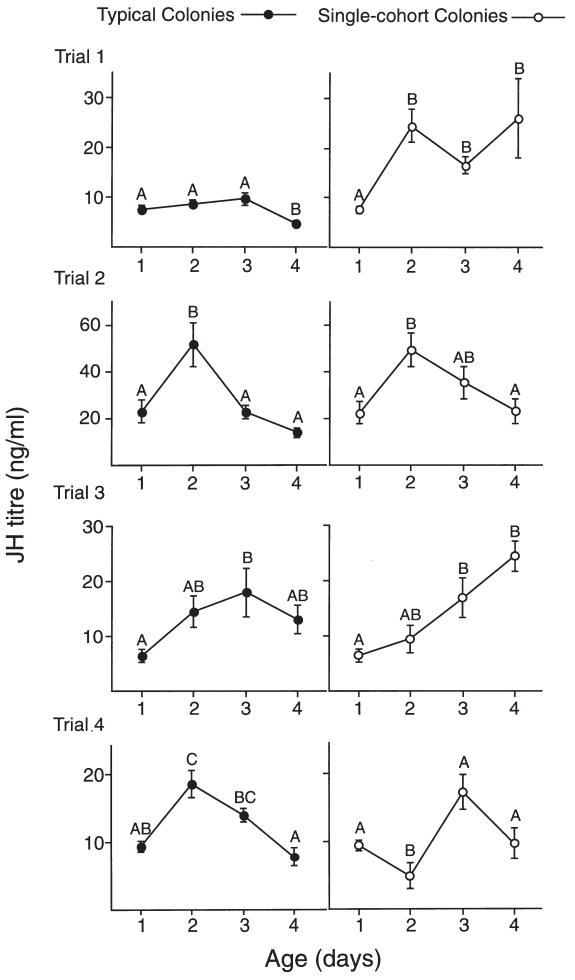

Fig. 4. Age-related changes in JH titres (mean±SE) during the first 4 days of adulthood. *N*=10 bees per day. Data points topped with different letters are significantly different at the 5% level, Tukey's HSD test (after ANOVA showed $P<0.05$ for each colony).

The function of this peak is not known. There are no differences in timing or magnitude between typical or single-cohort colonies, so it does not appear to be associated with differences in the rate of behavioural development. Perhaps it plays some organisational role in honey bee behavioural maturation. Sullivan et al. (1996) found that allatectomised bees were more likely to disappear while taking orientation flights. These bees were allatectomised at 1 day of age, and radioimmunoassay confirmed low and barely detectable levels following surgery, indicating that they did not experience this peak. Future studies can explore the role of this peak by allatectomising workers before and after the time when the JH peak occurs.

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