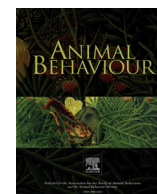




Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Special Issue: Kin Selection

Nurse bee behaviour manipulates worker honeybee (*Apis mellifera* L.) reproductive developmentYing Wang^{a,*}, Osman Kaftanoglu^a, M. Kim Fondrk^b, Robert E. Page Jr.^a^a School of Life Sciences, Arizona State University, Tempe, AZ, U.S.A.^b Department of Entomology, University of California, Davis, CA, U.S.A.

ARTICLE INFO

Article history:

Received 15 October 2013

Initial acceptance 3 January 2014

Final acceptance 4 February 2014

Available online 14 March 2014

MS. number: ASI-13-00865

Keywords:

Apis mellifera

body mass

division of labour

food delivery

honeybee

larval development program

nonreproductive caste

nurse feeding program

ovariole number

reproductive development

The evolution of nonreproductive castes is a fundamental question in evolution biology. The honeybee *Apis mellifera* L. has a reproductive division of labour: the queen is the primary egg-layer in a colony and has more than 200 ovarian filaments (ovarioles), whereas a worker normally does not reproduce and has fewer than 20 ovarioles. The number of ovarioles influences worker foraging behaviour and the propensity to become an egg-layer in the absence of the queen, suggesting that reproductive regulatory networks evolved with foraging division of labour in honeybee workers. Cooperation between nurse bee feeding behaviour and larval developmental programming results in the differentiation of queens and workers along with variation in ovariole number, body mass and foraging behaviour. Here, we tested how nurse bees affect ovariole number and body mass in workers, and how larvae respond to food delivery during different larval life stages. Our findings demonstrate that nurses control larvae growth and ovariole number by temporally manipulating food delivery and that the response of larvae to food differs with larval life stage and genotype. Body mass of larvae was more sensitive to nutrition during the first to the fourth instar (L1–L4), whereas ovariole number was more sensitive during the fifth instar (L5). Overall, we were able to decouple the nurse feeding program and the larval development program in honeybees. We conclude that nurse feeding behaviour during L5 is critical for modulating ovariole number in workers.

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Social insects have fascinated natural historians for millennia; however, for Darwin (1859/1998), they presented difficulties for his theory of evolution by natural selection. Darwin struggled with the evolution of nonreproductive workers in social insects and with the further differentiation of nonreproductive workers into differentiated castes. How could sterile castes evolve if reproductive success drives evolution? Darwin also struggled with the evolution of complex social behaviour, especially the construction and near perfection of honey comb by 'a crowd of bees working in a dark hive' (Darwin, 1859/1998, pp. 348–349). This was a two-part problem for Darwin for which he invoked family-level selection as an explanation. Hamilton (1964a, 1964b) proposed inclusive fitness as a mechanism for the first part (the evolution of loss of personal reproductive success), while most assume that colony-level (family) selection, which is inclusive of inclusive fitness, is responsible for the evolution of social organization for nutrition,

reproduction and protection, the traits of a superorganism (Hölldobler & Wilson, 2008, p. 8; Wilson, 1971, pp. 147, 317–318).

Selection on the insect superorganism affects multiple organizational levels, including the group, the individual and the inclusive fitness component derived from genetic relatedness, and it shapes the interaction structure of nestmates that is responsible for social behaviour (Hölldobler & Wilson, 2008; Page, 2013; Wade, 1985). The effects of selection, however, ultimately must be expressed in the anatomy, physiology and behaviour of the individual colony members and result from changes in developmental processes. Control of developmental processes is shared among nestmates of similar and different life stages and coevolves (Linksvayer, 2006; Linksvayer & Wade, 2005). An example is the coevolution of the feeding behaviour of worker honeybees (nurses) and larval development resulting in the development of a honeybee queen or worker (Leimar, Hartfelder, Laubichler, & Page, 2012). Honeybees go through eight development stages: egg, five larval instars with a prepupal stage at the end of the fifth instar (L5), where larvae no longer eat, a pupal stage, and finally becoming an adult (Winston, 1987). Nurse bees feed the larvae proteinaceous glandular secretions throughout development. Nurse bees differentially feed

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larvae and thereby control the differential developmental responses of the larvae, resulting in a queen or worker phenotype. The timing, quantity and sugar content of the food provided to larvae modulate the production of developmental hormones such as juvenile hormone (JH) and ecdysteroids, which affect tissue differentiation at the final moult (Leimar et al., 2012). However, the feeding program for worker larvae is more complex than that of queens (Leimar et al., 2012).

Of special interest is how the ovariole number in workers is determined during larval development, and what drives differences between high- and low-strain workers in the structure of their ovaries (Amdam, Csondes, Fondrk, & Page, 2006; Rueppell et al., 2011; Wang et al., 2009; Wang et al., 2012). Ovaries are composed of filamentous ovarioles in which eggs are produced. Honeybee workers have ovaries that are greatly reduced compared to queens (on average, fewer than 20 ovarioles for workers versus 200–250 for queens; Linksvayer et al., 2011), but can still be activated and produce eggs. Ovariole number has been shown to regulate foraging behaviour in wild-type bees (Wang, Kaftanoglu, Siegel, Page, & Amdam, 2010) and was differentially selected as a consequence of selection for high and low pollen hoarding (producing high and low pollen-hoarding strains), suggesting that reproductive regulatory networks were co-opted by natural selection during the evolution of foraging division of labour (Amdam, Norberg, Fondrk, & Page, 2004; Amdam & Page, 2010; Page & Amdam, 2007; Page, Rueppell, & Amdam, 2012; West-Eberhard, 1996). High-strain worker bees, on average, have more ovarioles than do low-strain workers, and they forage earlier in life and are more likely to collect larger loads of pollen. Similar relationships are seen in wild-type bees (Siegel, Freedman, & Page, 2012; Siegel, Kaftanoglu, Fondrk, Smith, & Page, 2012; Wang et al., 2009) and in the closely related honeybee species, *Apis cerana* Fabricius (Rueppell, Hunggims, & Tingek, 2008): workers with more ovarioles tend to collect more pollen.

Queen and worker differentiation is controlled by nurse bee feeding behaviour. The number of ovarioles in adult worker and queen honeybees is determined by JH levels, which is modulated by the food that nurses deliver during later larval instars (Antoniali & da Cruz-Landim, 2009; Capella & Hartfelder, 1998; Dedek, Hartfelder, Aumeier, Rosenkranz, & Engels, 1998; Schmidt Capella and Hartfelder, 2002). At this time, queen and worker larvae both have a similar number of ovariole primordia (Hartfelder & Steinbruck, 1997). Ovarioles are lost through programmed cell death (PCD) beginning in L5. Higher JH titres protect ovarioles from PCD (Capella & Hartfelder, 1998; Leimar et al., 2012). Recent studies on high and low strains have suggested the ovariole number in workers is modulated by both nurse feeding behaviour and an intrinsic developmental program. High-strain bees raised by low-strain nurses have more ovarioles, and low-strain bees raised by high-strain nurses have fewer ovarioles, compared to when members of each strain are raised by their own sibling nestmates, suggesting that low-strain nurses provide more or higher-quality food than high-strain nurses (Linksvayer, Fondrk, & Page, 2009; Linksvayer et al., 2011). However, the fact that high-strain bees have more ovarioles than low-strain bees even when cross-fostered shows that a genetic component in larval development also plays a role in determining ovariole number.

Body mass is an indicator of developmental nutritional status in different species. In general, there is a linear relationship between body mass and ovariole number in honeybees (Leimar et al., 2012). However, body mass and ovariole number can be dissociable and independently selectable. For example, high-strain bees are smaller than low-strain bees but have more ovarioles (Linksvayer et al., 2011; Wang et al., 2009). Likewise, Africanized honeybees are smaller than bees of European origins (Daly & Balling, 1978; Hunt,

Guzman-Novoa, Fondrk, & Page, 1998; Sylvester & Rinderer, 1987) but, on average, have more ovarioles (Linksvayer, Rueppell, et al., 2009). Linksvayer et al. (2011) showed that the relationship between body mass and ovariole number depends on the genotype of the larva and to some extent the genotypes of the nurse bees providing for the larvae.

Based on the above arguments, we asked how nutrition affects body mass and ovariole number of workers at different larval life stages, and how nurse bees temporally manipulate food delivery to regulate body mass and ovariole number of developing bees. We hypothesized that the effects of nutrition on developmental body mass and ovariole number differ in honeybee larvae. Specifically, food quantity or quality should have a greater effect on ovariole number during L5, the critical stage for rescuing ovarioles from programmed cell death, than it does during the first to the fourth instar (L1–L4). Low-strain nurses feed larvae more and/or better-quality food than high-strain nurses (Linksvayer, Fondrk, et al., 2009; Linksvayer et al., 2011); therefore, we used high- and low-strain bees to manipulate larval feeding. First, we tested the effects of food deprivation during L5 on body mass and ovariole number in wild-type bees (experiment 1). Next, we tested the effects of food deprivation on high-strain, low-strain and wild-type bees to see whether they would respond differentially as a consequence of differences in genotype (experiment 2). Then, we fed larvae more food than they would normally receive from nurse bees by extending the feeding period into the postcapping, starvation period of larval development to determine whether they are physiologically able to continue growth during the late L5 stage (experiment 3). And finally, we cross-fostered high- and low-strain larvae during L1–L4 and L5 and tested whether the effects on worker body mass and ovariole number at emergence differed by larval life stage (experiment 4).

METHODS

The first three experiments were performed in 2012–2013 at Arizona State University. The fourth experiment was conducted during May and June in 2013 at the University of California Harry H. Laidlaw Honey Bee Research Facility in Davis, California, where high and low pollen-hoarding strains are stored as well.

Experiment 1

Two wild-type colonies were used for testing the effect of food deprivation. The queen of each colony was caged on one side of a frame by using a 16 × 32 cm queen excluder push-in cage for approximately 24 h in the original hive while she laid eggs. One week (180 h) after caging the queens the larvae derived from the eggs had entered the L5. One half of the larvae were caged again by using 160 × 160 × 5 mm wire screen push-in cages for 12, 15 and 18 h. To prevent the larvae from being fed by the nurse bees, double screens were used. The other half of the larvae were used as controls (0 h). Screens were removed after the appropriate interval and larvae were allowed to complete development in the presence of nurse bees. The combs containing treatment and controls were removed from the hive 20 days after caging the queen. At this time they were developing pupae. Brood areas were covered with 160 × 160 × 15 mm push-in cages to separate treatment and control groups, then the combs were placed in an incubator at 34 °C and 60% RH. The next day, bees began emerging under the screens and were collected into separate containers for determining body mass, as wet weight, and ovariole number. Multiple regression was used to determine the

relationships between duration of food deprivation, body mass and ovariole number.

Experiment 2

Two wild-type, two high-strain and two low-strain colonies (Page & Fondrk, 1995) were randomly selected. The technique described above for experiment 1 was used for caging queens and starving the L5 larvae. The larvae were starved for 10 h and kept in the hives before they emerged. The newly emerged bees were weighed and dissected to determine body mass and ovariole number. Factorial ANOVA was used to determine the treatment effect and genotype effect on body mass and ovariole number.

Experiment 3

Two wild-type queens were caged for 24 h to obtain same-aged larvae. When the larvae reached to L5, they were carefully grafted on an artificial diet (53% royal jelly, 12% glucose, 12% fructose, 1% yeast extract, and 22% water; Kaftanoglu, Linksvayer, & Page, 2010; Patel et al., 2007). Larvae were allowed to feed for one extra day. 'Overfed' larvae were moved into 24-well cell culture plates and placed in an incubator at 34 °C and 60% RH until emergence as adults. Controls were same-aged bees that were raised in their maternal colony and put into the same incubator before they emerged. We determined body mass and ovariole number of newly emerged bees in each treatment. Factorial ANOVA was used for data analysis. Treatment and hive were independent factors, and body mass or ovariole number was the dependent factor, respectively.

Experiment 4

Honeybees were from a pollen-hoarding selection program that has been under continuous selection for 42 generations, two generations per year (Page & Fondrk, 1995). Three high-strain colonies and three low-strain colonies were used as donors for the cross-fostering experiment. Donor colonies were chosen based on the amount of stored pollen present.

In general, we used a cross-fostering design in which high- and low-strain focal larvae were reared in high- and low-strain colonies for varying amounts of time, resulting in six combinations of focal individuals (Linksvayer, Fondrk, et al., 2009). We transferred combs containing larvae between high- and low-strain colonies to produce treatments where larvae were cross-fostered between strains for either L1–L4 or L5. Controls were not cross-fostered (see Fig. 1 and Supplementary Table S1 for more details).

Three replicates, each with a different pair of high and low colonies matched for size and colony condition were used in this study. Queens from the donor high and low colonies were caged on combs to obtain eggs for the study. Once the required eggs had been secured, two nucleus colonies (nuc A and nuc B) were assembled from each donor hive composed of four frames of mixed-age adult worker bees, one frame of emerging brood, one frame of open brood, one frame of honey and one frame containing 75 g of pollen. These nucs were nursery colonies for developing larvae. Later on the same day each nuc was provided with a Bee Boost lure (PheroTech, Delta, BC, Canada), a commercial artificial blend of queen mandibular pheromone that simulates queen presence.

To ensure that all nucs used as nurseries had sufficient populations of young nurse bees, extra frames of mature brood from high and low colonies were placed together in a hive body in an incubator overnight at 34 °C and 60% RH. The following day, hives

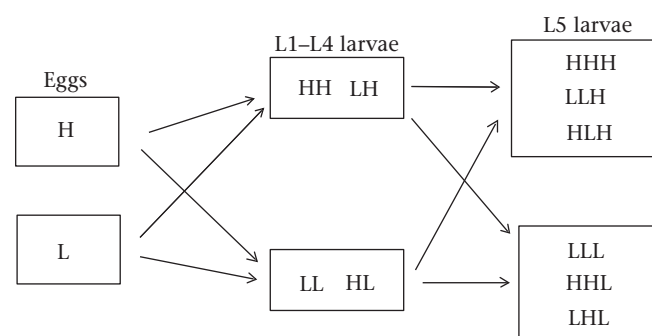


Figure 1. Diagram of the cross-fostering experiment of honeybees at different larval stages. Eggs from high (H) and low (L) colony sources were fostered in high and low colonies for stages L1–L4 and for stage L5. Boxes on the left show the strain of the eggs; boxes in the middle show the genotype of nurse bees during L1–L4; boxes on the right show the genotype of nurse bees for L5. Letter designations show the treatments for the larvae. For example, following a control bee from the high strain (H) to the genotype of the nurses for L1–L4 (HH) to the genotype of the nurses for L5 (HHH). A high-strain larva cross-fostered during L5 would be HHL. A low-strain larva cross-fostered only during L1–L4 is designated LHL, etc. The detailed treatment combinations can be found in [Supplementary Table S1](#).

were evaluated and equalized for worker population and all brood was removed from the nucs. Additional combs with emerging bees were placed in the incubator to provide bulk young bees for test colonies. Bulk newly emerged bees obtained from the incubator were brushed from combs and divided between test nucs by strains. Approximately equal numbers of workers of the appropriate strain were added to the nucs. This procedure was repeated for 3 days.

Each comb of focal eggs was split into six sections with approximately equal numbers of eggs. Each section of comb and/or frame was identified as to colony source and colony rearing environment. On the initial day of the test, each recipient nuc included the following: older larvae from the parent donor colony in half of the comb, one section of mature eggs from a high-donor colony and one section of mature eggs from a low-donor colony along with the aforementioned honey, pollen and bees. On day 2 of the experiment, a comb of eggs from the parent donor colony was inserted into each nuc and allowed to develop for the duration of the experiment to serve as a control. On day 3, nursery colonies were evaluated again for worker population and food stores, and no differences were found between them. On day 4 of the experiment, when the focal brood was probably in transition from L4 to L5, some sections of combs were switched. Therefore, larvae that started in a low-pollen worker rearing environment (L1–L4) finished in a high-pollen worker rearing environment (L5) and sections of brood that started in a high-pollen worker rearing environment were switched to a low-pollen worker environment (Fig. 1, Supplementary Table S1).

Frames and comb sections with colony-reared mature pupae were removed from the nucs 24 h before the workers emerged and placed in individual cages in incubators overnight at 34 °C and 60% RH. All sections of combs were identified as to colony source and colony rearing environment. Wax cells full of pollen or honey were covered by aluminium foil, so that newly emerged workers did not have access to food. We weighed 25 newly emerged focal individuals and counted the number of ovarioles in each. We used one-way ANOVA across all treatment groups because the sample size of bees in some subcolonies was too small for a full factorial design. Subcolonies showed large variation in body mass and ovariole number, so we performed sign tests to confirm the main directions of variation that were tested by one-way ANOVA.

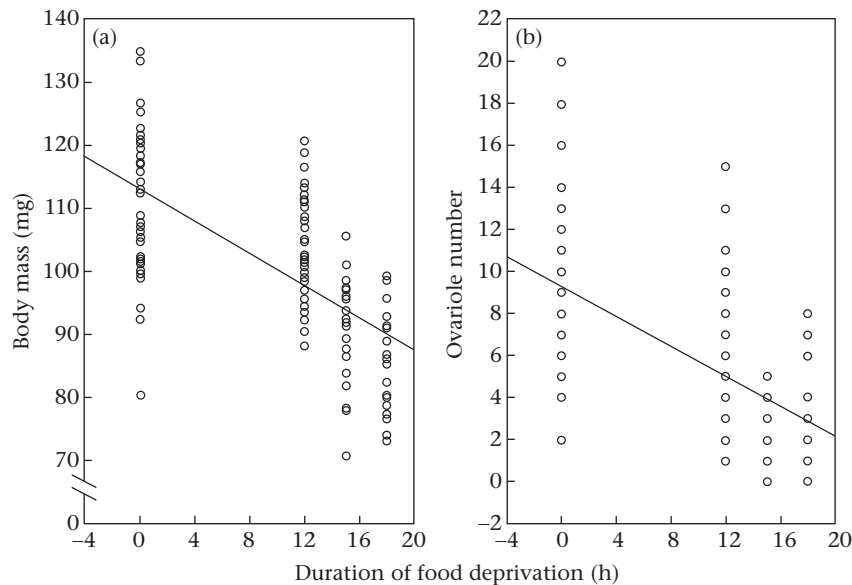


Figure 2. Relations between duration of food deprivation and (a) body mass and (b) ovariole number in experiment 1.

RESULTS

Experiment 1

Body weight ($R^2 = 0.42$, $N = 126$, $P < 0.001$) and ovariole number ($R^2 = 0.35$, $N = 126$, $P < 0.001$) scaled linearly with the duration of food deprivation (Fig. 2). Body weight and ovariole number were correlated ($R^2 = 0.16$, $N = 126$, $P < 0.001$; Fig. 3). Larvae lost 1.3 mg of body mass and 0.35 ovarioles per hour of food deprivation. Larvae lost 0.12 ovarioles per milligram of body mass. These results suggest that the timing of food deprivation influences body mass and ovariole number in adult workers.

Experiment 2

High-strain, low-strain and wild-type colonies used in this experiment were drawn at random from available colonies. Although the mean body mass and the number of ovarioles differ between strains, they also vary among colonies within strains, and

the distributions overlap broadly (Linksvayer, Fondrk, et al., 2009; Linksvayer et al., 2011). In addition, ovariole number varies seasonally and with individual colony conditions. Therefore, it is not unusual to have overlapping distributions as shown here when comparing the results of individual high- and low-colony controls.

High-strain, low-strain and wild-type larvae responded to 10 h of starvation during L5 with a loss in body mass and a reduction in the number of ovarioles (factorial ANOVA: $N = 30$ – 40 , treatment effect on body mass: $P < 0.001$; treatment effect on ovariole number: $P < 0.001$; genotypic effect on body mass: $P < 0.05$; genotypic effect on ovariole number: $P < 0.01$; Fig. 4). Ovariole number was correlated with body mass in all six colonies of this study ($P < 0.01$; Fig. 5). Number of ovarioles per milligram of body mass ranged between 0.10 and 0.24 for the different colony sources, with a mean of 0.16. Larvae lost 1.17 mg of body mass and 0.35 ovarioles per hour of food deprivation. Our findings here suggest that the effects of food deprivation on body mass and ovariole number are consistent across genotypes and that genotype is an important contributor to body mass and ovary development.

Experiment 3

Providing food beyond the normal larval capping time resulted in adult wild-type workers with greater body mass (factorial ANOVA: $N = 24$ – 38 , $P < 0.001$; Fig. 6). This extra feeding resulted in heavier adult bees from two replicate hives (post hoc Fisher LSD test: hive 1: $P < 0.001$; hive 2: $P = 0.068$). The response to extra feeding differed between the two test hives, resulting in a significant hive*treatment interaction ($P = 0.008$), suggesting that genotype influenced the response of larvae to the extra feeding treatment.

Additional food also resulted in bees with more ovarioles (factorial ANOVA: $P = 0.005$), although the effect was not as strong as for body mass (Fig. 6). The adult bees with extra feeding had more ovarioles in hive 1, but no statistically significant increase in ovariole number was found for hive 2 (post hoc Fisher LSD test: hive 1: $P = 0.007$; hive 2: $P = 0.187$). There was a significant relationship between body mass and ovariole number in hive 1 ($R^2 = 0.15$, $P = 0.002$). Bees from hive 1 gained 0.07 ovarioles for every additional milligram of body mass. Similarly, bees from hive 2 gained 0.08 ovarioles per milligram of body mass.

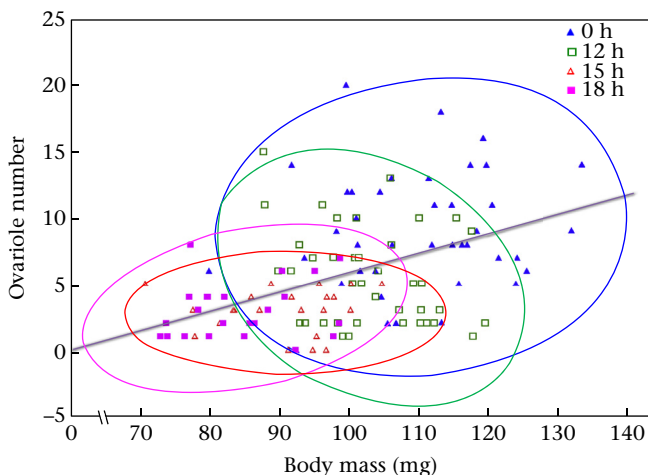


Figure 3. Relation between ovariole number and body mass, with 95% ellipses, for food deprivation treatments of 0, 12, 15 and 18 h in experiment 1.

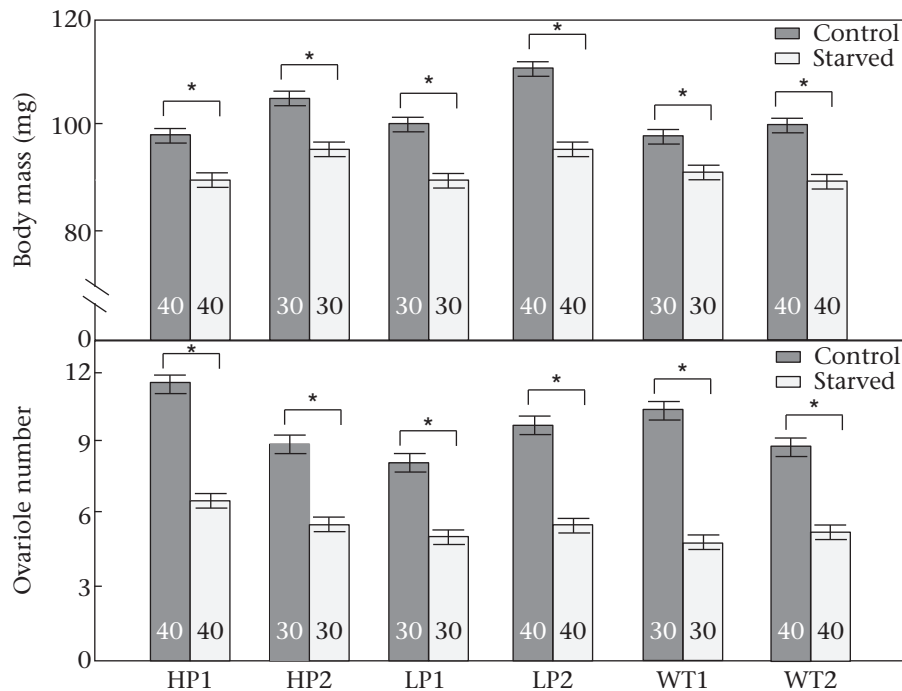


Figure 4. Effect of food deprivation on body mass and ovariole number of workers from high (HP1 and HP2), low (LP1 and LP2) and wild-type (WT1 and WT2) genetic strains in experiment 2. Asterisks indicate significant difference between treatment groups within each colony ($P < 0.05$). Bars represent means \pm SE. Sample sizes are shown at the bottom of each bar.

Experiment 4

We compared body mass and ovariole number of 925 bees derived from a total of six colony sources where the queens were mated with three drones each, two subcolonies derived from each source and two to three treatment conditions for each subcolony. However, we were not able to perform a full factorial design to test all three treatments in all subcolonies because of larval availability (see details of combinations in [Supplementary Table S1](#)). Altogether we had a total of 35 combinations of source, colony conditions and fostering treatments, with significant larval genotypic variation in

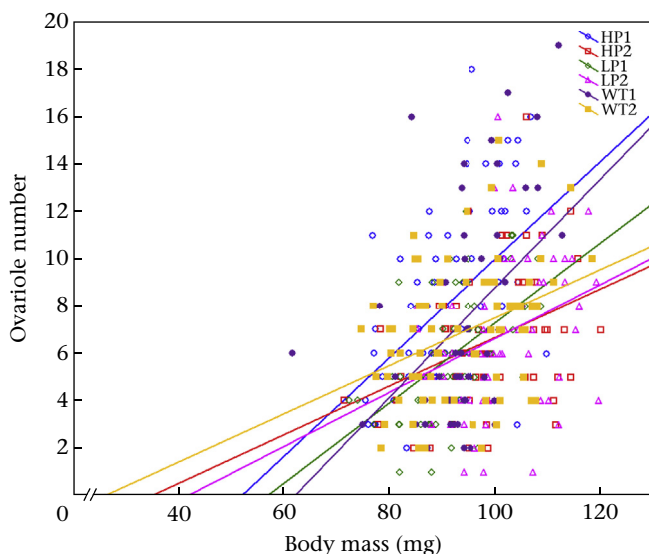


Figure 5. Relation between body mass and ovariole number in colonies from each genetic strain in experiment 2. Strain designations as in [Fig. 4](#).

each. Thus, we tested a broad representation of genotypes and larval rearing conditions. As we predicted, low-strain bees raised by high-strain nurse bees in general weighed less and had fewer ovarioles than when they were raised by their own sibling nurses. High-strain bees weighed more and had more ovarioles when raised by low-strain nurses ([Fig. 7](#)).

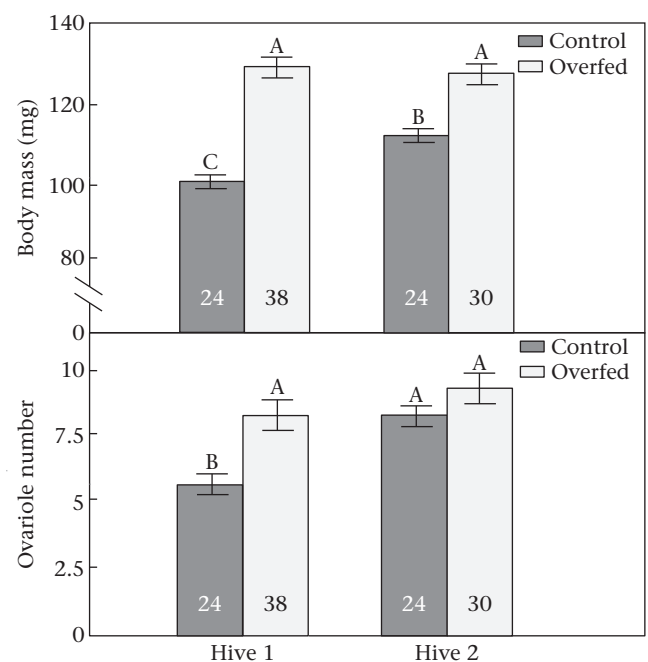


Figure 6. Effects of extra feeding during L5 on body mass and ovariole number in experiment 3. Different letters above bars indicate statistically significant differences ($P < 0.05$) based on post hoc Fisher LSD tests. Bars represent means \pm SE. Sample sizes are shown at the bottom of each bar.

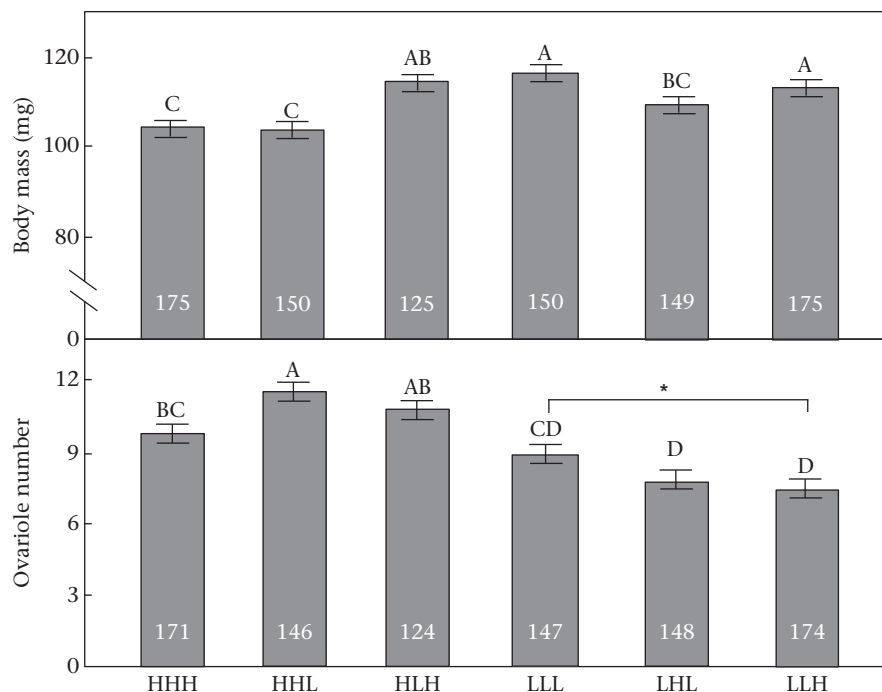


Figure 7. Effects of the cross-fostering program (see Fig. 1, Supplementary Table S1) on body mass and ovariole number in experiment 4. Different letters above bars indicate statistically significant difference ($P < 0.05$) derived from one-way ANOVA and post hoc Fisher LSD tests. The asterisk indicates a statistically significant difference ($P < 0.05$) between LLL and LLH when tested among the low-strain groups. Bars represent means \pm SE. Sample sizes are given inside bars.

Body Mass

Nurse bee genotype significantly affected body mass (one-way ANOVA: $N = 124$ – 175 , $P < 0.001$; Fig. 7). Overall, body mass varied among treatments as expected: low-strain bees were heavier than high-strain bees (post hoc Fisher LSD test: $LLL > HHH$: $P < 0.001$). High-strain bees reared in low-strain colonies during L1–L4 were much heavier than the high-strain bees reared in their own colonies (post hoc LSD test: $HLH > HHH$: $P < 0.001$) and heavier than the high-strain bees reared in low-strain colonies after L4 (post hoc LSD test: $HLH > HHL$: $P < 0.001$). However, there was no difference in the body mass between the high-strain controls and high-strain bees reared in low-strain colonies after L4 (post hoc LSD test: $HHH = HHL$: $P = 0.805$). Low-strain bees reared in high-strain colonies during L1–L4 weighed less than low-strain controls (post hoc LSD test: $LHL < LLL$: $P < 0.001$) and low-strain bees reared in high-strain colonies after L4 (post hoc LSD test: $LHL < LLH$: $P = 0.013$). The weight of low-strain bees reared in their own colonies (L1–L5) did not differ from that of low-strain bees reared in their own colonies during L1–L4 (post hoc LSD test: $LLL = LLH$: $P = 0.223$). These results are consistent with our previous findings that high- and low-strain nurses differ in their feeding behaviour. In addition, the results also suggest that body mass is more sensitive to nutrition during L1–L4 than during L5.

A comparison of high-strain and low-strain controls (HHH and LLL) between replicate subcolonies (nucs derived from the same colony source) showed significant variation in body mass between bees of the same source reared in different subcolonies (Supplementary Table S2). This demonstrates the sensitivity of larval development as a consequence of differences in colony environment even when the genotypes of the nurse bees are the same, in this case siblings. In addition, we tried to make the two subcolonies from each source as similar as possible. Overall, five subcolonies demonstrated significant differences among treatments. Four of the five subcolonies varied significantly in the

direction predicted based on the genotypes of the nurse bees that reared them.

Because of the significant variation in subcolonies, sign tests of the rank orders of all treatment combinations from all replicates were performed (Table 1), which confirmed the same relationship shown in Fig. 7. Adult workers raised by low-strain nurse bees for four or more instars were heavier than workers raised by high-strain nurses. These results are consistent with our previous findings that low-strain nurses increase body mass of workers they raise while high-strain nurses decrease it. In addition, the results also suggest that the body mass of honeybees is more sensitive to nutrition during L1–L4.

Table 1

Sign test results for body mass expectations based on specific treatment comparisons for replicates of experiment 4

Source/subcolony	Expected vs observed difference in body mass*		Larval strain
	HLH>HHH	HHL>HHH	
PS4A	+	na	H
PS4B	na	+	H
PS8A	+	–	H
PS8B	+	+	H
RR6A	+	+	H
RR6B	+	–	H
	LLL>LHL	LLL>LLH	
RR9A	na	–	L
RR9B	+	+	L
WS6A	–	–	L
WS6B	+	–	L
RR3A	+	+	L
RR3B	+	+	L
Sign rank probability	0.01	0.50	

H: high pollen-hoarding strain; L: low pollen-hoarding strain.

* Expectations were that low-strain nurses would produce larger adult workers than high-strain nurses. Differences in the ordered direction of treatment means were either in the predicted direction (+) or not in the predicted direction (–). 'na' means that the comparison could not be made because of missing treatment groups.

Ovariole Number

Nurse bee genotype significantly affected ovariole number (one-way ANOVA: $P < 0.001$; Fig. 7). Looking at the total data set, high-strain bees tended to have more ovarioles than low-strain bees. High-strain bees reared by low-strain nurses during L5 had significantly more ovarioles than the high-strain controls (post hoc Fisher LSD test: $HHL > HHH$; $P = 0.002$). However, there was no difference in ovariole number when high-strain bees were reared by low-strain nurses during L1–L4 (post hoc LSD test: $P = 0.221$). Although low-strain bees did not demonstrate significant treatment effects when high and low sources were analysed together, an analysis of low-strain sources showed that low-strain bees had significantly fewer ovarioles when raised by high-strain nurses during L5 than did the low-strain controls (one-way ANOVA, post hoc LSD test: $LLH < LLL$; $P = 0.027$). This result is consistent with that of the high strain, suggesting that food quantity and/or quality during L5 is critical for ovariole development in workers. In addition, our results indicate that low-strain nurses increase ovarioles of the bees they feed, and high-strain nurses decrease ovariole number. This result in ovariole number is also consistent with our finding for body mass, which may be a consequence of more and less food being provided by low- and high-strain nurses, respectively (Linksvayer, Fondrk, et al., 2009; Linksvayer et al., 2011).

Source replicates (subcolonies) did not vary significantly in any comparison (Supplementary Table S3). It appears that the direct effect of colony environment on ovariole number is less than that on body mass. Larvae assigned to three subcolony replicates varied significantly with treatment, although not in a consistent way.

Sign tests of the ranks of all the combinations in replicates demonstrated the same relationship (Table 2) shown in Fig. 7. The genotype of nurses during L5 had a significant effect on ovariole number in adult workers, and this effect was stronger than that of nurse bees during L1–L4.

High-strain workers gained 0.2 and 5.7 ovarioles per milligrams of body mass gained when larvae were raised by low-strain nurses during L1–L4 and L5, respectively. Low-strain bees lost 0.2 and 0.8 ovarioles per milligram of body mass lost when reared by high-strain nurse bees during L1–L4 and L5, respectively. These results clearly demonstrate that ovariole number is more sensitive to nutrition during L5.

Table 2

Sign test results for the number of ovarioles expected based on specific treatment comparisons for replicates of experiment 4

Source/subcolony	Expected vs observed difference in ovariole number		Larval strain
	HHL>HHH	HHL>HHH	
PS4A	–	na	H
PS4B	na	+	H
PS8A	+	+	H
PS8B	+	+	H
RR6A	–	+	H
RR6B	–	+	H
	LLL>LHL	LLL>LLH	
RR9A	na	+	L
RR9B	+	+	L
WS6A	+	+	L
WS6B	+	–	L
RR3A	+	+	L
RR3B	+	+	L
Sign rank probability	0.172	0.006	

See Table 1 for details.

DISCUSSION

Colony-level selection on honeybees has led to complex developmental and behavioural interactions resulting in caste and behavioural differentiation. These interactions and the subsequent changes in colony phenotype are hallmarks of superorganism (Hölldobler & Wilson, 2008). Here, our study demonstrates a social interaction between developing larvae and nurse bees that results in variation in body mass and number of ovarioles. Developing larvae are sensitive to the timing, quantity and quality of food delivered by the nurse bees. However, the composition of glandular secretions fed to queen and worker larvae is the same (Schmitzová et al., 1998; Shuel & Dixon, 1959, 1960). Queen-destined larvae are fed ad libitum a surplus of glandular secretions containing royal jelly proteins and a constant amount of sugar (nectar and honey) throughout development. The quantitative and qualitative feeding program for worker larvae, however, involves four temporal stages, apparently designed to restrict their body size and reproductive organs, and results in the differentiation of worker phenotypic characters. Initially, they are fed ad libitum, like queens, however, the food they receive has less sugar (Asencot & Lensky, 1976, 1985, 1988). The extra sugar fed to queens is believed to increase juvenile hormone (JH) and respiration in the queen larvae (Antoniali & da Cruz-Landim, 2009; Asencot & Lensky, 1984; Nijhout & Wheeler, 1982). The JH is probably involved in the differentiation of target tissues that differ between the worker and queen phenotypes (Leimar et al., 2012). For the next stage, beginning in mid L3, larvae are restricted in the amount of food they receive, presumably affecting the gain in body mass. In mid L4, nurses increase the sugar concentration of the food to approximately the same as queens, which is essential for the larvae to enter the final moult. If the sugar is not increased, the larvae will fail to moult and will die (Asencot & Lensky, 1988; Kaftanoglu et al., 2010). Then, during mid L5, worker larvae are capped in their cells without food for about 2 days prior to moulting into pupae. Cells containing queen larvae are capped with a surplus of food on which they continue to feed until they moult into prepupae.

Developmental plasticity of larvae in body mass and number of ovarioles was demonstrated by our food deprivation and food excess experiments (experiments 1–3). Food deprivation during L5 resulted in a linear decrease in both body mass and ovariole number. In experiment 1, 1.28 mg of body mass and 0.12 ovarioles were lost for every hour that L5 larvae were not in contact with nurse bees. Results from experiment 2 were comparable, with an average of 1.17 mg and 0.16 ovarioles lost per hour, demonstrating that L5 is an active growth period, demanding protein, and that the allocation of resources into body mass and reproductive tissues is remarkably consistent. Workers continue to gain body mass and develop more ovarioles if allowed to feed beyond the normal starvation initiation time. Experiment 3 shows that workers can gain an additional 15.4–36.1 mg and 1.3–2.5 ovarioles when allowed to feed for the extra days. Assuming 48 h of additional feeding, they gain 0.23–1.5 mg per hour of additional feeding and 0.07–0.08 ovarioles per milligram, showing that nurse bees influence development (restrict body mass and ovary development) by capping cells before development is complete.

Nurse bee behaviour and/or feeding physiology, which are determined by their genotypes, coevolves with larval development. Artificial selection for pollen hoarding resulted in two genetic strains that differed in many phenotypic traits, including body mass, ovariole number and the developmental relationship between body mass and ovariole number. Using these colony-level selected strains, we found that high-strain larvae gained significantly more ovarioles per milligram of body mass when they were fed by low-strain nurses than when they were raised by high-strain

workers, suggesting differences in food delivery by nurses (Linksvayer et al., 2011). Differences could be quality, quantity, timing, or any combination. Confirming previous results (Linksvayer, Fondrk, et al., 2009; Linksvayer et al., 2011), high-strain nurses fed larvae less food, but high-strain larvae dedicated more to reproductive tissues (Leimar et al., 2012). Cross-fostering larvae between strains resulted in direct effects of nurses on body mass and ovariole number, a consequence of differences in nurse bee feeding behaviour. However, differences in the feeding response of larvae remained between strains even when reared together in the same hives at the same time, showing that the larval genotype also affects developmental programming. Therefore, our results suggest that colony-level selection affects the coevolution of adult behaviour genotype and larval developmental genotype.

The allocation of food resources for body mass and ovaries is also shared between a larva and the nurse bees. When fed *ad libitum* in the laboratory, high- and low-strain larvae develop differently with regard to ovarioles per milligram of body mass. The scaling relationship also changes on the basis of the genotype of the nurse bees when larvae are fed in the hive (Linksvayer et al., 2011). Manipulation of the number of ovarioles per milligram of body mass could be attained by the temporal delivery of food, as observed in experiment 4. High-strain bees raised by low-strain nurses during L1–L4 were significantly larger but did not differ from high-strain controls in the number of ovarioles; therefore, they had fewer ovarioles per milligram of body mass. High-strain larvae raised by low-strain nurses during L5 did not differ in body mass from high-strain controls, but they had significantly more ovarioles, and hence more ovarioles per milligram of body mass. Whereas, low-strain larvae raised by high-strain nurses during L1–L4 weighed less than low-strain controls, but they did not differ in ovariole number; low-strain bees raised by high-strain nurses in L5 did not differ in body size from low-strain controls, but they had even fewer ovarioles. These findings clearly show that worker larvae developmental programs of body mass and ovariole number are partly independent: body mass increases throughout L1–L5, whereas the number of worker ovarioles is determined during L5. Our interpretation is that L5 is the critical stage for the rescue of ovariole from programmed cell death (PCD) by JH, and food restriction may be a key factor for nurses to control worker ovariole number through regulating JH level. Additionally, it is interesting to note that overfeeding beyond the normal feeding interval during L5 had less effect on both body mass and ovariole number than did food deprivation, and the effect of overfeeding on ovary development was less than that on body mass. Previously, it has been shown that applying JH on worker larvae at L5 can reverse worker phenotypes to queen phenotypes, including ovarioles (Capella & Hartfelder, 1998). Therefore, our result may suggest that JH level is more sensitive to food deprivation than it is to overfeeding.

Amdam et al. (2004) suggested that reproductive regulatory networks of honeybee ancestors were co-opted by natural selection during the evolution of foraging division of labour. Bees that are more reproductively activated, those with larger, more activated ovaries, forage more for protein, in this case, pollen. They presented evidence showing that pollen-foraging honeybee workers tend to have larger, more reproductively activated ovaries than do nectar foragers. During our selection program for pollen hoarding, colonies were indirectly selected as parents that had workers with more and fewer ovarioles that collected more and less pollen (the high and low strains, respectively). Differences in ovariole number and, hence, reproductive state, occurred as a consequence of changes in the feeding behaviour of the nurse bees and the ontogeny of the larvae. A critical selection target was JH titer in L5 (Amdam, Page, Fondrk, & Brent, 2010), resulting in differences in the number of ovarioles rescued from apoptosis (Capella &

Hartfelder, 1998). Hence, two evolutionarily ubiquitous developmental ‘modules’, nutrition-sensitive PCD (Edvardsson, Hunt, Moore, & Moore, 2009; Leimar et al., 2012; Terashima, Takaki, Sakurai, & Bownes, 2005) and the relationship between reproductive state and foraging behaviour (Amdam & Page, 2010; Atchley, Weaver, & Eckel, 2005; Clarke & Ossenkopp, 1998; Clements, 1992; Klowden, 1990), were co-opted by colony-level selection. Feeding behaviour of nurse bees provides another level of developmental control on foraging division of labour.

Overall, our results show that phenotypic plasticity of honeybee body mass and ovariole number is derived from the complex behavioural and physiological interactions between individuals in different life stages. The adult behaviour and larval physiology coevolve as a consequence of selection on a colony-level trait, stored pollen, and represent true traits of a superorganism (Hölldobler & Wilson, 2008).

Acknowledgments

We thank Dr Gro Amdam for helpful comments.

Supplementary Material

Supplementary material for this article is available, in the online version, at <http://dx.doi.org/10.1016/j.anbehav.2014.02.012>.

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