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Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen–worker dimorphism

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Abstract

Social evolution in honey bees has produced strong queen–worker dimorphism for plastic traits that depend on larval nutrition. The honey bee developmental programme includes both larval components that determine plastic growth responses to larval nutrition and nurse components that regulate larval nutrition. We studied how these two components contribute to variation in worker and queen body size and ovary size for two pairs of honey bee lineages that show similar differences in worker body–ovary size allometry but have diverged over different evolutionary timescales. Our results indicate that the lineages have diverged for both nurse and larval developmental components, that rapid changes in worker body–ovary size allometry may disrupt queen development and that queen–worker dimorphism arises mainly from discrete nurse-provided nutritional environments, not from a developmental switch that converts variable nutritional environments into discrete phenotypes. Both larval and nurse components have likely contributed to the evolution of queen–worker dimorphism.

Introduction

Ancestrally, bees are solitary and females are monomorphic for traits such as body size and ovary size (Michener, 2000). In contrast, in some highly derived social lineages, such as the honey bees (genus *Apis*), there is a reproductive division of labour characterized by strong queen–worker dimorphism for plastic traits such as body size and especially ovary size that depend on nutrition (Hölldobler & Wilson, 1990). Honey bee queens are approximately twice as large as workers in terms of mass, but queens can have more than 360 total ovarioles whereas workers typically have fewer than ten (Michener, 1974; Winston, 1987). Large queen ovaries enable a high egg-laying rate (more than 1500 day^{-1}) and are apparently an adaptation for large colony size and colony reproduction by fission (Winston, 1987). Thus, social evolution in bees leading to the elaboration of queen–worker dimorphism has involved dramatic changes in plasticity for body size and ovary size and the expressed allometric body–ovary size relationship.

Queen–worker caste dimorphism has often been regarded as a prime example of a polyphenism, environmentally induced alternative phenotypes (Wheeler, 1986). In honey bees, it has long been known that the expression of queen vs. worker traits depends mainly on larval nutrition (Weaver, 1955; Wheeler, 1986). However, evidence has accumulated in other social insect lineages that genotype also influence the expression of alternative caste phenotypes (Anderson *et al.*, 2008; Schwander *et al.*, 2010), and even in honey bees, there is evidence of genetic influences on the expression of caste and caste-related traits (e.g. Osborne & Oldroyd, 1999; Beekman *et al.*, 2000; Allsopp *et al.*, 2003; Linksvayer *et al.*, 2009b). The genetic modification of pre-existing

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developmental plasticity is thought to contribute to the evolution of polyphenisms (Moczek & Nijhout, 2002; Nijhout, 2003; Emlen *et al.*, 2007), and studying patterns of genetic variation for developmental plasticity and the expression of caste-related traits may provide insight into the evolution of queen–worker dimorphism.

Although previous studies of the developmental genetic basis of honey bee queen–worker dimorphism have focussed solely on larval genes involved in plastic developmental responses (Evans & Wheeler, 1999; Wheeler *et al.*, 2006; Barchuk *et al.*, 2007; Patel *et al.*, 2007; de Azevedo & Hartfelder, 2008), whether a larva develops into a queen or worker can also depend on genes expressed in nestmates that actively regulate the larval environment (Linksvayer & Wade, 2005). In particular, nurse workers constrain the range of nutritional environments experienced by larvae and thereby control the expression of phenotypic plasticity. Thus, the social insect developmental programme potentially has two distinct genetic components that are expected to co-evolve to shape expressed phenotypes (Wolf & Brodie, 1998; Linksvayer & Wade, 2005; Linksvayer, 2006, 2007; Linksvayer *et al.*, 2009a). Indeed, a range of studies provide evidence that social insect traits, including caste, are influenced by interactions between the genotypes of developing larvae and the genotypes of nurse workers (e.g. Rinderer *et al.*, 1986; Osborne & Oldroyd, 1999; Beekman *et al.*, 2000; Pankiw *et al.*, 2002; Allsopp *et al.*, 2003; Linksvayer *et al.*, 2009a,b; Jarau *et al.*, 2010). These studies suggest that approaches considering the contribution of social regulation of development can complement previous studies focused on differential gene expression in queen- and worker-destined larvae.

Here, we study the contribution of larval and nurse components of the developmental programme to variation between two pairs of honey bee lineages for the relationship between body size and ovary size in queens and workers. Our two pairs of study lineages are Africanized honey bees (AHB) from a population in south-central Arizona and European honey bees (EHB) from commercial US stocks, and the selected high- and low-pollen-hoarding strains of Page and Fondrk (1995) derived from commercial European US stocks. The two pairs of divergent lineages show similar differences in worker body size and ovary size but have diverged over very different evolutionary timescales: AHB and EHB are derived from lineages that initially diverged about 1 million years ago (Whitfield *et al.*, 2006), and the high- and low-pollen-hoarding strains have been produced by 33 generations of artificial selection over 20 years, for a colony-level trait, the amount of pollen stored in the colony. As a result of the artificial selection programme, high-pollen-hoarding worker bees are on average smaller in body size but have larger ovaries (more ovarioles), and similarly, AHB are smaller but with larger ovaries than EHB (Amdam *et al.*, 2006; Linksvayer *et al.*, 2009b). We use *in vivo* cross-fostering to determine how changes in the regulation of the social environment contribute to differences between lineages; then, we use *in vitro* rearing to study how the bee lineages respond to variation in the nutritional environment, independent of social regulation. Our results can provide insight into the evolution of developmental plasticity for body size and ovary size that contributes to the expression of queen–worker dimorphism.

Materials and methods

Colony sources

Eight colony sources were used: two AHB colonies established from swarms collected near Mesa, Arizona, two EHB colonies from commercial stocks and two colonies each from the high- and low-pollen-hoarding strains of Page and Fondrk (1995).

Colony-reared workers and queens

We created four pairs of colonies matched for size: two pairs consisting of a matched AHB colony and a EHB colony and two pairs consisting of a matched high-pollen-hoarding strain colony and a low-pollen-hoarding strain colony. Cross-fostering was carried out between colonies within each of these pairs (i.e. between AHB and EHB and between high- and low-pollen-hoarding strains), first to produce colony-reared workers and then colony-reared queens.

Queens of all genotypes (i.e. lineages and strains) were caged for approximately 24 h on frames matched for age, and then three days later, the frames with approximately 3-day-old eggs were cross-fostered into one rearing environment for each pair of matched genotypes (e.g. frames with eggs from EHB colony 1 and AHB colony 1 were fostered to EHB colony 1). The two frames were placed in the colony facing each other to minimize within-colony environmental differences experienced by brood on the two cross-fostered frames. Frames with colony-reared workers were removed from the hive 24 h before the workers began to emerge and placed in incubators. Any cells full of pollen or honey were covered by wax or foil so that newly emerged workers did not have access to food. Queens were then re-caged, and three days later, the frames cross-fostered into the alternate rearing environment (e.g. frames from EHB colony 1 and AHB colony 1 were fostered to AHB colony 1). Fifty newly emerged workers were collected and phenotyped for each combination of colony source and colony-rearing environment.

After producing colony-reared workers, colonies were prepared to produce queens. Queens were caged for approximately 24 h. Then, to facilitate queen production (Laidlaw & Page, 1997), colonies were supplemented with saturated sugar syrup solution on the third day, and

on the fourth day, queens were removed from the brood-rearing area of the colony. Fifteen larvae that were approximately 1 day old were grafted from each paired source into queen cell cups placed into each colony on frames as above. Frames with queen cells were removed from the hive 24 h prior to emergence and placed in incubators. We monitored the cells every 6 h and removed newly emerged queens for immediate weighing and subsequent ovary phenotyping. The whole procedure was then repeated a second time so that in total 30 larvae were grafted into queen cells cups and reared to adulthood for each combination of colony source and colony-rearing environment.

In vitro rearing

Individual larvae were reared *in vitro* in climate control chambers (34 °C, approximately 85% relative humidity during larval stages and 70% relative humidity during the pupal stages) following the protocol of Kaftanoglu *et al.* (Kaftanoglu *et al.*, 2010) in which each grafted larva was fed a single time. Approximately 24-h-old larvae (obtained after caging queens and checking larvae under a microscope to ensure they were approximately the same size across colonies and treatments) were grafted directly onto the surface of their food, kept in cells made from the conical bottoms of 50-ml graduated plastic centrifuge tubes (Corning). Larvae in the 'worker diet' treatment were fed 175 mg of liquid diet and larvae in the 'queen diet' were fed 250 mg of liquid diet; according to preliminary trials, these two quantities produced mainly normal-sized workers and normal-sized queens, respectively. A total of 96 larvae per colony per treatment were grafted (i.e. for a grand total of four genotypes*two replicate colonies*two treatments*96 larvae = 1536 larvae).

Cells were monitored daily and were removed as soon as the larva or pupa became discoloured or was drowned in food, or if there was any evidence of fungal growth. Mortality occurred mainly during the first day after grafting and during pupation (apparently usually due to drowning in food and due to fungal infection, respectively). Larvae that survived to the pupal stage generally consumed all the food in both treatments, and bees that survived pupation generally appeared to be healthy adults, with some individuals morphologically indistinguishable from colony-reared workers and queens.

Phenotyping

Newly emerged colony-reared workers were weighed to the nearest 0.1 mg using a Mettler-Toledo AB204-5 microbalance and both ovaries dissected and counted under a stereo microscope (after Linksvayer *et al.*, 2009a,b). Newly emerged colony-reared queens and *in vitro*-reared females were weighed, ovariole numbers counted and caste-defining external traits (degree of mandibular notch and corbicula) scored on a discrete 0, 1, 2, 3 scale. Individuals with only external worker morphological characters were dissected and ovariole number counted as for colony-reared workers. For counting the ovarioles of the remaining individuals that had at least some queen-like characters, we chose a more labour-intensive protocol because it is difficult to count ovariole number accurately for large queen-like ovaries with simple dissection. Briefly, specimens were fixed in alcoholic Bouin's fixative for at least one week (Presnell & Martin, 1997); after fixation, the abdomens of specimens were removed, washed in 95% ethanol, dehydrated in 95% n-butanol and 100% n-butanol, cleared in xylene and infiltrated with and embedded in Fisherbrand Paraplast X-tra tissue-embedding medium; next, abdomens were sectioned on a Surgipath Rotary microtome at 5 μm, stained with Gill's haematoxylin solution and counterstained with eosin solution, and mounted on gelatin-coated microscope slides (Presnell & Martin, 1997); finally, ovariole counts were made by counting the number of individual cross-sectioned ovarioles with an American Optical compound microscope. Ovariole counts were made blindly with respect to genotype and treatment, and further a qualitative ovary quality score (0, 1, 2 or 3) was recorded for each count based on the clarity of individual ovarioles on the slide. Ovariole counts with the lowest quality score (0) were excluded from subsequent analysis to minimize downward bias in ovariole counts due to low-quality sectioning or staining.

In order to categorize *in vitro* individuals further as being 'worker-like', 'queen-like', or 'intercastes', we converted ovariole counts into a discrete ovary size score from 0 to 3 where normal colony-reared workers would have a score of 0 and normal colony-reared queens would have a score of 3: 0 – ovariole number ≤ 50; 1 – ovariole number 50–85; 2 – ovariole number 86–124; or 3 – ovariole number ≥ 125. We added these ovary size scores to the scores for mandibular notch and corbicula to get a total 'queenliness' score. Individuals with values in the range of colony-reared workers (0–1) were defined as 'worker-like', individuals with values in the range of colony-reared queens (7–9) were defined as 'queen-like' and individuals with values in between colony-reared workers and queens (2–6) were defined as 'intercastes'.

Statistical analysis

All analyses were carried out in R version 2.10.1. Generalized linear mixed models (GLMM, using GLMER and GLMMPQL packages) with quasi-Poisson errors were used for worker ovariole number, and general linear mixed models (GLM, using the LME package) were used for worker and queen mass and queen ovariole number. Larval genotype (strain or lineage) and rearing environment were included as fixed factors, and colony replicate was included as a random factor (Linksvayer, 2007; Linksvayer *et al.*, 2009a).

Results

Colony-reared high- and low-pollen-hoarding strain workers

The mass of focal individuals depended on their genotype (GLM, $F_{1,396} = 8.21$, $P = 0.0044$) and the rearing environment (GLM, $F_{1,396} = 4.51$, $P = 0.034$). Confirming the results of a previous study (Linksvayer *et al.*, 2009a), we found that the ovariole number of high- and low-strain workers depends on an interaction between their own genotype and the rearing environment (see the electronic supplementary material, Fig. S1): focal individual genotype main effect (GLMM, $n = 391$, $z = -6.64$, $P < 0.001$; on average, low-strain individuals had 2.97 fewer ovarioles than high-strain individuals), rearing environment main effect (GLMM, $z = 7.71$, $P < 0.001$; on average, focal individuals reared by low-strain nurses had 4.03 more ovarioles than when reared by high-strain nurses), genotype × rearing environment interaction (GLMM, $z = -3.76$, $P < 0.001$; on average, low-strain focal individuals reared by low-strain nurses had 2.35 fewer ovarioles than expected).

To investigate how combinations of focal individual genotype and rearing environment affect the relationship between body size and ovary size, we first used a full model for ovariole number including focal worker genotype, rearing environment, focal worker mass, all interactions, as well as replicate. The full model indicated complex interactions between genotype, rearing environment and mass: there was a genotype × rearing environment effect (GLMM, $n = 391$, $z = 1.989$, $P = 0.0467$), but there were also rearing environment × mass (GLMM, $z = 2.433$, $P = 0.0150$) and genotype × rearing environment × mass effects (GLMM, $z = -2.33$, $P = 0.0199$) (Fig. 1). These results show that the relationship between worker body size and worker ovary size is conditional on worker genotype and the social environment. To disentangle these complex interactions, we looked separately within each genotype of focal individuals.

Within high-strain focal workers, the relationship between mass and ovariole number was conditional on rearing environment (GLMM, rearing environment × mass, $n = 193$, $z = 2.551$, $P = 0.0107$), indicating that high-strain larvae reared in different social environments had a different relationship between mass and ovariole number (Fig. 1). In contrast, for low-strain focal workers, no factors predicted ovariole number even after removing the nonsignificant rearing environment × mass interaction (GLMM, $n = 193$, rearing environment, $z = 1.69$, $P = 0.0916$; mass, $z = -1.53$, $P = 0.126$), indicating that for low-strain workers, there was no significant relationship between body size and ovary size in either rearing environment (Fig. 1). A model with replicate and worker mass showed that adult worker mass was positively associated with adult worker ovariole number when high-strain larvae were reared in a low-strain social environment (GLMM, $n = 98$, $z = 5.436$, $P < 0.001$), but there was no significant relationship for all other combinations of focal worker genotype and rearing environment (all $P > 0.05$).

Colony-reared Africanized honey bee and European honey bee workers

The mass of AHB and EHB workers depended on their own genotype (GLM, $F_{1,465} = 85.04$, $P < 0.0001$) and the interaction between genotype and rearing environment (GLM, $F_{1,465} = 8.67$, $P = 0.0034$); on average, EHB workers were larger (effect = 12.80 mg, SE = 1.52, $t = 8.43$), and the differences between AHB and EHB workers were less extreme in the EHB social environment (effect = −6.09 mg, SE = 2.07, $t = -2.95$) (see the electronic supplementary material, Fig. S2). Similarly to

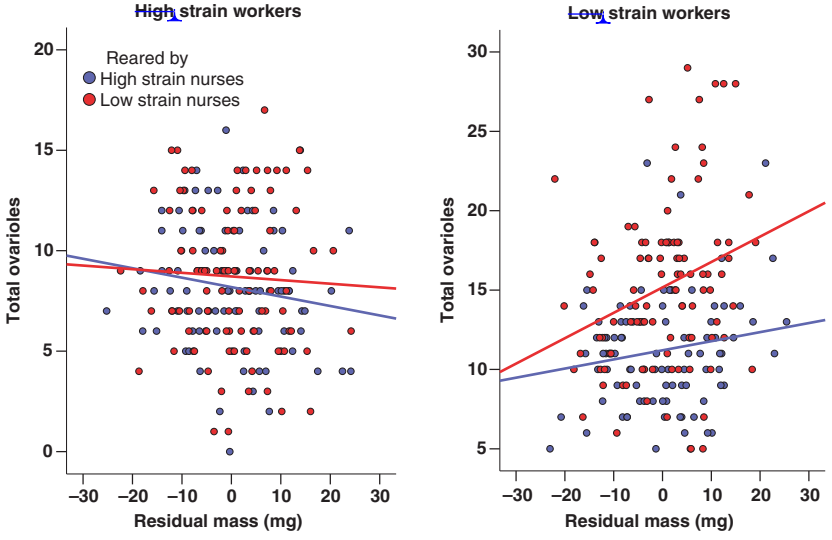

Fig. 1 Body–ovary size relationship for cross-fostered high- and low-pollen-hoarding workers. High strain workers (left panel) and low strain workers (right panel) were reared to adulthood in either a high-strain (blue) or low-strain (red) social environment. Residual mass is plotted after controlling for differences between replicate colonies.

the results above, the interaction means that worker body size differences between AHB and EHB depends on both worker genotype and the genotypic composition of the social environment. Total ovariole number of AHB and EHB workers depended only on their own genotype; AHB workers on average had 5.15 more ovarioles than EHB workers (GLMM, SE = 0.0477, d.f. = 445, $t = -10.42$, $P < 0.0001$) (see the electronic supplementary material, Fig. S2). There were no additional significant factors when mass and all two- and three-way interactions with genotype and rearing environment were added to the model. When AHB and EHB focal workers were examined separately, there was no significant interaction between rearing environment and mass (GLMM, $P > 0.05$); however, both showed the same pattern (see the electronic supplementary material, Fig. S3), and when AHB and EHB focal individuals were pooled, there was a significant interaction between rearing environment and mass (GLMM, SE = 0.00423, d.f. = 443, $t = 2.25$, $P = 0.025$). These results suggest that the mass–ovariole number relationship expressed by focal individuals depends on whether they were reared by AHB or EHB nurses.

Colony-reared high- and low-pollen-hoarding strain queens

For queen mass, there was a main effect of genotype (GLM, $F_{1,99} = 21.84$, $P < 0.0001$; effect = 22.3, SE = 4.81, $t = 4.64$, see the electronic supplementary material, Fig. S4). There were no genotype or social rearing environment effects on total ovariole number (GLM, $n = 103$, all $P > 0.05$) for high- and low-strains queens. When mass and the additional interactions were added to the model predicting ovariole number, there was a positive relationship between mass and ovariole number (GLM, $F_{1,98} = 7.14$, $P = 0.0088$), with on average an increase of 0.442 ovarioles for every milligram in mass (SE = 0.165, $t = 2.67$). When examining high- and low-genotype queens separately, there was no relationship between ovariole number and mass or rearing environment in low-strain queens (GLM, $P > 0.05$), but for high-strain queens, there was a significant positive relationship between mass and ovariole number (GLM, $F_{1,58} = 9.81$, $P = 0.0027$), with on average an increase of 0.684 ovarioles for every mg increase in mass (SE = 0.218, $t = 3.13$).

Colony-reared Africanized honey bees and European honey bees queens

For mass, there was a significant genotype × rearing environment interaction; EHB queens were smaller on average than AHB queens, but only when reared by EHB workers (GLM, $F_{1,152} = 4.43$, $P = 0.037$; effect = −15.83 mg, SE = 7.52, $t = -2.105$; see the electronic supplementary material, Fig. S5). For ovariole number in AHB and EHB queens, there were no effects of genotype or rearing environment (GLM, d.f. = 153, all $P > 0.05$). With all terms in the full model for total ovariole number, mass, genotype × mass, and rearing environment × mass were all significant (GLM, d.f. = 148, $P < 0.05$), but these effects were not stable, and when nonsignificant terms were sequentially removed, no factors remained significant. When we looked at each genotype separately, there were no significant effects for EHB queens (GLM, d.f. = 79, all $P > 0.05$). In contrast, for AHB queens, mass was positively associated with ovariole number (GLM, $F_{1,69} = 7.73$, $P = 0.0070$), with an increase of 1.18 ovarioles (SE = 0.338, $t = 3.49$) for every milligram increase in mass; there was a rearing environment × mass interaction (GLM, $F_{1,69} = 4.77$, $P = 0.032$), because there was a relationship between mass and ovariole number when AHB queens were reared in a AHB environment but not in a EHB environment. However, this relationship depended largely on a few individuals with small body and ovary size.

When pooling all genotypes, there was no relationship between body size and ovary size for hive-reared workers (Spearman's rank correlation; $\rho = -0.042$, $P = 0.20$, $n = 938$), but there was a positive body–ovary size relationship in hive-reared queens (Spearman's rank correlation; $\rho = 0.188$, $P = 0.002$, $n = 260$) (Fig. 2).

Laboratory-reared females

When the influence of social environment was removed by *in vitro* rearing, there were main effects of genotype (GLM, $F_{1,558} = 16.81$, $P < 0.0001$) and food treatment (GLM, $F_{1,558} = 207.85$, $P < 0.0001$), and a genotype × treatment interaction (GLM, $F_{3,558} = 17.62$, $P < 0.0001$; see the electronic supplementary material, Fig. S6) on

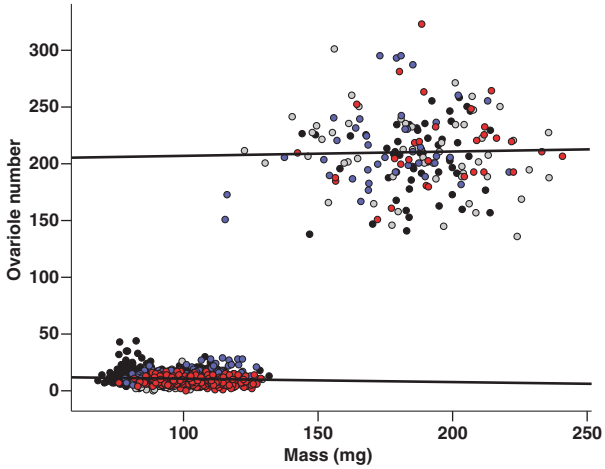

Fig. 2 Body–ovary size relationship across colony-reared workers and queens. Africanized honey bees (black), European honey bees (light grey), high (blue)- and low (red)-pollen-hoarding strains for workers (lower left cloud of points) and queens (upper right cloud of points).

mass. This interaction is a genotype × environment interaction (G × E) in which different genotypes display different patterns of phenotypic plasticity in response to the two different nutritional environments we provided. For ovariole number, there were main effects of genotype (GLMM, d.f. = 3, $\chi^2 = 5246.6$, $P < 0.0001$) and food treatment (GLMM, d.f. = 1, $\chi^2 = 3735.1$, $P < 0.0001$), and a genotype × treatment interaction (GLMM, d.f. = 3, $\chi^2 = 1035.8$, $P < 0.0001$; see the electronic supplementary material, Fig. S6). As for mass, this is G × E, indicating that the different genotypes respond differently to the nutritional environments for ovary size.

In the full model for ovariole number that includes mass, all main and two- and three-way interaction effects involving genotype, treatment, and mass were significant (GLM, all $P < 0.01$), indicating that the genotypes had different relationships between mass and ovariole number for each treatment (Fig. 3). Within the worker diet treatment, there was only a significant effect of mass (GLM, $F_{1,\ 286} = 14.34$, $P < 0.001$), indicating that all genotypes had a similar positive relationship between mass and ovariole number (Fig. 3). In contrast, within the queen diet treatment, there were significant effects of genotype (GLM, $F_{3,\ 216} = 4.35$, $P = 0.005$), mass (GLM, $F_{1,\ 216} = 97.5$, $P < 0.0001$) and genotype × mass interaction (GLM, $F_{3,\ 216} = 6.19$, $P < 0.001$), indicating that for the queen diet, the genotypes had different relationships between mass and ovariole number (Fig. 3). Similarly, when we used a regression model with genotype, mass, and their interaction, but without diet treatment, there was an effect of genotype (GLM, $F_{3,\ 453} = 7.92$, $P < 0.0001$), mass (GLM, $F_{1,\ 453} = 197.92$, $P < 0.0001$) and genotype × mass interaction (GLM, $F_{3,\ 453} = 11.72$, $P < 0.0001$). Figure 3 shows that high-strain individuals responded differently to the food treatment than the other genotypes; specifically, ovary size did not increase as rapidly with mass for high-strain bees fed the queen diet.

Over all genotypes and the two food treatments, ovariole number was strongly positively correlated with the degree of mandibular notch (a queen character; Spearman's rank correlation, $\rho = 0.801$, $P < 0.001$, $n = 516$), strongly negatively correlated with the degree of corbicula development (a worker character; Spearman's rank correlation, $\rho = -0.830$, $P < 0.001$, $n = 505$) and less strongly positively correlated with mass (Spearman's rank correlation, $\rho = 0.584$, $P < 0.001$, $n = 525$). Using scores for ovary size, degree of corbicula development and degree of mandibular notch development, we defined classes of individuals as worker or queen phenotypes if they fit within the ranges of hive-reared workers and queens, or 'intercastes' if they had intermediate values for these three traits. The four genotypes produced different proportions of workers, intercastes and queens when fed a 'queen diet' (Table 1; $\chi^2 = 76.84$, d.f. = 6, $P < 0.001$). Most notably, across the two *in vitro* treatments, only 6.2% of high-strain larvae developed into an intercaste phenotype, whereas 25% of low-strain larvae and on average 44% of EHB and AHB larvae developed into an intercaste or queen phenotype (Table 1). Furthermore, within the 'worker diet' treatment, there was variation between strains in the proportion of workers, intercastes and queens produced ($\chi^2 = 53.296$, d.f. = 6, $P < 0.001$); in particular, AHB produced a higher proportion of queens and intercastes (0.4, $n = 75$; see the left panel of Fig. 3 and note that there are many AHB females between approximately 100–125 mg but with more than 100 ovarioles) relative to EHB (0.174, $n = 23$), high strain (0.0313, $n = 96$) and low strain (0.0617, $n = 81$), indicating that AHB larvae were more likely to initiate the development of queen characters relative to the other strains, even under relatively low-nutrition conditions.

Discussion

Social evolution in the honey bees (genus *Apis*) has led to a dramatic elaboration of queen–worker dimorphism so that only two relatively discrete sets of trait constellations that span a fraction of the possible phenotypic space are naturally expressed. Our results demonstrate that differences between honey bee lineages in developmental

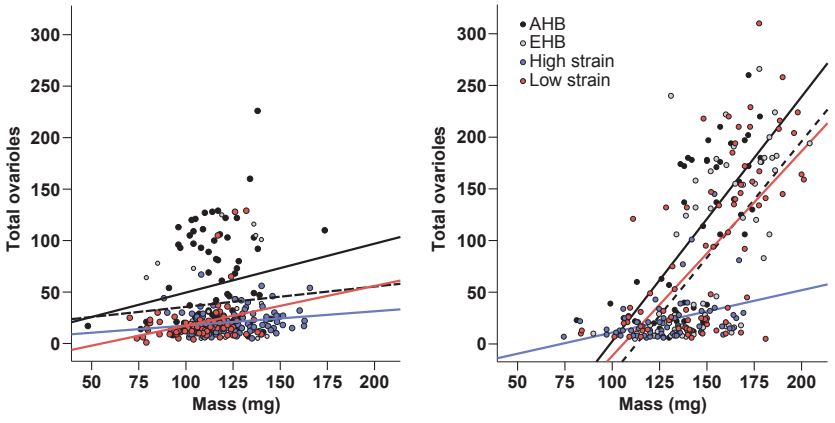

Fig. 3 Body–ovary size relationship across *in vitro*-reared females. Africanized honey bees (black), European honey bees (light grey), high-strain (blue) and low-strain (red) larvae reared *in vitro* on a worker (left panel) or queen (right panel) diet.

Table 1 Proportion of worker, intercaste and queen phenotypes produced by Africanized honey bees (AHB), European honey bees (EHB), high-, and low-pollen-hoarding strain larvae reared *in vitro* on a queen diet.

	Worker	Intercaste	Queen	*N*
AHB	0.551	0.305	0.144	118
EHB	0.576	0.2	0.224	85
Low strain	0.75	0.109	0.141	156
High strain	0.938	0.0621	0	161

High-strain larvae were less likely than the other strains to develop into intercaste or queen phenotypes. Queen and worker phenotypes were defined by ovary size, degree of mandibular notch and corbiculae, see Methods.

plasticity for body size and ovary size and body–ovary size allometry result from differences in the nutritional environment provided by nurse workers and differences in larval developmental responses to nutritional environmental inputs. Our interpretation of these findings is that our pairs of study lineages have diverged for larval and nurse components that control the expression of developmental plasticity and allometry. We suggest that these components were also available for selection to act on during the evolution of queen–worker dimorphism and that the evolution of queen–worker dimorphism involved the developmental integration and co-evolution of these components.

The expression of other polyphenisms besides queen–worker caste dimorphism also depends on social inputs, e.g. dispersal morph in the locust *Schistocerca gregaria* (Maeno & Tanaka, 2008), head morph in the salamander *Hynobius retardatus* (Michimae *et al.*, 2009) and tadpole morph in the spadefoot toad *Spea* spp. (Pfennig & Frankino, 1997; Martin & Pfennig, 2010). In some cases, both social inputs and developmental responses have been shown to be heritable and can respond to selection, e.g. male horn length in the horn-dimorphic dung beetle *Onthophagus taurus* (Moczek, 1998; Hunt & Simmons, 2002; Moczek & Nijhout, 2002). Thus, developmental evolution may often involve the co-evolution and integration of social inputs and plastic developmental responses.

Confirming our previous results (Linksvayer *et al.*, 2009a), worker ovary size differences between the high- and low-pollen-hoarding strains were determined by an interaction between the genotype of focal individuals and the genotypic make-up of the rearing environment. Similarly, body size differences between AHB and EHB workers and queens were determined by an interaction between genotype and social environment. These results emphasize that the expression of social insect phenotypes depend on the combination of focal individual genotype and the genotypic composition, or 'sociogenome' of the colony (Linksvayer, 2007; Linksvayer *et al.*, 2009a).

The relationship between body size and ovary size expressed by high-strain workers, but not low-strain workers, also depended on the rearing environment (Fig. 1). It seems that high-strain larvae are more sensitive than low-strain larvae, in terms of the resulting adult ovary size over the range of nutritional environments provided by low-strain nurses. Besides food quantity, high- and low-strain nurses may provide qualitatively different food or may differ in the timing of food delivery. These different parameters may depend on the specific signalling and response interactions that occur between different nurse–larvae genotypic combinations (Kolliker *et al.*, 2005; Linksvayer *et al.*, 2009a), resulting in changes in developmental plasticity and expressed allometry. Regardless of the mechanism, the differences we observed in worker allometry between the high and low strains indicate that the strains have diverged for expressed body–ovary size allometry, likely as a result of the artificial, colony-level selection programme (Linksvayer *et al.*, 2009a). Whereas high- and low-strain workers differed in their sensitivity to the rearing environment for their expressed body–ovary size relationship, AHB and EHB workers showed consistent changes in the body–ovary size relationship depending on the rearing environment (electronic supplementary material, Fig. S3). We also found evidence that the rearing environment may play a role in the different body–ovary size relationships expressed by the different queen genotypes.

Our *in vitro* feeding results showed that larvae of the different genotypes also respond differently to the same nutritional environment, when there was no potential for social control of development. Whereas low-strain, EHB and AHB larvae responded to the high food, 'queen diet' treatment with increased body mass and increased ovary size, high-strain larvae grew larger but did not respond with a similar increase in ovary size (Fig. 3). Indeed, the low strain, EHB and AHB produced many queen and queen-like phenotypes, whereas none of the high-strain larvae developed queen-like phenotypes (Table 1). These results show that the artificial colony-level selection programme has also shaped components of the developmental response of larvae to their nutritional environment that affect the body–ovary size relationship spanning the full range of possible female body sizes. The genotypes also differed in their likelihood to develop queen-like phenotypes when fed the 'worker diet', and in particular many AHB individuals developed queen-like traits. In a previous study, we found that some genotypes of colony-reared AHB workers had large queen-like ovaries, and furthermore, the expression of this large ovary phenotype was conditional on the social rearing environment (Linksvayer *et al.*, 2009b). Thus, segregating variation for caste-related traits, and body–ovary size allometry, may be relatively common in honey bee populations. Indeed, recent theory indicates that relatively high levels of genetic variation for caste can be maintained, even when variants are deleterious, by mutation–kin selection balance (Van Dyken *et al.*, 2011).

Within three of the four lineages, we found that body size and ovary size were not consistently correlated within queens and workers, suggesting that it is possible for these traits to be dissociated and independently canalized. The exception was the two sources of high-pollen-hoarding strain bees, which displayed different patterns of plasticity and allometry. The pollen-hoarding strain bees used were derived from generation 33 of artificial selection on commercial EHB stocks, demonstrating that plasticity and allometry can change rapidly. Similarly, studies with *Onthophagus* beetles using artificial selection (Emlen, 1996) and comparisons between natural and recently introduced populations show that allometries can evolve rapidly (Moczek & Nijhout, 2003). However, the rapid change in plasticity for high-pollen-hoarding strain worker traits may explain the disrupted queen development we observed in the high strain, suggesting that the developmental mechanisms linking the expression of queen and worker traits may constrain their independent evolution, at least over short evolutionary timescales (Tomkins & Moczek, 2009). Over longer evolutionary timescales, it may be possible for the relationship between worker body size and ovary size to evolve without disrupting queen development. Indeed, EHB and AHB lineages initially diverged approximately 1 million years ago (Whitfield *et al.*, 2006), and whereas EHB and AHB lineages show similar differences as the high- and low-pollen-hoarding strains in terms of worker body size and ovary size (i.e. both AHB and high-pollen-hoarding strains have smaller worker mass but larger worker ovaries), the EHB and AHB lineages both produced normal queen phenotypes when reared *in vitro* (Table 1).

It appears that high-strain females do not initiate queen development as readily as low-strain, EHB and AHB females, suggesting that high-strain larvae are less sensitive to nutritional cues that promote queen development. Why are high-strain larvae seemingly more sensitive to the rearing environment over the range of worker phenotypic space but less sensitive to the rearing environment over the broader range extending to queen phenotypic space?

We propose that our data can point to underlying developmental mechanisms that also allow us to explain the different sensitivities that high-strain larvae show across the worker and queen phenotypic spaces. Recent studies suggest that developmental growth is regulated in the holometabolous insects by specific sensing mechanisms for body size. Central to this system is insulin/insulin-like signalling (IIS), a nutrient-sensitive signalling cascade that, together with the target of rapamycin (TOR) complex, affects the synthesis and release of ecdysteroid moulting hormones and influence patterns of tissue- and organismal growth in many organisms, perhaps including the honey bee (Patel *et al.*, 2007; Walkiewicz & Stern, 2009). Organ size and body size are generally related, but high-strain workers retain a larger ovary inside a smaller body. High-strain bees also differ from low-strain bees in the expression of several IIS and TOR-associated genes (Wang *et al.*, 2009), and during early development, they show a different pattern of ecdysteroid release than low-strain workers (Amdam *et al.*, 2010). These findings suggest that the body–ovary allometry that sets high-strain bees apart from low-strain bees and EHB could emerge from a different sensing of body size during development.

Queen larvae grow faster and attain a larger final size than worker-destined individuals, so that successful sensing of a critical size during development is likely central to the expression of queen traits (Patel *et al.*, 2007). Although large size is attained by high-strain bees reared *in vitro*, these bees fail to express queen traits. This result is consistent with the suggestion that body size may be differently interpreted by the high selected strain during development.

Thus, we propose that the retention of a larger ovary in high-strain worker bees can be achieved by disrupting the body–ovary size relationship such that a larger ovary is achieved in a smaller body. The disruption of the body–ovary size relationship via changes in the sensitivity of body size and ovary size to the nutritional environment seems to have been coupled with nurse worker regulation of the rearing environment such that a limited range of worker phenotypes are produced under natural conditions. This disruption, however, also affects the queen phenotype in high-strain bees, in that it becomes more difficult to express. We propose that this phenotypic outcome has been maintained because the artificial selection programme acts more strongly on worker traits than queen traits. Only a handful of queens need to ever be produced and be functional to continue the artificial selection lines, resulting in relatively relaxed selection on queen traits. In contrast, worker traits mainly determine the amount of pollen collected and stored in the hive so that artificial selection on pollen hoarding likely acts more strongly on worker traits. Relatively relaxed selection on queen traits may also occur naturally in swarm-founding species, where queens do not initiate colonies alone.

We reared larvae *in vitro* on two discrete quantities of food, but we ended up with adult females that were more variable than colony-reared queens and workers, indicating that nurse bees are better at regulating expressed queen–worker dimorphism than we are (compare Figs 2 and 3). By feeding larvae continuously (instead of a single time in our *in vitro* protocol), nurse workers can tightly regulate available food over the course of development. Nurse workers can also regulate development by removing individuals that express intermediate phenotypes, so that only discrete queen and worker phenotypes are produced (Weaver, 1957; Woyke, 1971; Dedej *et al.*, 1998; Hatch *et al.*, 1999).

For many species that express a polyphenism, there is a step-like relationship between body size and the

polyphenic trait, so that only discrete alternate morphs are produced across the full range of body sizes (Nijhout, 2003). In contrast, our *in vitro* results suggest that discrete queen and worker morphs result mainly from discrete, nurse-controlled nutritional environments, not because of an underlying step-like relationship between body size and ovary size that converts variable larval nutrition into discrete alternate phenotypes. As shown in Fig. 3, a continuous range of ovary size and body size phenotypes was produced in the absence of social control, with the *in vitro* queen diet. Thus, under natural colony conditions, only the extreme ends of the full phenotypic space are normally expressed, and the relationship between body size and ovary size that we observed *in vitro* is typically suppressed, so that ovary size is relatively canalized over the range of worker and queen body sizes produced (see Fig. 2). The apparent lack of a steep step-like relationship highlights the importance of social control for ensuring the production of discrete queen and worker morphs and may also indicate that social control played an important role during the early evolution as well as elaboration of honey bee queen–worker dimorphism. That is, the evolution of a steep, step-like relationship between body size and ovary size may have been unnecessary precisely because there was already relatively strict social control. Further study of the relative importance of social control of larval development and larval developmental responses to social inputs in other social insect lineages will help to clarify the various roles that these components have played during the evolution of eusociality.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Body size and ovary size of cross-fostered high and low pollen hoarding strain workers.

Figure S2 Body size and ovary size of cross-fostered Africanized honey bees and European honey bees workers.

Figure S3 Body-ovary size relationship for cross-fostered Africanized honey bees and European honey bees workers.

Figure S4 Body size and ovary size of cross-fostered high and low pollen hoarding queens.

Figure S5 Body size and ovary size of cross-fostered Africanized honey bees and European honey bees queens.

Figure S6 Body size and ovary size of *in vitro* reared workers and queens.

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