EVOLUTION & DEVELOPMENT 12:5, 428–436 (2010)
DOI: 10.1111/j.1525-142X.2010.00429.x

Hormone response to bidirectional selection on social behavior

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SUMMARY Behavior is a quantitative trait determined by multiple genes. Some of these genes may have effects from early development and onward by influencing hormonal systems that are active during different life-stages leading to complex associations, or suites, of traits. Honey bees (*Apis mellifera*) have been used extensively in experiments on the genetic and hormonal control of complex social behavior, but the relationships between their early developmental processes and adult behavioral variation are not well understood. Bidirectional selective breeding on social food-storage behavior produced two honey bee strains, each with several sublines, that differ in an associated suite of anatomical, physiological, and behavioral traits found in unselected wild type bees. Using these genotypes, we document strain-specific changes during larval, pupal, and early adult life-stages for the central insect hormones juvenile hormone (JH) and ecdysteroids. Strain differences correlate with variation in female reproductive anatomy (ovary size), which can be influenced by JH during development, and with secretion rates of ecdysteroid from the ovaries of adults. Ovary size was previously assigned to the suite of traits of honey bee food-storage behavior. Our findings support that bidirectional selection on honey bee social behavior acted on pleiotropic gene networks. These networks may bias a bee's adult phenotype by endocrine effects on early developmental processes that regulate variation in reproductive traits.

INTRODUCTION

A bias toward a particular behavior often arises from the interaction of ensembles of genes (Anholt 2004), some of which may have pleiotropic effects. Selection on a specific behavior can, thereby, produce a suite of associated changes in other behavioral and physiological traits relying on the same genes or hormonal mediators (Flatt et al. 2005; McGlothlin and Ketterson 2008). Although elucidating such associations is challenging, it provides an opportunity to better understand how a suite of interlinked behavioral and physiological traits might have evolved (Zera et al. 2007). This approach is useful when examining complex behavioral adaptations, such as those observed in highly social animals (Robinson et al. 2008).

Honey bees (*Apis mellifera*) show behavioral biases that correlate with other traits. These bees are among the best studied social insects, with sequenced genome, gene repression tools, behavioral paradigms including sensory perception, learning and memory, and established selective breeding methods (Weinstock et al. 2006). Honey bees vary in preference for nectar and pollen, and artificial bidirectional selection for the amount of stored pollen (pollen hoarding) by colonies resulted in strains with different behavioral biases toward nectar and pollen collection (Page and Fondrk 1995). Pollen-hoarding behavior is a parental care (maternal provisioning) behavior in solitary and primitively social bees (Knerer 1969; Goodell 2003; Neff 2003; Camillo 2005), but in honey bees the behavior is expressed by "workers," a caste of facultatively sterile female helpers. The developmental separation of such sterile castes from reproductives is the defining step in evolution of advanced sociality (eusociality, reviewed by Pennisi 2009). Within the worker caste, correlated trait-differences occurred with the breeding scheme for pollen hoarding: compared with high pollen-hoarding strains that store a large surplus of pollen, low pollen-hoarding genotypes show lower locomotor activity in young adults, reduced gustatory sucrose sensitivity and vitellogenin (egg yolk protein) titers, and ovaries with fewer and less oogenic ovarioles (ovary filaments, reviewed by Amdam and Page 2010). Low-strain workers also initiate foraging later in life and collect and store more nectar (relative to pollen) than high-strain bees that are biased toward pollen collection.

The linkage between pollen hoarding and anatomical, physiological, and behavioral traits in workers has been documented repeatedly in unselected wild type bees as well as in strains selected for pollen hoarding (see Amdam and Page 2010 for a review). This suite of traits is referred to as a

"pollen-hoarding syndrome" (Page and Erber 2002; Sih et al. 2004). Because the traits shifted as one unit during breeding, the artificial selection can have acted on pleiotropic gene ensembles. Thus, we hypothesized that genetics of hormonal networks, which broadly affect insect development, reproduction, and maternal behavior, were co-opted by natural selection during social evolution to provide a mechanistic basis for honey bee foraging specialization and division of labor (Amdam et al. 2004; Pennisi 2009). Artificial selection on foraging biases, thereby, results in a coordinated response in workers that involves many characters including reproductive traits. It is not possible to confirm these connections without comparative studies between closely related solitary and social species (Amdam et al. 2009), but it is possible to test whether strains bidirectionally selected for pollen hoarding are characterized by different hormonal expression during development. Identification of such differences would support that behavioral specialization can be achieved by selection on developmental networks.

Using pollen-hoarding strains, we here address this possibility by examining the expression of the two major systemic hormones of insects, juvenile hormone (JH), and ecdysteroids. These endocrine factors regulate insect development and reproduction (see Goodman and Granger (2005) and Raikhel et al. (2005) for reviews), and are implicated in mechanisms of social insect division of labor (reviewed by Hartfelder and Emlen 2005). In honey bees, JH is synthesized by the *corpora allata* (gland complex behind the brain), while ecdysteroids are first produced in the prothoracic glands. The latter organs degenerate in pupae before adult eclosion (Hartfelder 1993). Thereafter, it is assumed that active gonads are the primary ecdysteroidogenic organs, as is true for many adult insects (Lafont et al. 2005).

We first tested JH titers in late (4th–5th) instar larvae, where ovary size is determined in honey bees: unless a threshold level of JH is present, apoptosis reduces the number of ovarioles in each larval ovary from 150+ (in queens) to 2–12 (in workers, Rembold et al. 1974; Schmidt-Capella and Hartfelder 1998). Although the difference is slight by comparison, high pollen-hoarding genotypes average twice as many ovarioles as low-strain bees (Amdam et al. 2006, 2007). Thus, we predicted that high-strain larvae have higher JH titers. Thereafter, we monitored the pupal stage, where hormone changes can alter developmental progression and pigmentation (Bitondi et al. 1998; Zufelato et al. 2000; Santos et al. 2001). We predicted that strain-specific patterns of development were reflected in different timing or quantities of hormone. The prediction was tested by matching major transitions to hormone levels. Finally, we examined early adulthood when worker maturation is completed. Less is known about the hormone changes of this life-stage (Velarde et al. 2009), but we could test whether titers correlated with ecdysteroid release from the ovary, despite the presumed inactivity of this organ in workers. The last experiment also monitored vitellogenin, a behavioral affector protein in honey bees that interacts with JH during adulthood (Guidugli et al. 2005; Nelson et al. 2007).

We found significant differences in JH and ecdysteroids levels between high and low pollen-hoarding strain bees during larval, pupal, and early adult life-stages. Our data strongly suggest that artificial selection on honey bee food-storage behavior acted on the genetic basis of JH expression to influence the larval retention process of ovarioles and the adults' ovary size. Ovary size may in turn dictate adult ecdysteroidogenesis. Correlations between worker ovary size, adult hormone sensitivity, and foraging biases are already established (Amdam et al. 2006, 2007). We conclude that the response to selection on honey bee pollen-hoarding behavior could be brought about by modification of early developmental processes.

MATERIALS AND METHODS

Honey bees

Larval and pupal stage worker bees were reared from newly laid eggs obtained from queens bidirectionally bred over 26 generations for colony pollen-hoarding behavior (Page and Fondrk 1995). This artificial selection follows a circular breeding plan with three to five sublines within each strain. Sublines are outcrossed periodically (every three to four generation) to the wild type source population (Californian commercial stocks, see Page and Fondrk (1995) for further details). Queens ($n = 6$) representing three sublines for each strain laid eggs for 6 h. Hatched larvae were cofostered in common wild type nursing colonies. Using this method, one additional queen of each strain produced workers for a replicate study of JH during the first 4 days of pupal development.

Sample collection

Larvae were analyzed 6–10 days after egg-laying, while newly pupated individuals were collected after 13 days and incubated at 33°C and 80% humidity in the laboratory. Larvae sampled from colonies and pupae obtained from laboratory incubation were anesthetized on ice, and hemolymph extracted with graduated glass micropipettes. For each of two independent replicates of the experimental design, about 20 bees were sampled daily as a mix from the subline sources that represented each pollen-hoarding strain (142 larval and 386 pupal samples total). A subset of workers was also allowed to emerge, marked (Testors paint) on the thorax to distinguish genotype, and then cofostered in wild type colonies. From each strain, about 20 adult individuals were sampled on each of the 4 successive days, totaling 147 samples.

Quantification of JH titer

To monitor changes in JH during worker development, daily titers of JH III (only JH homolog in honey bees, Hagenguth and Rembold 1978), were measured using GC–MS (Bergot et al. 1981). A hemolymph volume of 2–4 µl was used from each bee. Sample contamination by tissue fragments and foregut content was

avoided. Hemolymph was diluted in 200 µl 50% acetonitrile (HPLC grade) and stored at −80°C until analysis. Samples were prepared for analysis as described by Brent and Vargo (2003). JH d_3-methoxyhydrins were analyzed on an HP 6890 Series GC equipped with a 30 m × 0.25 mm Carbowax Econo-Cap GC column (Alltech, Grace, Deerfield, IL, USA) coupled to a HP 5973 N inert MSD/DS running in SIM mode, with helium as the carrier. Two ions for JH III d_3-methoxyhydrin, found at m/z 76 and 225, were monitored to ensure JH specificity. Resulting peaks were compared with calibration curves of known quantities of standards containing d_3-methoxyhydrins of JH III and JH III ethyl ester.

Quantification of ecdysteroid titer

Ecdysteroid titers were determined using a radioimmunoassay (Warren et al. 1984; Zera and Bottsford 2001). Individual bees, separate from those used for JH sampling, provided 2–4 µl of hemolymph per sample. Hemolymph was diluted in 200 µl methanol (80%, HPLC grade) then stored at −80°C until analysis. Duplicate 10 µl aliquots of each sample were incubated overnight with 100 µl of [^{3}H]-20-hydroxyecdysone stock (1 mg/ml, Perkin-Elmer, Waltham, MA, USA) in Borate Buffer, and 100 µl of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH) at 4°C on an orbital shaker. The antiserum is cross-reactive for ecdysone, ecdysterone, 20-hydroxyecdysone, and makisterone A (Warren et al. 1984), the dominant compound in adult honey bees (Feldlaufer and Hartfelder 1997). Standard competition curves were generated with 20-hydroxyecdysone stock (Sigma-Aldrich, St. Louis, MO, USA) in quantities of 15.6–2000.0 pg. After 18 h incubation, 20 µl cleaned Protein A Solution (Pansorbin, CalBiochem, EMD Chemicals, Inc., Gibbstown, NJ, USA) was added to each tube to precipitate the complex during 1 h of incubation at room temperature. Samples were centrifuged at 5000 *g* and the remaining pellet washed twice with 100 µl borate buffer. Incorporation of microlabel was determined by scintillation counter. Ecdysteroid concentration was estimated by nonlinear regression, adjusting for antibody cross-reactivity (Warren et al. 1984; Feldlaufer and Hartfelder 1997).

Quantification of vitellogenin titer

To determine vitellogenin protein content, 1 µl of hemolymph was dissolved in 10 µl of Tris buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidin, 0.7 µM pepstatin, 8 µM chymostatin, 10 µM leupeptin, 0.8 µM aprotinin; Sigma-Aldrich) and processed as described before (Seehuus et al. 2006). Individual samples of 5 µg of hemolymph protein were subjected to one-dimensional SDS electrophoresis using 7% polyacrylamide gels, where honey bee vitellogenin occurs as a single band of 180 kDa (Wheeler and Kawooya 1990). Vitellogenin was titered with a β-galactosidase standard (Sigma) as described previously for *A. mellifera* (Lin et al. 1999; Seehuus et al. 2006). To ensure that vitellogenin was present in amounts insufficient for quantification by SDS-PAGE, Western blots were used to characterize samples from pupae and newly emerged workers (Amdam et al. 2003).

Assessment of developmental pacing

Daily until adult emergence, 10 individuals per strain were briefly removed from the incubators for photo-documentation of development. Pupal development was matched to one out of six stages, based on eye color and progressive pigmentation of the cuticle as distinguishing characters (Rembold et al. 1980; Michelette and Soares 1993). Stages were white eyed (Pw), pink-eyed (Pp), brown eyed (Pb), brown-eyed with light cuticular pigmentation (Pbl), brown-eyed with medium cuticular pigmentation (Pbm), and brown-eyed with dark cuticular pigmentation (Pbd).

Exogenous application of JH to pupae

We tested whether a surge of JH in high-strain Pb pupae (15 days postoviposition, see "Results") influenced developmental pace by artificially boosting JH in low-strain Pb pupae. A set of high-strain pupae was used as untreated reference for pace. Queens were caged on empty combs for 6 h and eggs transferred to wild type nursing colonies. Developing bees were collected 15 days later and divided into five treatment groups of 21 pupae each. Following established protocols (Rembold et al. 1974; Bitondi et al. 1998; Zufelato et al. 2000), four groups of low-strain pupae received topical applications to their second abdominal tergite of either 0.001, 0.01, or 0.1 µg JH III (Sigma-Aldrich) in 2 µl acetone (Honeywell, Burdick & Jackson, Muskegon, MI, USA), or just solvent (vehicle control). One group was left untreated. Pupae were next placed in 100 × 20 mm Optilux® Petri dishes lined with filter paper, folded to ensure an upright dorsal posture that facilitates normal development. Dishes were incubated at 34°C and 70% RH. Two days pre-eclosion, pupae were transferred into 24-well microtiter plates lined with filter paper to limit movement. Beginning 24 h before the expected emergence time, pupae were observed every 3 h to determine time of emergence, as defined by fully developed wings and walking behavior.

Quantification of ecdysteroid release

Following hemolymph collection for ecdysteroid titration, the same adult individuals were placed in bee saline (Huang and Otis 1991) for removal of brain and ovaries. Organs were cleaned of attached tissues and washed twice, once in bee saline and once in the incubation medium, then incubated separately for 18 h at 33°C in 50 µl of sterilized Grace's medium. Brain served as a negative control, as there is no evidence that the insect nervous system produces steroid hormones (Warren et al. 1984). After incubation, medium and organs were homogenized with a sonicator, then extracted twice with 250 µl chilled 90% methanol. Insoluble material was removed by centrifugation at 5000 *g* for 10 min at −4°C. Samples were lyophilized, resuspended in 200 µl methanol, and stored at −80°C until analyzed. Ecdysteroids were titered by radioimmunoassay, as described above. Per microgram rates of ecdysteroid release were calculated by averaging the mass of 10–17 pooled organs of each tissue type. To avoid possible adverse effects of weighing on the biosynthetic capacity of tissues, different organs were used.

Statistics

Differences in developmental time were analyzed with a Student's *t*-test. ANOVA was used to determine if hormone or vitellogenin

titer varied by subline or strain genotype. No subline effects were observed, and thus subline was nested within the reported results for strain genotype. This approach maximized phenotypic variation within each strain such that only robust differences between strain genotypes would be detected in the statistical analyses. Planned comparisons were made at the time points where titer curves suggested a divergence within or between strains. For time points with normally distributed data, Student's *t*-tests were used. When data did not conform to normality, as determined by Levene's test, Mann–Whitney *U* tests were used. Dunnett's method was used for multiple comparisons. The data on developmental times for control and JH-treated pupae were analyzed across all groups using a Kruskal–Wallis ANOVA on ranks. Depending on normality of the data, either ANOVAs or Mann–Whitney *U* tests were used for post hoc comparisons of groups. Analyses were performed with Sigmastat v. 3.5.

RESULTS

JH titers during larval development

Because honey bees achieve large ovary sizes by elevated JH in 4th–5th instar larvae, and because high pollen-hoarding strain workers have larger ovaries than low-strain bees on average, we predicted that high-strain 4th–5th insert larvae have higher JH titers than those of the low strain. We tested this possibility in a between-strain comparison 6–13 days postoviposition, that is, from the late 4th larval instar to pupation. In support of our prediction, we found that JH was elevated in high-strain larvae 6–10 days after oviposition (Fig. 1). This difference was significant on days 8 ($P<0.05$, $n=12, 20$) and 10 ($P<0.05$, $n=14, 20$), corresponding to the early feeding and late feeding/spinning stages of the 5th larval instar, respectively. To validate that this increased JH level correlated with significantly larger ovaries, individuals from the same worker cohorts were dissected as adults. As expected and shown before (Amdam et al. 2006, 2007), workers of the high-strain genotype averaged significantly more ovarioles per ovary (5.75 ± 0.30, $n=20$) than low-strain bees (3.90 ± 0.22; $P<0.001$, $n=20$).

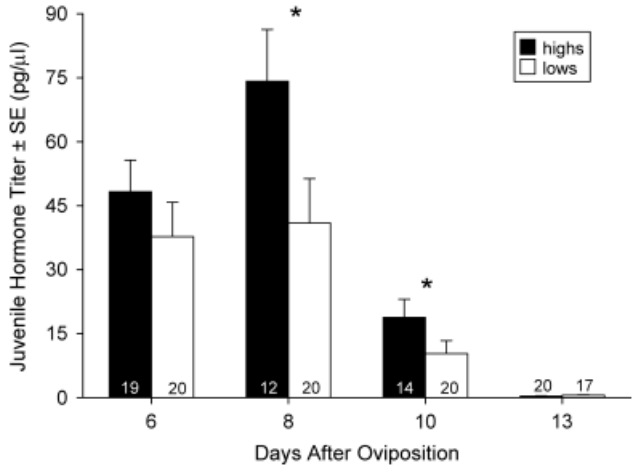

Fig. 1. The mean (± SE) JH titer of worker honey bees during larval development. Data are shown for cofostered high (black bars) and low (white bars) pollen-hoarding strain larvae obtained from three sublines per strain. Strains diverged on days 8 and 10, corresponding to the early feeding and late feeding/spinning stages of the 5th larval instar, respectively. Sample sizes are given and asterisks (*) indicate significance (Mann–Whitney *U*-test, $P<0.05$).

Pacing of pupal development

Daily photodocumentation revealed that the developmental trajectories of the pollen-hoarding strains initially were the same. Individuals molted from prepupae into the first pupal stage 13 days after being oviposited, and the timing of changes in eye- and cuticle pigmentation were similar for the next 5 days, that is, through the Pbm stage (Fig. 2). Strain differences became evident at the subsequent Pbd stage. Low-strain workers were characterized by accelerated pupal pigmentation, and their initial locomotor activity and full expansion of wings occurred 13 h earlier than in the high-strain genotypes. Low-strain bees began emerging as adults at the end of day 19 (▲ indicator, Fig. 2), after 1 day in Pbd stage, whereas high-strain workers emerged 18 h later (SE ± 1.39, $n=20$, $P<0.001$). All adults of both strains completed emergence with 24 h.

JH and ecdysteroid titer changes during pupal development

The finding of significant differences in pacing of pupal development led us to predict that pollen-hoarding strains differ in hormone expression as pupae. In accordance with this expectation, we established that strain differences were evident for both JH and ecdysteroids. During the pupal stage, JH was initially low in both strains (Fig. 3A), consistent with previous findings from wild type bees (Rembold 1987). The strains diverged during the Pb stage (15 days postoviposition) when, unlike low-strain workers, high-strain bees exhibited a significantly elevated titer (15.15 ± 1.12 pg/µl, $n=24$) relative to the Pp (6.81 ± 0.70 pg/µl, $P<0.001$, $n=24$) and Pbl1 stages (7.72 ± 0.78 pg/µl, $P<0.001$, $n=24$). This strain difference was verified by independent replication (see "Materials and Methods"); again high-strain titers rose during Pb (12.67 ± 1.06 pg/µl, $n=9$) relative to Pp (4.59 ± 0.51 pg/µl, $P<0.001$, $n=10$) and Pbl1 (5.34 ± 1.06 pg/µl, $P<0.001$, $n=9$), while there was no change in the JH of low-strain bees. Another difference arose at Pbd1, when JH increased in low-strain pupae (Fig. 3A, 9.16 ± 1.11 pg/µl, $n=30$) but not in high strains (5.64 ± 1.03 pg/µl, $P<0.05$, $n=20$). High-strain bees did not show an equivalent concentration until the following day (8.22 ± 1.62 pg/µl, $P=0.263$, $n=21$), when high-strain pupae entered the second half of an extended Pbd stage (Fig. 2). In accordance with evidence from wild type

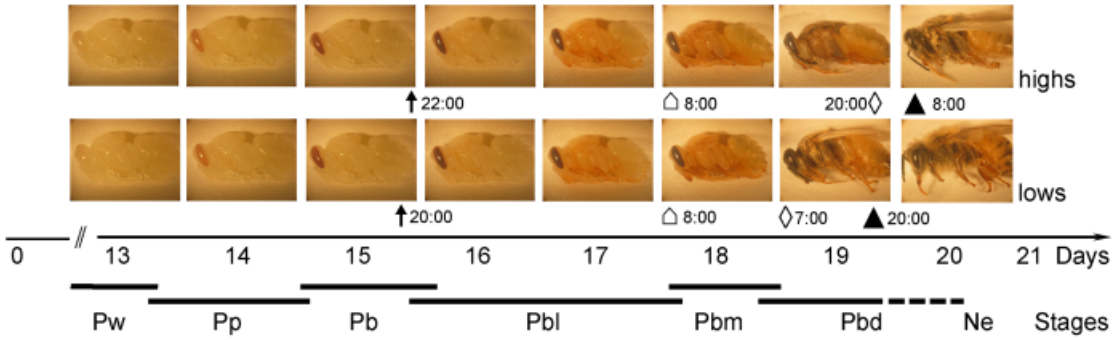

Fig. 2. Development timeline for worker honey bee pupae of high (highs) and low (lows) pollen-hoarding strains. The photographed individuals represented the average phenotype at each developmental time point with bees from three sub-lines per strain. After cofostering of larvae in wild type nursing colonies, pictures were taken at 24-h intervals from pupation. There are six discrete stages of pupal development, categorized by changes in body pigmentation: white (Pw), pink pigmented eyes (Pp), brown pigmented eyes (Pb), light pigmented body (Pbl), medium dark pigmented body (Pbm), and dark pigmented body (Pbd). The average duration range for each stage is indicated by the underlying bar. Key developmental events, including 24-h clock times, are also noted: ↟ 1st appearance of yellow cuticle pigmentation; ⌂ 1st full pigmentation of the antennae; ◊ 1st locomotor activity and full wings; ▲ 1st adult emergence (newly emerged, Ne stage).

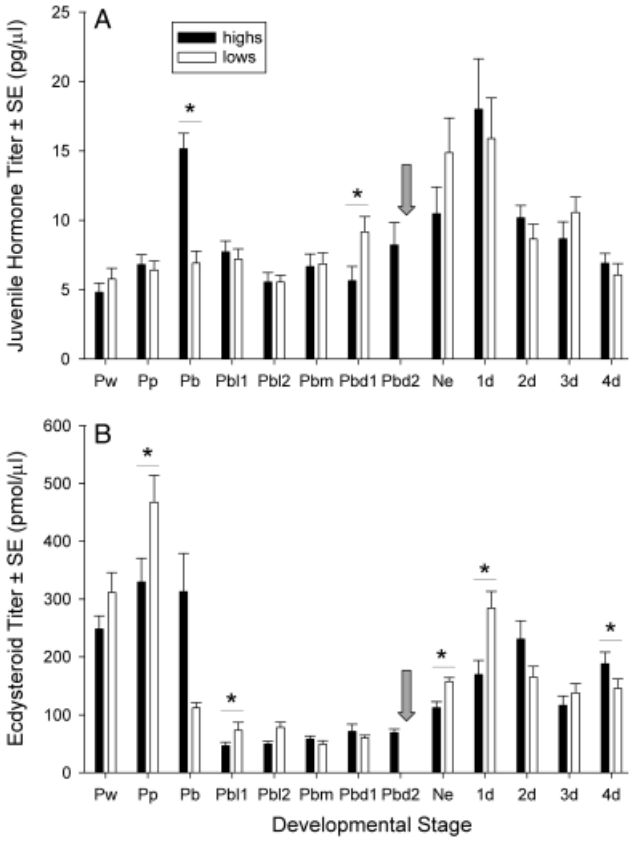

Fig. 3. Titer dynamics of (A) JH and (B) ecdysteroids (20-hydroxyecdysone equivalents) in cofostered worker honey bees of high (black bars) and low (white bars) pollen-hoarding strains. Bees were sampled from the white pupal stage (Pw, Day 13, Fig. 2), at adult emergence (Ne), and for 4 days posteclosion (1–4 days). Low-strain workers emerged 1 day earlier and did not have a second day of Pbd stage (gray arrows). Daily means (± SE) combine two replicate sets of each strain, with 17–30 bees per data point. Significant differences (Mann–Whitney *U*-test, $P<0.05$) are denoted by asterisks (*).

bees (Zufelato et al. 2000; Santos et al. 2001; Barchuk et al. 2002), these data suggest that JH signaling during the Pbd stage promotes the transition from pupa to adult.

For the pupal ecdysteroid titer, the profile was similar between the genotypes, but all major changes occurred at least 1 day earlier in the low strain (Fig. 3B). As observed in wild-type bees (Feldlaufer et al. 1985; Zufelato et al. 2000), the ecdysteroid titer increased during the first few days of pupal development. The highest mean (± SE) ecdysteroid concentration occurred during the Pp stage and was greater in low-strain genotypes (467.5 ± 43.0 pmol/µl, $n = 24$) than in high-strain bees (329.5 ± 44.0, $P<0.05$, $n = 23$). The strains also differed in the timing of the subsequent decline in ecdysteroid titer: a significant drop occurred between the Pp and Pb stages in the low strains ($P<0.001$, $n = 23$, 24) and between the Pb and Pbl1 stages in the high-strain bees ($P<0.001$, $n = 19$, 24). Thereafter, ecdysteroid levels remained low for several days.

Effect of Pb stage JH titer on development pacing

We considered the possibility that the temporal increase in JH at the Pb stage might slow subsequent developmental pacing, as seen at eclosion in high-strain bees (Figs. 2 and 3A). To test this hypothesis, we treated low-strain pupae with endogenous application of JH in acetone. We found that the untreated low-strain reference group, the acetone (vehicle) control and the 0.001 µg JH-treated group (lowest JH concentration) developed at similar rates ($P = 0.330$, $n = 16$–20). This pace was significantly faster than the untreated high-strain reference group ($P<0.001$, $n = 13$–20, Fig. 4). Our results confirmed the effect of genotype on developmental pace (Fig. 2), and showed that vehicle did not influence this trait. However, in contrast to our assumption, the higher JH concentrations accelerated, not

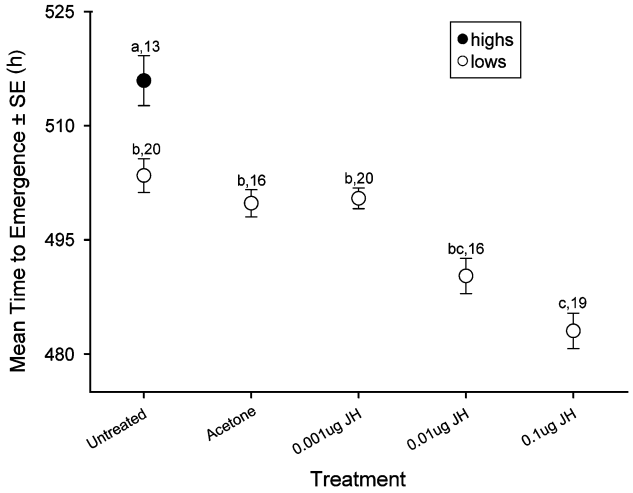

Fig. 4. Mean (± SE) time in hours (h) from oviposition to adult emergence for low pollen-hoarding strain worker bees (white circles) given one of several treatments at 15 days postoviposition (Pb stage): an untreated reference, acetone (solvent control), or an increasing dosage of JH III. High-strain bees were included as a reference for developmental pace (black circle). Letters indicate significant difference ($P<0.05$) by Dunn's method. Sample sizes are given.

slowed, the bees' development (Fig. 4). These effects of JH were consistent in wild type bees and in titrations up to 10 µg (O. Kaftanoglu, C. S. Brent, R. E. Page, unpublished data). Our results suggest that the elevated JH level of high-strain pupae (Fig. 3A) does not delay development around the time of adult emergence (Fig. 2 vs. Fig. 4).

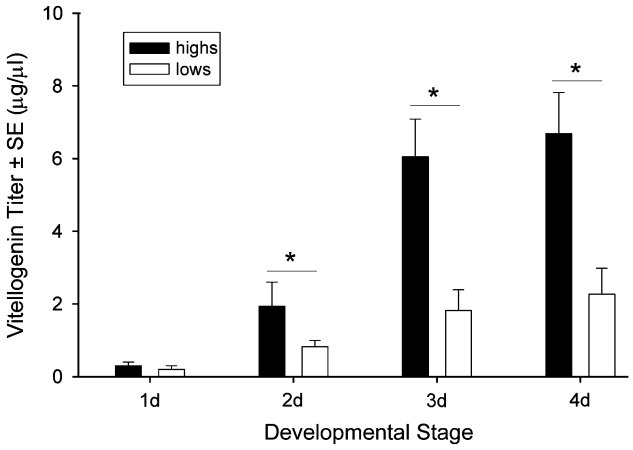

Fig. 5. Mean (± SE) hemolymph vitellogenin titer (in β-galactosidase equivalents) of co-fostered high (black bars) and low (white bars) pollen-hoarding strain worker bees during their first 4 days posteclosion. Bars represent data from two replicates, with 9–10 workers per strain. Significant differences between the two genotypes (ANOVA, $P<0.05$) are denoted by asterisks (*).

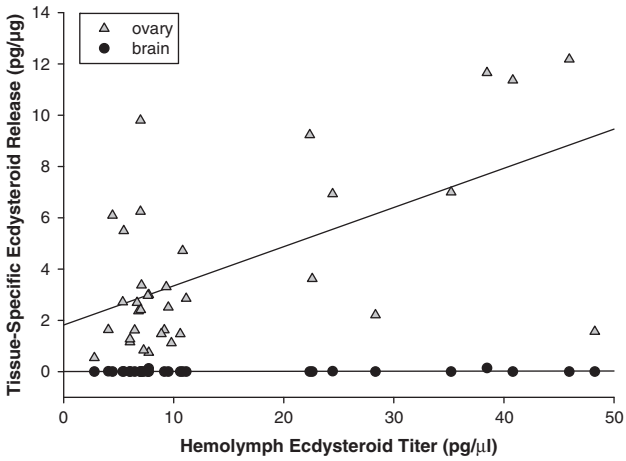

Fig. 6. Scatter plot showing the correlation between individual hemolymph ecdysteroid titers and ecdysteroid release rates (both in 20-hydroxyecdysone equivalents) from the brain (negative control) and ovaries of honey bee workers. Only the ovarian ecdysteroid release is significantly associated with the workers' circulating hormone level.

JH, ecdysteroid, and vitellogenin titer changes during early adulthood

From emergence (Ne stage, Fig. 3A) and onward, both strains exhibited very similar patterns of JH expression. The highest titers occurred on the day following emergence (1 day) in low (15.89 ± 2.92 pg/µl, $n = 17$) and high-strain bees (18.00 ± 3.63 pg/µl, $n = 20$, $P = 0.964$), after which titers gradually declined. This initial spike and subsequent decrease in JH can enhance vitellogenin synthesis in newly emerged workers (Barchuk et al. 2002). In our study populations, vitellogenin levels did indeed increase as JH titers declined (Fig. 3A vs. Fig. 5). Hemolymph vitellogenin concentrations were significantly influence by genotype ($P<0.00005$, $n = 45$) and worker age ($P<0.00001$, $n = 10$–20). Also, the increase by age was significantly stronger in high-strain bees (Fig. 5), confirming previous findings (Amdam et al. 2004, 2007).

During this same developmental period, ecdysteroid titers were significantly greater in low-strain genotypes at emergence (157.3 ± 6.8 vs. 112.4 ± 10.2, $P = 0.002$, Fig. 3B) and the day after (284.6 ± 28.8 vs. 169.3 ± 24.4, $P = 0.005$), with peak concentrations occurring 1 day earlier in low-strain bees compared with high-strain genotypes (1 day, 21 days postoviposition; 284.5 ± 27.8 pmol/µl, $n = 22$, vs. high strain, 2 days, 23 days postoviposition; 239.1 ± 34.9 pmol/µl, $n = 14$).

Ovarian production of ecdysteroids in young adults

Our data on ecdysteroid levels in young adults indicated dynamic regulation over several days. Given that the prothoracic glands degenerate before eclosion (Hartfelder 1993), we

predicted that the principle source of these ecdysteroids was the ovaries. Our results conformed to this prediction. While only trace amounts of ecdysteroids were released from brain (negative control, mean ± SE, 0.01 ± 0.01 pg/μg, $n = 30$), bees from both high and low pollen-hoarding strains released substantial quantities of the hormone from their ovaries. A significant increase in this release occurred between emergence and the 1 day stage (2.04 ± 0.33 vs. 8.68 ± 1.55 pg/ig; and 2.87 ± 0.33 vs. 12.87 ± 7.51 pg/ig, for highs and lows, respectively, $n = 10$), corresponding with hemolymph titers (Fig. 3B). Furthermore, while ecdysteroid release from brain did not correlate with circulating hormone levels ($R = -0.06$, $P = 0.75$, $n = 30$), release from the ovaries was significantly associated with the hemolymph titer ($R = 0.41$, $P < 0.05$, $n = 36$, Fig. 6). These data support the hypothesis that the different circulating levels of ecdysteroid hormone in young pollen-hoarding strain bees (Fig. 3B) result directly from ovarian endocrine activity.

DISCUSSION

JH and ecdysteroids are central regulators of insect development and reproduction, and as integrators of morphology, physiology, and behavior their signaling can result in complex trait correlations (Zera et al. 2007; Suzuki and Nijhout 2008). The robust hormonal differences shown here for honey bee genotypes bidirectionally selected for pollen-hoarding behavior correlate with a well-established behavioral syndrome (as defined by Sih et al. 2004). Although many of the specific phenotypic effects of these hormone differences remain to be determined, our results represent a step forward in the understanding how changes in a complex suite of interrelated traits can result from selection on a social behavior.

One of the pronounced differences between the honey bee genotypes was found during late larval development, when a higher JH titer was observed in high pollen-hoarding bees. Based on a previously demonstrated relationship between JH and ovarian morphology (Schmidt-Capella and Hartfelder 1998), we hypothesize that this titer difference enhances ovariole number in high strains. We further document that the ovaries of adult worker bees produce ecdysteroids (Fig. 6), which along with other ovarian factors can influence JH biosynthesis (K-A. Nilsen, B. Stay, G. V. Amdam, unpublished data; see also Elliott et al. 2006; Flatt et al. 2008). Thus, differences in ovariole number established by JH signaling in larvae may cumulatively influence JH, ecdysteroid, and vitellogenin titers during later development and after adult emergence, to regulate the observed suite of traits associated with each pollen-hoarding strain. Consistent with these speculations, we found a hormonal difference in late stage pupae (Pbd1), where low-strain individuals showed elevated JH titers 1 day sooner, coinciding with their earlier eclosion. After emergence, the genotypes differed in ecdysteroid titers—likely established by their ovaries—as well as vitellogenin produced by fat body (functional homolog to liver and white fat in vertebrates). Similar changes in developmental pacing and endocrine events can be provoked by hormone application on wild type bees (Zufelato et al. 2000; Barchuk et al. 2002).

We also observed endocrine differences between pollen-hoarding strains during early pupal development. Ecdysteroid titers peaked higher and dropped sooner in the low strain (Fig. 3B), but there was no observable external change in development (Fig. 2). High-strain bees expressed more JH at the Pb stage (Fig. 3A), and because JH is a developmental pacemaker in insects (Goodman and Granger 2005) we postulated that this event could delay adult eclosion. However, we found that pharmacological JH doses accelerated development while a lower dose had no effect. We propose, therefore, that JH titer changes during the Pb stage (within systemic concentrations) do not influence developmental pace, but may instead and in conjunction with ecdysteroids affect the organization of the central nervous system, which is sensitive to hormones during pupal development (Malun et al. 2003). Although this effect remains to be demonstrated, nervous system differences are expected between pollen-hoarding strains as they differ in nutrient perception, a significant predictor of foraging behavior (Pankiw and Page 2000; Pankiw et al. 2004). Perhaps not coincidentally, honey bee gustatory sensitivity correlates with reproductive traits: wild type workers with higher sucrose responsiveness have more ovarioles and higher *vitellogenin* mRNA levels (Tsuruda et al. 2008). Similar pleiotropic effects have been demonstrated in *Drosophila melanogaster*, where natural polymorphisms in the *foraging* gene, contribute to differences in feeding preference, locomotory activity (Pereira and Sokolowski 1993), sensory perception, and memory (Mery et al. 2007).

The genes that responded to artificial selection on honey bee pollen-hoarding behavior have not been fully identified. However, genome mapping studies have detected four quantitative trait loci (QTL) for pollen hoarding, *pln* 1–4, which reveal that the pollen-hoarding syndrome is connected by a highly epistatic and pleiotropic network of genes (reviewed by Amdam and Page 2010). Genetic linkage between *pln* regions and reproductive traits in workers is supported by genome mapping of QTL for worker ovary activation (Oxley et al. 2008) and ovary size (Wang et al. 2009), as well as studies of candidate genes such as the *insulin receptor substrate* (*IRS*), the *hormone receptor-like 46* (*HR46*) and *phosphoinositide-dependent kinase 1* (*PDK1*) (Wang et al. 2009, 2010). *IRS* and *PDK1* are central components of insulin/insulin-like signaling pathways, which influence the endocrine physiology and life-history of insects (Flatt et al. 2005). In honey bees, *IRS* is required for JH synthesis in larvae (N. Mutti, A. Dolezal, G. V. Amdam, unpublished data), and affects foraging behavior in adults (Wang et al. 2010). Insulin/insulin-like signaling can

also interact with honey bee vitellogenin, which has direct effects on foraging bias and JH synthesis (Guidugli et al. 2005; Nelson et al. 2007). These findings support a role of endocrine signaling in the genetic basis of the pollen-hoarding syndrome (Amdam et al. 2007).

To determine the specific roles of the endocrine differences presented here will require extensive hormonal manipulations and monitoring of several modes of physiological and behavioral expression. Still, our current results strongly suggest that artificial selection changed honey bee pollen-hoarding behavior by acting on developmental gene networks pleiotropically linked to female reproductive traits. We proposed before that natural selection can have acted on the same networks during evolution of insect sociality (Amdam et al. 2004; Amdam and Page 2010). We recognize that artificial selection can operate differently on functional and developmental constraints than natural selection (reviewed in Brakefield 2003; Schwenk and Wagner 2004). Yet, the presence of a consistent suite of traits in selected strains and wild type suggests that these associations are available for natural selection to act on to produce adaptive foraging biases and food-storage behavior in honey bee colonies (Amdam and Page 2010).

Acknowledgements

We thank K Hartfelder and T Flatt for comments, A Dolezal, O Kaftanoglu, K Norberg and C Ozturk for assistance, and LI Gilbert for H-22 antibody. Research was funded by U.S. Department of Agriculture (NRI-CSREES 2003-01620), National Institute on Aging (NIA P01 AG22500), Norwegian Research Council (180504, 185306, 191699), U.S. National Science Foundation (0615502), and The Pew Scholars Program in the Biomedical Sciences.

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