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The Genetic Architecture of the Behavioral Ontogeny of Foraging in Honeybee Workers

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ABSTRACT

The initiation of foraging during the life course of honeybee workers is of central interest to understanding the division of labor in social insects, a central theme in sociobiology and behavioral research. It also provides one of the most complex phenotypic traits in biological systems because of the interaction of various external, social, and individual factors. This study reports on a comprehensive investigation of the genetic architecture of the age of foraging initiation in honeybees. It comprises an estimation of genetic variation, the study of candidate loci, and two complementary quantitative trait loci (QTL) maps using two selected, continually bred lines of honeybees. We conclude that considerable genetic variation exists between the selected lines for this central life history component. The study reveals direct pleiotropic and epistatic effects of candidate loci (including previously identified QTL for foraging behavior). Furthermore, two maps of the honeybee genome were constructed from over 400 AFLP markers. Both maps confirm the extraordinary recombinational size of the honeybee genome. On the basis of these maps, we report four new significant QTL and two more suggestive QTL that influence the initiation of foraging.

RECENT developments in quantitative genetics and genomics allow for genetic analyses of increasingly complex traits in a variety of organisms. This development is of unprecedented value for all biological disciplines, as most phenotypic variation of interest is determined by genetic and environmental components that often interact in elaborate ways. In addition to a quantification of the overall genetic variation, single gene effects and their interactions need to be identified because the sum of the constituent factors might be not only greater but also different from its parts (Brodie 2000). Behavioral and life-history traits can be regarded as prime examples of complex, polygenic traits that often cannot be reduced to simple components (Finch and Rose 1995).

Social insects constitute a particularly interesting system to consider the causation of such polygenic traits because their social environment adds an additional level of complexity that is itself partially under genetic control (Wolf 2000; Pankiw *et al.* 2002). The honeybee (*Apis mellifera* L.) has become a model in several research areas for a variety of reasons (Robinson *et al.* 1997; Menzel 1999; Page *et al.* 2002). One of the most important aspects of honeybee biology is the intriguing and well-described temporal division of labor among its workers: Young workers specialize in particular duties in the nest, while older workers forage for resources outside the nest (Winston 1987).

This transition of individual honeybees from hive bees to foraging initiation has been the focus of much theoretical (Beshers and Fewell 2001) and empirical (*e.g.*, Robinson 2002) research effort. While successful modeling can fruitfully guide further research, model interpretation ultimately requires knowledge of the underlying causation of this behavioral phenomenon (Robinson *et al.* 1997). Some progress in understanding the mechanisms of foraging initiation has been made by investigating hormones (Sullivan *et al.* 2000), neurotransmitters (Schulz and Robinson 2001), and candidate genes (reviewed by Robinson 2002). However, an extensive genetic dissection of this central life-history trait has never been attempted in spite of its high potential to reveal genetic structure and causative candidates.

The onset of foraging is a complex trait with interacting endogenous developmental processes (Robinson *et al.* 1994), potential neurogenic (Toma *et al.* 2000) or neuro-endocrine pacemakers (Sullivan *et al.* 2000; Schulz and Robinson 2001), and social (pheromonal) influences (Huang and Robinson 1992; Pankiw *et al.* 1998; LeConte *et al.* 2001). Furthermore, environmental conditions that influence the colony status, such as food shortage (Schulz *et al.* 1998), influence the individual onset of foraging. It has been shown that individual factors, such as octopamine, interact with

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other factors in a nontrivial manner (Barron *et al.* 2002; Schulz *et al.* 2002). Such interactions may occur even between different levels of organization (cell-organ/individual-colony; Page and Erber 2002) and constitute the backbone of theoretical models for the onset of foraging (Huang and Robinson 1992; Beshers and Fewell 2001; Amdam and Omholt 2003). Thus, an array of parameters makes many genes potentially influential. Genetic variability for the age at foraging initiation has been shown repeatedly in honeybees (Calderone and Page 1988; Robinson *et al.* 1989; Page *et al.* 1991; Giray *et al.* 2000; Brillet *et al.* 2002).

We studied the phenomenon of foraging initiation using a comprehensive, quantitative genetic approach, exploiting pronounced differences in the age of foraging initiation (or age of first foraging: AFF) between two lines of honeybees. These lines had previously been found to differ significantly with respect to AFF (Pankiw and Page 2001), although they were selected on the basis of their pollen-hoarding behavior (Page and Fondrk 1995). Bees selected for high pollen-hoarding behavior were found to initiate foraging earlier than both unselected bees and bees selected for low pollen-hoarding behavior (Pankiw and Page 2001).

This correlated selection response, which pertains to other traits such as sucrose sensitivity (Page *et al.* 1998) and learning behavior (Scheiner *et al.* 2001), prompts the question whether the behavioral and life-history differences between the selected lines are genetically correlated (by close linkage or pleiotropy) or the two selected lines have been fixed by chance for alternative alleles at different loci (each independently influencing a single trait).

In this study, we generated four experimental groups with different genetic composition from the two selected lines to investigate the genetic architecture of the AFF in worker honeybees. Our specific aims were: (1) to quantify overall genotypic effects, (2) to test for genetic effects of genetic markers linked to previously identified QTL affecting foraging behavior (Hunt *et al.* 1995; Page *et al.* 2000) and to the candidate genes *Amper* (*period* ortholog, GenBank accession no. AF159569) and *Amfor* ("PKG" ortholog, GenBank accession no. AF469010) that have both been associated with the initiation of foraging (Toma *et al.* 2000; Ben-Shahar *et al.* 2002; Robinson 2002), and (3) to construct genomic linkage maps in reciprocal backcross populations to screen the honeybee genome systematically for new QTL that affect the age of onset of foraging behavior. To understand the genetic architecture of complex traits, the study of such multiple segregating populations is far more informative than results from a single experimental population (Asíns 2002).

MATERIALS AND METHODS

Experimental groups: A high and a low pollen-hoarding line set up by Page and Fondrk (1995) were used as genetic sources for the experiment. These lines were derived from strains established in 1990 and maintained under continuous, bidirectional selection for pollen-hoarding behavior. At the beginning of the experiment, the strains were in their eighteenth generation, maintained by circular inbreeding among lines and three outcrossings to commercial hives of similar (high pollen-hoarding or low pollen-hoarding) phenotype.

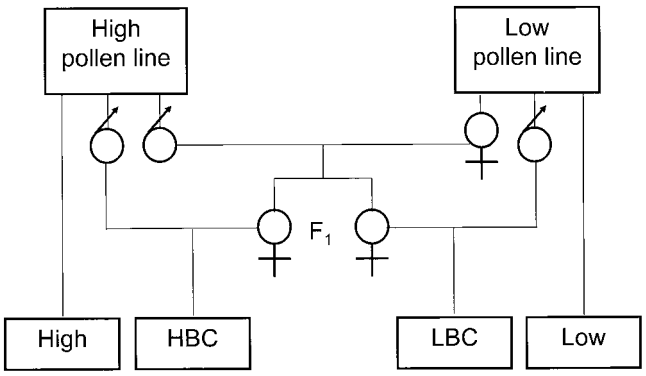

Figure 1.—Breeding scheme used in the experiment to generate two reciprocal backcross populations and two populations from the original source lines (Page and Fondrk 1995). HBC, high backcross; LBC, low backcross; High, high pollen hoarding; Low, low pollen hoarding.

We generated hybrid supersister queens (Page and Laidlaw 1988) by instrumentally inseminating a virgin queen from a low line ("C") with semen of a single drone from one high line ("Q1"). Eggs of this queen were grafted into queen cups to produce hybrid queen offspring (Laidlaw and Page 1997). Subsequently, these hybrid queens were instrumentally inseminated with sperm of either a high-line male or a low-line male. Two of these queens were selected to generate the high backcross (HBC) and low backcross populations (LBC). Worker offspring of the corresponding high- and low-line queens (eighteenth generation) represented our two other experimental groups (high and low, Figure 1).

Experiment: All four source queens were simultaneously caged on empty comb within their respective colonies to induce maximal, simultaneous egg-laying behavior. Twenty-four hours prior to emergence, these combs were transferred into individual cages in temperature- (34°) and moisture (50% relative humidity)-controlled incubators. Newly emerged bees of each source were color marked on the thorax with a unique enamel color and transferred within 6 hr to a common, unrelated bee colony (common hive environment).

Returning foragers from this host colony were monitored daily for 2 hr during peak foraging (Pankiw and Page 2001). Each marked returning forager was captured and assigned a unique number, and her date of capture was recorded (which corresponded to her AFF). Our observational period proved long enough to capture the vast majority of marked bees flying on any specific day, and we avoided collection during orientation flights (Pankiw and Page 2001).

Evaluations of candidate markers: DNA was purified from whole bees with a CTAB lysis and a single phenol/chloroform extraction (Hunt and Page 1995). After quantification of the resuspended DNA with a DNA fluorometer (Hoefer Scientific Instruments, San Francisco), the DNA concentration was adjusted to 100 ng/μl. Specific primers were used to PCR amplify previously identified sequence-tagged-site (sts) markers (Hunt and Page 1995; Page *et al.* 2000) that are linked to identified QTL (*pln1*, *pln2*, and *pln3*) or to candidate genes (*Amfor* and *Amper*). The QTL markers were within 15 cM of their respective "QTL-peak," and the candidate gene markers

TABLE 1

The five candidate genome regions investigated for their effect on the age at foraging initiation in both backcross populations

Genome region	sts marker (with reference)	PCR reagents	PCR thermoprofile	Backcross
pln1	stsD8-.33f (Hunt *et al.* 1995)	200 μM dNTPs 0.3 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 51°, 60 sec Elongation: 73°, 120 sec 35 cycles	HBC
	stsD8-.33f (Hunt *et al.* 1995)	200 μM dNTPs 0.3 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 51°, 60 sec Elongation: 73°, 120 sec 35 cycles	LBC
pln2	sts-tyr (cut with *Taq*I) Forward primer: GTGTCACCAATGATCAAGGATT Reverse primer: AGCCTGTCATGTCGTAGTCCTC	200 μM dNTPs 0.7 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 60° (5×)/50° (30×), 60 sec Elongation: 72°, 120 sec	HBC
	sts-tyr (cut with *Taq*I) Primer sequences: see above	200 μM dNTPs 0.7 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 60° (5×)/50° (30×), 60 sec Elongation: 72°, 120 sec	LBC
pln3	RAPD Y15 (Hunt *et al.* 1995)	100 μM dNTPs 0.5 μM primers 2 mM $MgCl_2$ 0.25 units Taq	Denaturation: 94°, 60 sec Annealing: 35°, 60 sec Ramp (35°–72°): 120 sec Elongation: 72°, 120 sec 45 cycles	HBC
	sts-q4-ecap (cut with *Mbo*I; R. E. Page, M. Beye and D. I. Nielsen, unpublished results)	200 μM dNTPs 0.5 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 68° (8×), 63° (30×) 60 sec Elongation: 72°, 120 sec	LBC
Amfor	sts-pkg-ecap (cut with *Alu*I) Forward primer: CCAAGACGTTCTGCTGGGTTGTC Reverse primer: TATACACGGCCATAATCGCGATCG	200 μM dNTPs 0.3 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 70° (5×)/65° (5×)/60° (30×), 60 sec Elongation: 72°, 120 sec	HBC
	sts-pkg-ecap (cut with *Rsa*I) Primer sequences: see above	200 μM dNTPs 0.3 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 70° (5×)/65° (5×)/60° (30×), 60sec Elongation: 72°, 120 sec	LBC
Amper	NA	NA	NA	HBC
	sts-per-ecap (cut with *Alu*I or *Rsa*I) Forward primer: GCGCACTTCGAATATATTGCAT CATATACC Reverse primer: GCATCATTCATTTGCAAAATGG CTGACC	200 μM dNTPs 0.5 μM primers 2 mM $MgCl_2$ 0.5 units Taq	Denaturation: 94°, 60 sec Annealing: 60° (5×)/55° (5×)/50° (30×), 60 sec Elongation: 72°, 120 sec	LBC

Site-specific markers are listed with conditions for PCR amplification and the restriction enzyme (used according to the manufacturers' recommendations) that was used to score the polymorphism.

were designed from the immediate sequence of the respective gene.

Table 1 lists these candidate loci with information on primers and PCR conditions. When PCR amplification did not result in a presence/absence polymorphism, the amplified DNA sequences were screened for polymorphisms (segregating markers) with >20 restriction endonucleases. The restriction enzymes used in the final analyses are also given in Table 1. All candidate loci were evaluated on 20 × 25-cm agarose gels [0.64% agarose, 1% synergel (Diversified Biotech), 0.5×

TBE] and ethidium bromide staining. We tested each marker's effect on the AFF by a two-way ANOVA (marker genotype × backcross population) and corresponding posthoc tests. Furthermore, we tested for epistasis among candidate loci by complete multiway ANOVAs.

QTL analyses: *AFLP marker generation:* A mapping population of 182 individuals from the HBC group and a second mapping population of 94 individuals from the LBC group were generated, choosing individuals with extreme phenotypes in both cases. The AFLP core reagent kit (Invitrogen Life Technologies) was used according to the manufacturer's recommendations (with reaction volumes reduced to 50%) for the double restriction (*Eco*RI and *Mse*I) and ligation of the adaptors. The preamplification involved 24 cycles of denaturation (94°/60 sec), annealing (56°/60 sec), and elongation (72°/60 sec). The product was diluted 1:50 to serve as template for a second, selective PCR. Selective primers contained two specific nucleotides and their sequences are given in the APPENDIX. The *Eco*RI primers were labeled with [γ-^{33}P]ATP and used at a final concentration of 85 ng/ml. The unmodified *Mse*I primers were used at 625 ng/ml.

The products of the selective amplifications were run out on denaturing, 6% polyacrylamide gels (35 cm × 45 cm × 0.4 mm), along with radioactively labeled Sequamark size marker (Research Genetics, Hunstville, AL). Gels were dried on filter paper and exposed for 24–96 hr to Biomax MR film (Kodak). Polymorphic bands were identified visually, sized, and scored for all individuals. Finally, all films were rescored once to check for scoring errors. For most markers, linkage phase was established by comparison with the genotype of the grandfather of the mapping populations (Figure 1).

Map construction: Linkage groups in both marker data sets were identified with Mapmaker 3.0b (LANDER and BOTSTEIN 1989). We used the Kosambi mapping function and 37.5 cM/LOD 3 as linkage criteria between markers (HUNT and PAGE 1995). Linkage groups identified by two-point analysis were subsequently scrutinized by multipoint analysis and exhaustive tests of local map permutations to find the best marker order within linkage groups (LINCOLN *et al.* 1993). An ordered data set was generated and the data checked for potential PCR artifacts indicated by double crossovers (HUNT and PAGE 1995).

The linkage maps from the HBC and LBC were compared to establish homology of markers and linkage groups. The nature of AFLP markers (codominant only if a length polymorphism is involved, dominant in the more common case of presence/absence polymorphism) makes this process difficult and we resorted to a third map, constructed from the haploid male offspring of one of the hybrid queens (our unpublished results). In theory, both the HBC and the corresponding LBC marker can be scored in the haploid drones to establish homology between corresponding markers. Thus, we used the linkage groups of the male map to determine homology for HBC and LBC linkage groups without match in the direct LBC-HBC map comparison.

We used two criteria to establish homology between linkage groups in the HBC and LBC maps: (1) Two linkage groups were regarded as homologous when both contained three or more markers in the same order that were generated with, respectively, the same primer combination and (2) two linkage groups were regarded as homologous when both contained two or more markers that had exact matches in the same linkage group in the male map.

QTL analysis: All markers were initially screened for an association with the age of first foraging using simple one-way ANOVAs. As data were bimodal and significance levels would have to be corrected for multiple comparisons, these tests were used only to sort markers according to their effect size. Additionally, we ordered the HBC and LBC marker sets according to the results of the linkage mapping and analyzed both data sets with the computer programs MapQTL 4.0 (VAN OOIJEN *et al.* 2002) and QTL Cartographer (BASTEN *et al.* 1994, 2002). Interval mapping (JANSEN 1993) was used, and as both programs resulted in almost identical genome profiles of the LOD score, only the results of MapQTL are shown here. The genome-wide significance threshold for LOD scores was adopted from HUNT *et al.* (1995; see also LANDER and BOTSTEIN 1989), and these scores were compared to empirically determined values (CHURCHILL and DOERGE 1994).

RESULTS

Overall genetic differentiation: The age of first foraging was significantly different among the high (mean 16.1 ± SD: 5.9; $n = 185$), HBC (17.1 ± 7.8; $n = 623$), LBC (20.8 ± 6.1; $n = 511$), and low (26.7 ± 5.5; $n = 165$) groups ($F_{(3.1480)} = 109.08$, $P < 0.0001$, Figure 2). Tukey's HSD posthoc test revealed highly significant differences ($P < 0.001$) among all individual groups except between the high and the HBC groups (NS). The proportion of phenotypic variation attributable to genotype was measured over all groups by regression analysis ($r^2 = 0.16$, $F_{(1.1482)} = 277.55$, $P < 0.0001$; Figure 2). When only the two original lines (high and low genotype groups) were included, $r^2 = 0.46$ ($F_{(1.348)} = 300.19$, $P < 0.0001$). The regression equations were Y [days] $= -0.40X$ [days] + 24.1 [days] and Y [days] $= -0.68X$ [days] + 26.7 [days], respectively. Y represents the AFF and X the proportion of genome that originates from the high line.

Although both backcrosses matured faster than expected on the basis of a linear additive model ($\delta_{HBC} = 1.77$ days, $\delta_{LBC} = 3.41$ days), this deviation was not significant. As expected, the random recombination of genomes in both backcross populations resulted in higher phenotypic variance than in the source strains (high = 35.08, low = 30.21, HBC = 61.22, LBC = 36.69, Figure 2).

Candidate genes: Effects of single candidates in both backcross populations were evaluated by two-way ANOVA (marker genotype × backcross population). The effect of backcross population was highly significant (F-values > 13, P-values < 0.001) in all statistical tests, with the HBC group having a lower AFF (17.10 ± 6.06) than the LBC group (20.76 ± 7.82). For *pln1*, a significant interaction between marker genotype and backcross population was found ($F_{(1.725)} = 7.46$, $P = 0.0065$). This was due to an effect of the *pln1* marker in the HBC ($F_{(1.389)} = 7.22$, $P = 0.0075$), but not in the LBC group ($F_{(1.344)} = 0.95$, $P = 0.3293$). No other marker effects were detected (*pln2* overall, $F_{(1.707)} = 0.73$, $P = 0.394$; interaction, $F_{(1.707)} = 0.57$, $P = 0.452$; *pln3* overall, $F_{(1.657)} = 3.454$, $P = 0.0635$; interaction, $F_{(1.657)} = 0.136$, $P = 0.713$).

Similarly, the *Amfor* marker results did not have a significant effect (overall, $F_{(1.696)} = 3.272$, $P = 0.0709$; interaction, $F_{(1.696)} = 0.303$, $P = 0.582$). The *Amper* marker

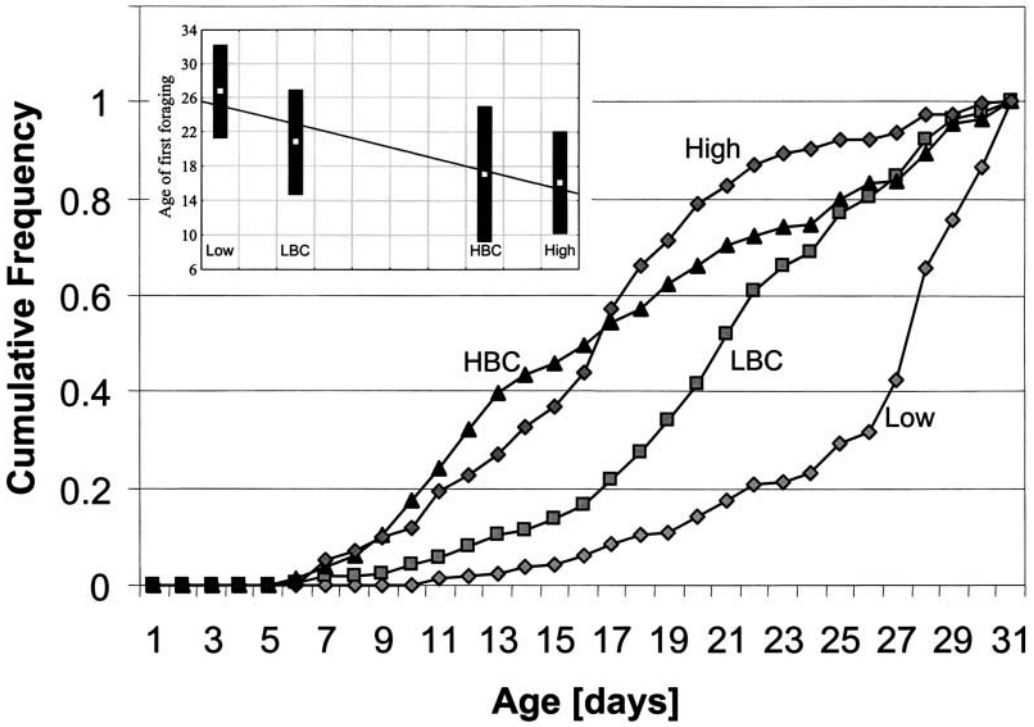

FIGURE 2.—The age at the onset of foraging (age of first foraging) strongly differed between the experimental groups. The cumulative hazard plot of foraging initiation is shown. Inset: Means and SD of the AFF (in days) of the four groups and the regression of AFF on genotype.

did not have any effect in the LBC group ($F_{(1.293)} = 0.138$, $P = 0.711$). It could not be evaluated in the HBC group due to lack of variability.

The multifactorial ANOVA did not indicate any epistatic effects in the HBC, but revealed interaction effects in the LBC population: A significant two-way interaction was found between the *pln1* and the *pln3* marker ($F_{(1.165)} = 4.966$, $P = 0.0272$), together with two significant three-way interactions, *pln1* × *pln2* × *pln3* ($F_{(1.165)} = 4.476$, $P = 0.0359$) and *pln1* × *pln2* × *Amper* ($F_{(1.165)} = 8.382$, $P = 0.0043$).

Genomic maps: Overall, 438 AFLP markers were generated in the HBC. Of these, 387 markers could be scored reliably and were linked to one of 35 separate linkage groups (three or more linked markers). The total map size was 3897 cM. In the "low" backcross, 435 markers were generated. These were assembled into 38 linkage groups that comprised 396 markers. The total map size was 3702 cM and 39 markers were unlinked. When adding 37.5 cM for every linkage group in excess of the expected 16 (SANDERSON and HALL 1948), the map size estimates were increased to 4610 and 4527 cM, respectively. Comparisons between the HBC, LBC, and the male map allowed us to combine several linkage groups in one map that had homologies to a single linkage group in one of the other maps. Thus, 16 linkage groups in the HBC map were condensed to 7, and 11 groups in the LBC were combined into 5, resulting in 26 and 32 linkage groups, respectively.

The map comparisons also revealed a reasonable degree of homology between the HBC and LBC maps. Fifteen linkage groups (containing 320 markers in the HBC and 299 markers in the LBC) could be established as homologous between the two backcross maps. This corresponded to 79 and 75% of the total genetic map of the HBC and LBC, respectively.

QTL analysis: The preliminary ANOVA analyses in the HBC and LBC indicated a few markers of strong effect on the AFF of the focal honeybee workers. Table 2 lists, for both backcrosses, the three markers with the strongest effect on AFF. Three of these six most promising markers did not map to any linkage groups and are therefore not reflected in the QTL described below.

Interval mapping revealed two major QTL for age of first foraging in the HBC (AFF1 and AFF2, Figure 3) and two in the LBC (AFF3 and AFF4, Figure 4). The LOD score of AFF1 was 4.2 and that of AFF2 was 3.5, explaining 16.3 and 14.2% of the total variance, respectively. AFF3 had a LOD score of 3.3 and AFF4 of 3.2, explaining 23.1 and 17.0% of the total variance, respectively. On the basis of the calculated significance threshold of 3.0 (LANDER and BOTSTEIN 1989; HUNT *et al.* 1995), these four QTL are significant. However, bootstrapping indicated a LOD threshold for significance (3.2 for HBC and 3.4 for LBC) slightly higher than the theoretically calculated one.

Both QTL in the HBC had effects in the expected direction; *i.e.*, the allele for fast behavioral maturation was inherited from the high line. For the two QTL identified in the LBC, the opposite was true: In both cases the allele for fast behavioral maturation was inherited from the low line (Table 3). The two QTL identified in the HBC were located on different linkage groups from the two significant QTL in the LBC. Therefore,

TABLE 2

The three markers with highest *F*-scores (correlated best with the age of first foraging) in the ANOVA analyses in the HBC and LBC

	HBC			LBC	
Marker	ANOVA	Interval mapping[a]	Marker	ANOVA	Interval mapping[a]
E5M3445	$F_{(1.173)} = 213.6$	—	E5M7580	$F_{(1.77)} = 17.3$	—
E1M4270	$F_{(1.143)} = 37.6$	4.2	E3M3299	$F_{(1.89)} = 15.9$	—
E3M5324	$F_{(1.82)} = 15.3$	2.5	E6M8348	$F_{(1.81)} = 14.1$	3.20

Marker abbreviations reflect the fragment size appended to the primer combination (*e.g.*, E1M2348 indicates a marker based on a 348-bp fragment amplified with the primers E1 and M2; for primer sequences; see APPENDIX).

[a] Interval mapping LOD score of the peak nearest to the marker (determined with the computer package MapQTL). Markers found to be unlinked are reported without interval mapping LOD score.

the four reported QTL are independent and identify separate genomic regions. The maximum LOD score of the homologous region in the opposite backcross was 0.00 for AFF1, 0.27 for AFF3, and 0.17 for AFF4. For AFF2, no homologous region was identified in the LBC map.

Additionally, we found one putative QTL in the HBC, characterized by marker E5M3323 (LOD score 2.5), and one in the LBC, associated with the marker E9M1363 (LOD score 2.0). Both loci are unrelated to any of the reported significant QTL because they are located on different linkage groups (Figure 5).

DISCUSSION

Many aspects of the division of labor, central to the organization of social insect colonies, can be explained by individual response thresholds (BESHERS and FEWELL 2001) that are linked to genotypic differences (*e.g.*, ROBINSON *et al.* 1989). The onset of foraging is probably the most important and most prominent event in the age-related division of labor in honeybees. PANKIW and PAGE (2001) reported significant genotypic effects on the initiation of foraging between two lines of honeybees from *A. mellifera*, which were selected on the basis of pollen-hoarding behavior (PAGE and FONDRK 1995). This study confirmed and significantly extended their results by quantifying the genetic differentiation of these lines and studying the underlying genetic architecture. Our results provide the foundation for the future characterization of genes with major causal effects on the rate of behavioral maturation in honeybees.

We quantified the proportion of the variance that is attributed to differences in genotype and evaluated dominance effects by calculating the dominance deviation (FALCONER and MACKAY 1996) in both reciprocal backcrosses. The quantification of genetic and environmental effects in a common hive environment is problematic because social interactions may cause genotype × genotype interactions (CALDERONE and PAGE 1991; WOLF 2000; RÜPPELL *et al.* 2001; PANKIW *et al.* 2002). Although social interactions influence the rate of behavioral maturation (HUANG and ROBINSON 1992, 1996), we believe our estimate is little affected by $G \times G$ interaction because the experimental groups constituted only a very small part of the social environment (*i.e.*, altogether 1500 bees were introduced into a hive of ~30,000 bees).

The amount of genetic variation in AFF is fairly high for a central life-history parameter (MOUSSEAU and ROFF 1987). This could be due to the fact that a certain amount of variability in the AFF is advantageous at the colony level and thus genetic variation is maintained (PAGE *et al.* 1995). On the other hand, the pronounced genotypic differences in behavioral ontogeny are a correlated response to selection on the amount of pollen hoarding (PAGE and FONDRK 1995; PANKIW and PAGE 2001) and adaptive explanations for the maintenance of genetic variability do not need to be invoked. The genetic correlation between AFF and foraging behavior (see also PANKIW 2003) is not trivial. It suggests that the artificial selection regime has modified fundamental pathways of general influence on behavior that are constrained by pleiotropy and epistasis (see below). Incorporating the backcrossed populations expectedly decreased the amount of phenotypic variation that can be explained by genotype because of the inherent genetic heterogeneity of the backcrosses. As neither the HBC nor the LBC population demonstrates a bimodal distribution of the age at onset of foraging, we could further conclude that multiple segregating factors were involved in the pronounced genetic differentiation of the AFF between the high and low line.

Several molecular pathways have been suggested to control, or at least influence, the rate of behavioral development in honeybee workers and thus their age at foraging initiation (HUANG and ROBINSON 1992, 1996; SCHULZ and ROBINSON 1999, 2001; SULLIVAN *et al.* 2000; TOMA *et al.* 2000; BEN-SHAHAR *et al.* 2002; BLOCH *et al.* 2002; AMDAM and OMHOLT 2003; HUMPHRIES *et al.* 2003). In this study, we failed to find a significant direct effect of two genetic markers located near the candidate

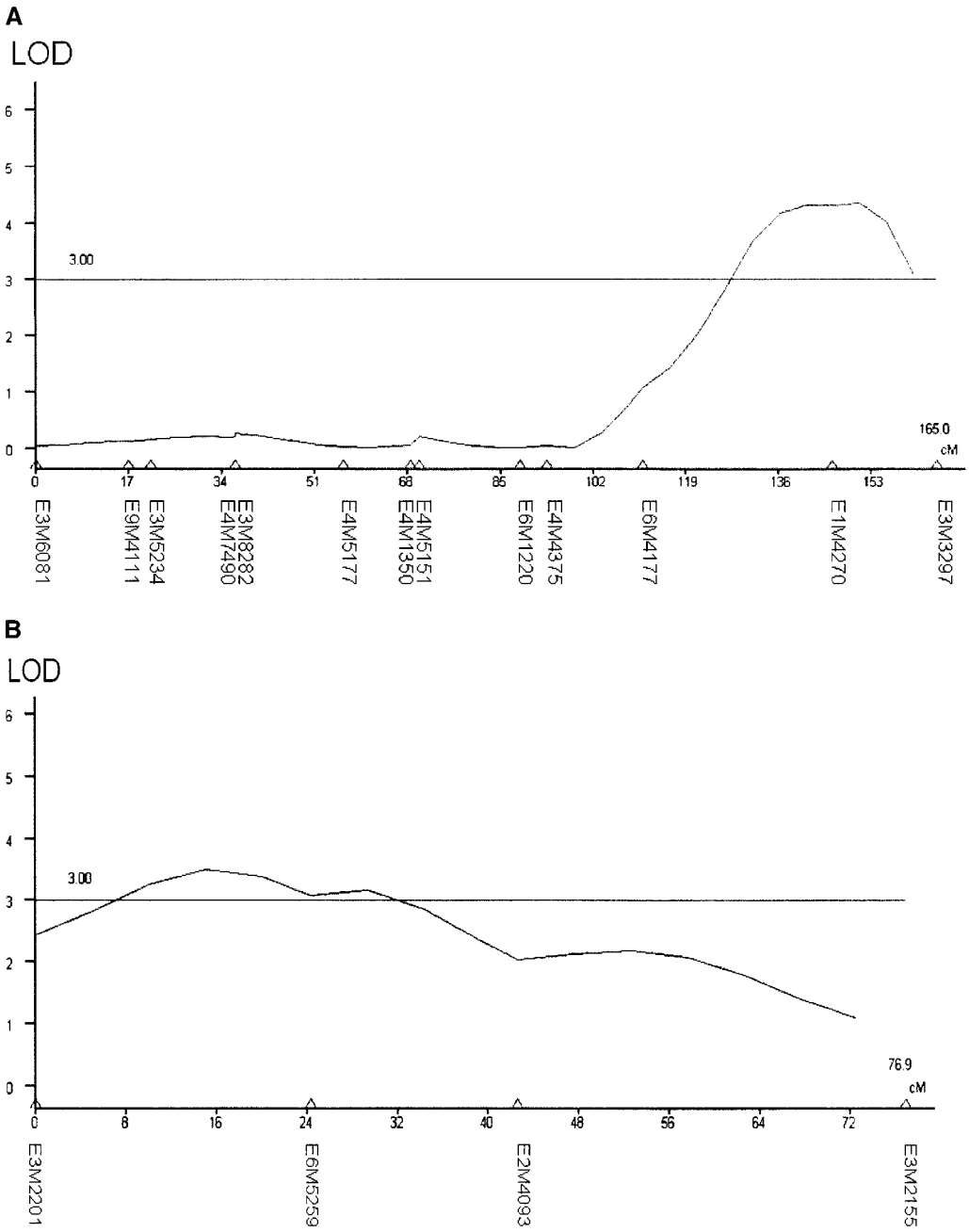

FIGURE 3.—Two regions in the high backcross showed LOD scores higher than the significance threshold. The *x*-axis represents the linkage group with recombinational distances between markers. We called these regions (A) AFF1 and (B) AFF2 to indicate their effect on the age of first foraging.

genes *Amfor* (BEN-SHAHAR *et al.* 2002) and *Amper* (TOMA *et al.* 2000) that have been suggested to be associated with or involved in the initiation of foraging (ROBINSON 2002).

Our results do not disprove the involvement of *Amper* or *Amfor* in the initiation of foraging. However, the pronounced differences in the rate of behavioral maturation between the two investigated strains cannot be explained by variability in the genomic region of *Amper* or *Amfor* but must be predominantly due to genetic variation in other genomic regions. The gene *Amfor* has been identified by sequence similarity to the foraging gene (*For*) in *Drosophila melanogaster* and it encodes a cGMP-dependent protein kinase that has potential for a variety of cellular functions (BEN-SHAHAR *et al.* 2002). It has been implicated in the initiation of foraging mainly because its expression increases independently of age with the onset of foraging (BEN-SHAHAR *et al.* 2002). On the basis of our results we cannot support the hypothesis that *Amfor* influences the initiation of foraging, but this may be due to a lack of effectual genetic variability in our experimental crosses.

However, the *Amper* marker contributes to the variability in the onset of foraging in a complex way, interacting with *pln1* and *pln2*. It has been shown previously that the onset of foraging is normally preceded by an upregulation of the activity of the *Amper* gene (TOMA *et al.* 2000). The increase in gene activity also correlates

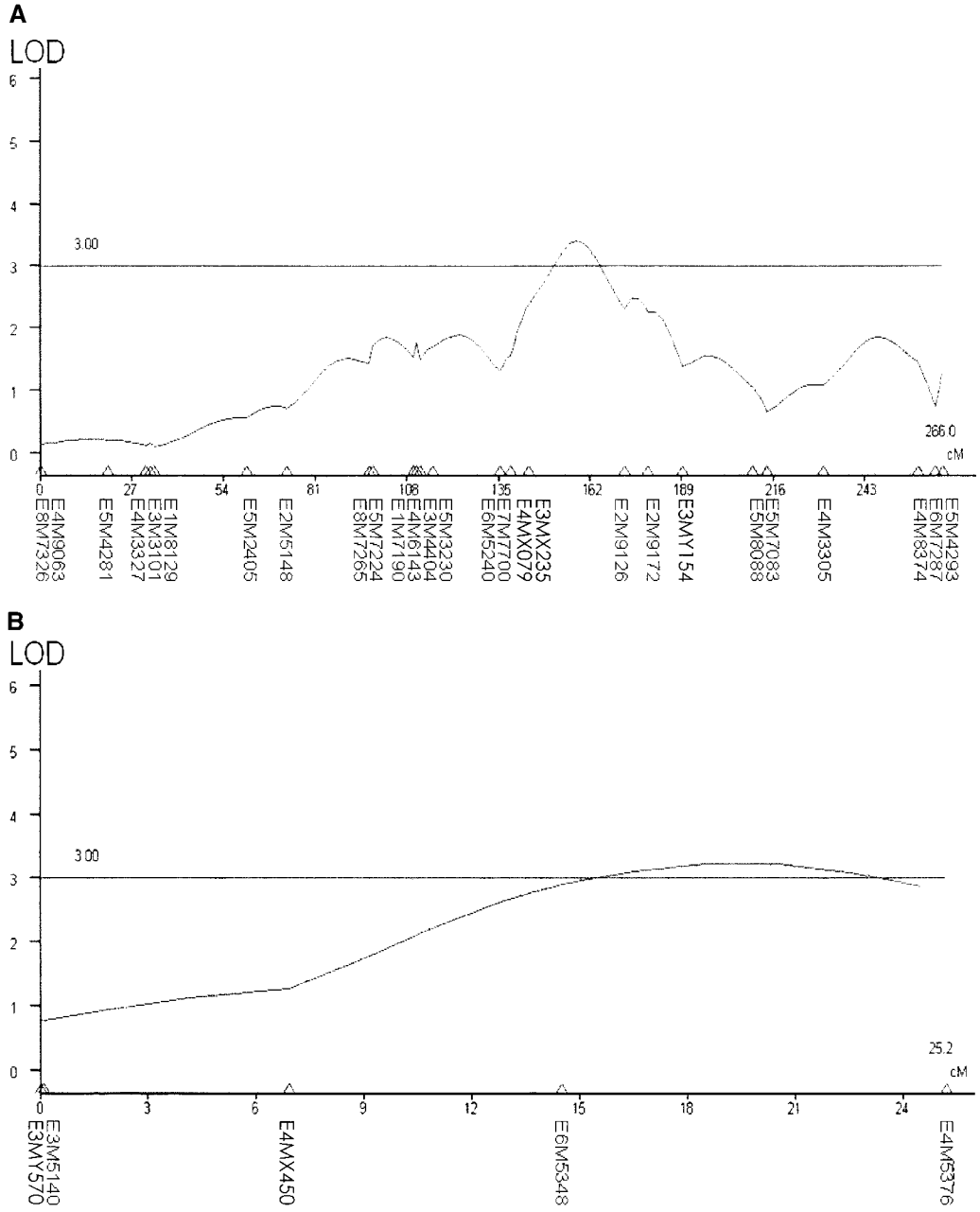

FIGURE 4.—Two regions in the low backcross showed significant LOD scores. These were called (A) AFF3 and (B) AFF4, and they have different genomic locations from the two QTL shown in Figure 3.

with the rise of endogenous circadian rhythmicity (TOMA *et al.* 2000). The interaction effect demonstrated in this study fits well with the view that *Amper* could facilitate the effect of other factors that lead to the initiation of foraging. For example, rhythmic bees that are more active during the day are more exposed to recruitment

TABLE 3

Effects (mean ± SD) of the ALFP markers most closely linked to the LOD peak of the four significant QTL identified in this study

QTL	Backcross population	Most closely linked marker	Individuals with allele from high line	Individuals with allele from low line
aff1	HBC	E1M4270	17.5 ± 11.8	28.8 ± 5.5
aff2	HBC	E6M5259	16.6 ± 10.4	20.8 ± 10.5
aff3	LBC	E2M9126	23.4 ± 10.3	16.1 ± 10.0
aff4	LBC	E6M8348	25.8 ± 9.2	17.5 ± 10.2

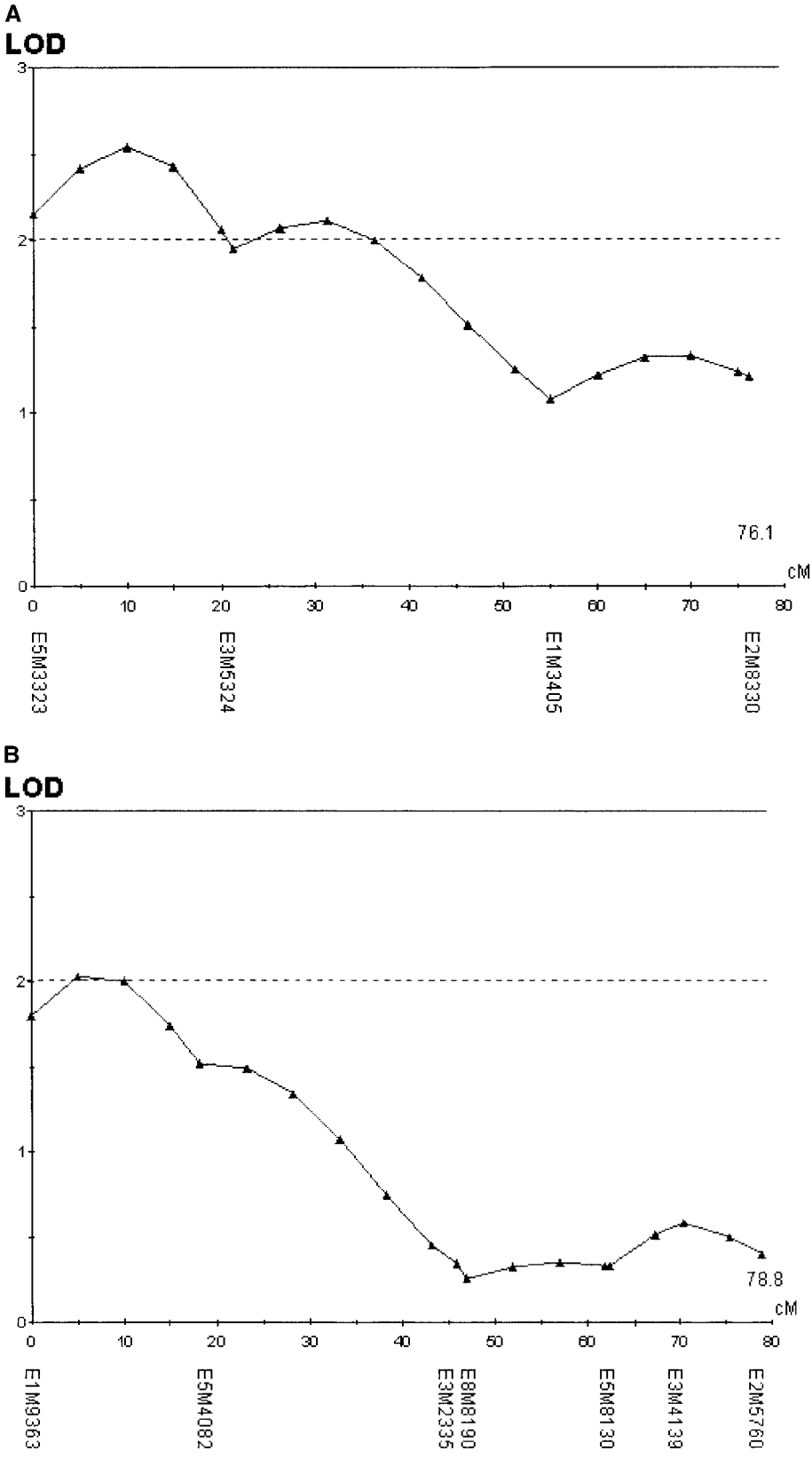

FIGURE 5.—One additional putative QTL was detected in each backcross. The LOD scores of these QTL did not reach the significance threshold but both have a notable effect on AFF, explaining 9.8 and 13.3% of the total variation in HBC and LBC, respectively.

from incoming foragers. Under this scenario, it seems plausible that *Amper* has a modulatory effect on the age of first foraging that can be rendered insignificant by extreme social conditions (BLOCH *et al.* 2001).

We regarded the QTL *pln1*, *pln2*, and *pln3* that were identified previously for their effect on pollen hoarding and foraging (HUNT *et al.* 1995; PAGE *et al.* 2000) as potential candidates to influence the rate of behavioral

maturation. Our rationale for this hypothesis was the phenotypic correlation of pollen hoarding and fast maturation (Pankiw and Page 2001; Pankiw 2003). One of these loci, *pln1*, showed a direct pleiotropic effect on the age of first foraging. The allele inherited from the "low" line caused individuals to forage, on average, 2.7 days earlier. The fact that the "fast maturation" allele is inherited from the "slow maturation" line may seem counterintuitive, but such results are fairly common in artificial selection programs (*e.g.*, Burke *et al.* 2002). Correspondingly, the *pln1* allele from the low line exerts an effect that is contrary to the phenotype of its strain of origin on the amount of stored pollen, pollen-foraging behavior (Hunt *et al.* 1995; Page *et al.* 2000), and sensitivity to sucrose (our unpublished data). This result suggests a genetic architecture with nonadditive gene interactions (Omholt *et al.* 2000; see below) and additional factors with opposite effects (*e.g.*, AFF1 and AFF2). A complex genetic architecture with nonlinear interactions is further supported by several (epistatic) interaction effects among our candidate loci found in the LBC. Epistasis may be the rule in natural genomic architectures of complex traits (Templeton 2000). However, its detection is severely limited in most genome-wide QTL studies (Frankel and Schork 1996). This is also true for our QTL maps in the HBC and LBC, in which we did not test for any epistasis between the newly generated markers. Thus, we want to suggest the possibility (high likelihood) of more undetected epistatic interactions (that involve some of our newly generated markers) as a cautionary note.

Our results demonstrate that the phenotypic correlations between foraging-choice behavior and the initiation of foraging (Pankiw and Page 2001; Pankiw 2003) are reflected in genetic correlations measured at individual loci (*pln1*). This can be due to tight linkage or pleiotropic effects of individual QTL, both of which have critical implications for adaptive evolution (Maynard Smith *et al.* 1985; Hawthorne and Via 2001).

The high recombination rate of the honeybee genome (Hunt and Page 1995; Beye *et al.* 1999) makes genetic linkage as a basis for genetic correlations less likely in honeybees than in many other organisms. Additionally, the genetic correlation between the rate of behavioral maturation and pollen-hoarding and foraging behavior has persisted through three outcrossing events in our current selection lines, and it also has been found in a previous, independent selection experiment (Hellmich *et al.* 1985; Calderone and Page 1991). The pleiotropic link between the maturation rate and foraging behavior is further corroborated by independent studies of Africanized and European honeybees (Pankiw 2003). Pleiotropy suggests a common proximate basis for pollen hoarding, foraging behavior (Hunt *et al.* 1995; Page *et al.* 2000), and the rate of behavioral maturation. Genes involved in the perception of brood pheromone (*e.g.*, Briand *et al.* 2002) thus are one class of candidate genes for our QTL because brood pheromone affects foraging initiation (Le Conte *et al.* 2001) and foraging choice (Pankiw *et al.* 1998) of honeybee workers. However, the extensive pleiotropy, together with the demonstrated epistatic interactions, may also be a signature of components of more central cellular networks (Omholt *et al.* 2000), such as the cAMP-dependent second messenger cascade (Humphries *et al.* 2003).

Our analysis of 783 linked markers indicated four significant QTL for the rate of behavioral maturation, two in each backcross. These four QTL all differ from each other and they also differ in the size of their effects. Comparison with results from the HBC and earlier mapping experiments (Hunt *et al.* 1995; Page *et al.* 2000) convinced us to not demote AFF3 and AFF4 from "significant" to "suggestive" QTL on the basis of the bootstrapped significance values. Although AFF3 and AFF4 have lower LOD scores than AFF1 and AFF2, their effect size (and percentage of variation explained) is actually larger. Furthermore, all previously reported honeybee-foraging QTL (even though they had lower LOD scores than any of the AFF-QTL) could be subsequently verified. This makes us also optimistic about the two additional QTL reported in Figure 5. Three of the markers with the strongest effects on AFF were unlinked to any linkage groups in our genetic maps and are therefore not reflected in our current QTL model. However, they may constitute additional QTL in unmapped regions of the genome.

We conclude that there are several sections of the honeybee genome with major influence on the age at onset of foraging. The systematic study of candidate genes in these regions will be greatly facilitated by the results of the honeybee genome project (http://www.hgsc.bcm.tmc.edu/projects/honeybee/). After collection of possible candidates genes, fine-scale mapping, genetic association studies (Hirschhorn *et al.* 2002), and functional analyses (*e.g.*, Amdam *et al.* 2003; Beye *et al.* 2003) promise further progress toward our mechanistic understanding of the rate of behavioral maturation in honeybees (Phillips 1999; Flint and Mott 2001).

As mentioned above, it is not uncommon in experimental crosses between selected lines to find allelic effects that are opposite to the expectation (see, for example, Hunt *et al.* 1995 for *pln1* and Page *et al.* 2000 for *pln3*). Our newly isolated QTL showed a clear pattern of allelic effects. In both QTL detected in the HBC, the allelic effect was "correct": The allele that promoted fast behavioral maturation was inherited from the high line that matures faster overall. The allelic effects were also consistent with their origin in all three unlinked markers of major effect on AFF. However, for both QTL detected in the LBC, the pattern was the reverse: The allele that promoted fast behavioral maturation was inherited from the "low" line. There are three potential explanations

for this phenomenon: epistasis, overdominance, or random fixation of alleles of contrary effect.

We have found evidence for epistasis in this study, as well as in other studies of the high and low pollen-hoarding lines (R. E. Page, T. Pankiw, M. Beye and D. I. Nielsen, unpublished results). Thus, interactions among loci seem to be the rule, rather than the exception, in the genetic differentiation between the selected honeybee lines studied here. Epistasis can mask additive genetic effects and allow alleles to be maintained in spite of selection against their additive effect (Brodie 2000; Wade 2002). Thus, epistasis is a potent explanation for our results that the additive effects of alleles of AFF3 and AFF4 seem to contradict their origin. However, on the basis of this observation, it is impossible to rule out the alternative explanation of overdominance (AFF3 and AFF4 heterozygote individuals may be older than either class of homozygotes when initiating foraging) because we had no control over genetic background independently of the three genotypes at each QTL. In the case of overdominance among alleles from the high and the low line, the artificial selection within the low line would not have operated against the alleles of high (that is, fast) effect. The third explanation, random fixation (within a selection line) of alleles whose effects are contrary to the overall direction of selection (facilitated by dominance), is also possible but less likely. Although the population size of our selection lines was not very large (Page and Fondrk 1995), the initial selection was strong (Page and Fondrk 1995) and both lines underwent three outcrossing events before this experiment.

The second striking overall result from our QTL mapping was the lack of QTL correspondence between the two maps. We did not find any significant effect in homologous regions of the QTL identified in the alternative backcross. Genomic regions that affected AFF in the HBC did not affect AFF in the LBC and vice versa. This finding could likewise be explained by epistatic interactions or within-locus (dominance) interactions but is incompatible with the notion of overdominance. The smaller sample size of the LBC *vs.* the HBC may also help to explain why HBC QTL were not found in the LBC (but not vice versa). In sum, epistasis seems to be the most likely explanation for the patterns of QTL effects in this study. To demonstrate epistasis and confirm individual QTL, further investigations of the AFF in the two selection lines are needed, involving selected, marker-assisted crosses.

We do not want to exclude the possibility that there are more genetic factors than reported here (Beavis 1994) that influence the rate of behavioral maturation. In fact, our results indicate a number of additional regions with possible effects, only two of which we have listed in this article. The restricted sample size, especially in the LBC, has probably led to the omission of QTL with less pronounced effects and inflated the effects of the reported QTL (Beavis 1994). Some QTL might also have been missed because our coverage of the honeybee genome is incomplete. Despite the >3700 cM covered in each map, our markers are not grouped in 16 linkage groups, as would be expected on the basis of cytological findings. This disparity indicates gaps in our genetic map. However, independent maps (Hunt and Page 1995; Page *et al.* 2000) indicate similar, slightly smaller, map sizes, which suggests that our gaps are small and represent only a minor portion of the honeybee genome.

In conclusion, our selected lines provide an important tool for understanding the rate of behavioral maturation that leads to differences in the age at foraging initiation, a central parameter in the division of labor in social insect colonies. Our experiment supports the view that the substantial genetic variability for this parameter is accessible for identification and characterization. There are several loci with major effects, and nonadditive complexity seems to play a major role. The identified QTL can be regarded as causally responsible for differences in the rate of behavioral maturation, rather than as a correlate of the initiation of foraging (Ohashi *et al.* 1999; Kucharski and Maleszka 2002; Robinson 2002). While we could exclude direct effects of two candidate genes, this study, together with the honeybee genome project and emerging molecular tools in the honeybee, provides an important basis for the exciting prospect of identifying unexpected or unknown genes that could play a central role in social organization.

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APPENDIX

AFLP primers used

Primer	Sequence
E1	AGACTGCGTACCAATTC–AA
E2	AGACTGCGTACCAATTC–AC
E3	AGACTGCGTACCAATTC–AG
E4	AGACTGCGTACCAATTC–CA
E5	AGACTGCGTACCAATTC–CC
E6	AGACTGCGTACCAATTC–CG
E7	AGACTGCGTACCAATTC–TA
E8	AGACTGCGTACCAATTC–GG
E9	AGACTGCGTACCAATTC–GC
EX	AGACTGCGTACCAATTC–TG
M1	GATGAGTCCTGAGTAAC–AA
M2	GATGAGTCCTGAGTAAC–AC
M3	GATGAGTCCTGAGTAAC–AG
M4	GATGAGTCCTGAGTAAC–AT
M5	GATGAGTCCTGAGTAAC–CA
M6	GATGAGTCCTGAGTAAC–CC
M7	GATGAGTCCTGAGTAAC–CG
M8	GATGAGTCCTGAGTAAC–CT
M9	GATGAGTCCTGAGTAAC–GA
MX	GATGAGTCCTGAGTAAC–GC
MY	GATGAGTCCTGAGTAAC–TA