Pleiotropy, Epistasis and New QTL: The Genetic Architecture of Honey Bee Foraging Behavior

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Abstract:

The regulation of division of labor in social insects, particularly in the honey bee (*Apis mellifera* L.), has received considerable attention from a number of biological subdisciplines, including quantitative and behavioral genetics, because of the high complexity of the behavioral traits involved. The foraging choices of honey bee workers can be accurately quantified, and previous studies have made the foraging behavior of honey bees one of the best studied naturally occurring behavioral phenotypes. Three quantitative trait loci (QTL) have been identified that influence a set of foraging variables, including the concentration of nectar collected and the amount of pollen and nectar brought back to the hive. This study extends previous genetic investigations and represents the most comprehensive investigation of the genetic architecture of these foraging variables. We examined the effects of markers for the three established QTL and for one further candidate gene (*Amfor*), in two reciprocal backcross populations. These populations were also used to carry out two new QTL mapping studies, with over 400 Amplified Fragment Length Polymorphism (AFLP®) markers in each. We detected a variety of effects of the genetic markers for the established QTL and the candidate gene, which were mostly epistatic in nature. A few new QTL could be detected with a variety of mapping techniques. Our results add complexity to the genetic architecture of the foraging behavior of the honey bee. Specifically, we support the hypotheses that *pln1, pln2, pln3*, and *Amfor* are involved in the regulation of foraging behavior in the honey bee and add some new factors that deserve further study in the future.

Article:

Studies of quantitative, complex traits have become a major research focus in biology because most traits of interest are quantitative in nature, including many human diseases (Flint and Mott 2001). The study of quantitative trait loci (QTL) has been promoted by theoretical (Doerge et al. 1997; Liu 1998; Phillips and Belknap 2002) and experimental (Kristensen et al. 2001) advances. In conjunction with the sequence data generated by genome projects, QTL analyses have a high potential to reveal the genetic architecture of complex traits (Barton and Keightley 2002; Mackay 2001) and propose candidate genes for further study (Flint and Mott 2001; Phillips 1999).

Before the advent of modern quantitative genetics, behavioral geneticists faced the choice between in-depth studies of mutants generated in the laboratory (Fischbach and Heisenberg 1984) and crude estimates of the heritability of naturally occurring behavior (e.g., Milne 1985). However, the synergistic combination of these two approaches, detailed studies of naturally occurring behavior, is needed to explain the ecology and evolution of behavioral genes (Boake et al. 2002). Behavioral traits provide classic examples of complex traits (Plomin et al. 1997) because the brain, as central mediator of behavior in most organisms, is extraordinarily complex, integrating many developmental, physiological, and biochemical systems (Fischbach and Heisenberg 1984; Greenspan 2001). Today, several research programs have been established that seek to reveal the detailed genetic architecture of naturally occurring behavioral variants and characterize the responsible genes (e.g., Hawthorne and Via 2001). One of the earliest cases of QTL detection for a naturally occurring behavior was a study on honey bee food storage and foraging behavior (Hunt et al. 1995).

There are many aspects of honey bee foraging behavior, but the primary characteristic of a honey bee's foraging is represented in her choice of resource. Bees carry pollen, nectar, or both, as well as propolis (plant resin) or water, back to the hive (Winston 1987). Scouts and bees that do not find acceptable resources return empty. The main foraging effort is directed toward food resources, so the fundamental choice is between nectar and pollen. It has been repeatedly demonstrated that genotype (e.g. Calderone and Page 1988), colony resources (e.g., Fewell and Winston 1992), and brood stimuli (e.g., Pankiw et al. 1998) affect foraging choice, but resource conditions encountered by the foragers also play a major role (Waddington and Kirchner 1992; Waddington et al. 1998).

The first study of the genetic architecture of honey bee foraging (Hunt et al. 1995) revealed two major QTL (*pln1* and *pln2*) that explained 59% of the variation in quantities of pollen stored by honey bee colonies (hoarding) of two selected strains (Page and Fondrk 1995). The mapping population constituted a backcross to the high-pollen-hoarding line after the second generation of bi-directional selection. In an independent verification study after five generations of selection, both QTL demonstrated effects on individual foraging choices (nectar versus pollen foraging) when returning workers were collected in a narrow time window (3 days). Additionally, the genotype for the *pln2* marker explained a significant proportion of the variation in the sugar concentration of nectar collected (Hunt et al. 1995).

As an extension of the first study, Page et al. (2000) performed two additional QTL mapping experiments, a backcross between the pollen-hoarding selection lines (Page and Fondrk 1995) after the fifth generation and a cross between a queen of European descent and a male (drone) from an Africanized source (Winston 1992). A new QTL (*pln3*) was mapped in the backcross population and independently confirmed. Additionally, *pln2* demonstrated a significant effect on individual pollen-load size (workers collected over 1–3 days) in the backcross experiment and on colony-level pollen hoarding in the European-African cross (Page et al. 2000). Thus, at least three major genetic factors governing foraging choices seemed to vary in honey bee populations. The effects of these QTL on foraging behavior have been somewhat variable, but in general they have been persistent across experiments (Page et al. 2000), in spite of large environmental variation of resources available (Steffan-Dewenter and Kuhn 2003) and colony-level regulation mechanisms on foraging behavior (Dreller et al. 1999; Fewell and Winston 1992).

We studied the foraging choices made by honey bees over time in a comprehensive QTL analysis to gain a more complete understanding of the underlying genetic architecture, specifically including possible interactions between genotypic and environmental control (Beekman et al. 2003). After 18 generations, we generated reciprocal backcrosses (backcross to the "high" line: HBC; backcross to the "low" line: LBC) from the above-mentioned, high- and low-pollen-hoarding strains (Page and Fondrk 1995). The foraging choices of bees from both backcrosses were studied as they initiated foraging in a common hive environment over a 30-day time interval. We tested for effects of the three previously identified QTL and one candidate gene. This candidate gene, *Amfor* (GenBank accession no. AF469010), represents the honey bee orthologue of the *for* gene in *Drosophila melanogaster*, and in both systems the encoded cGMP-dependent protein kinase (PKG) is associated with foraging behavior (Ben-Shahar et al. 2002; Pereira and Sokolowski 1993). Specifically, it suggested itself as candidate because of its widespread effects on sensory sensitivity (Ben-Shahar et al. 2003; Renger et al. 1999; Shaver et al. 1998).

Additionally, we performed QTL analyses of different foraging variables in both backcrosses in order to search for further genetic factors influencing honey bee foraging behavior. To test for interaction between the time of foraging (which may reflect changing resource conditions) and foraging choices, we performed these analyses with and without correction for time trends, where appropriate.

METHODS AND MATERIALS
Crosses
Two strains of honey bees were selected for high and low pollen-hoarding behavior from commercial stocks (Page and Fondrk 1995). Through 18 generations, strains were maintained through cyclic inbreeding among five lines and three outcrossings to unrelated, commercial stocks of similar phenotype. A queen was raised from the "C" low-pollen-hoarding line and artificially inseminated with semen of a single male (Laidlaw and Page 1997) from the "Q1" high-pollen-hoarding line. Queens were raised from the offspring of this F_1-hybrid queen and backcrossed to males of the respective high line and low line (Laidlaw and Page 1997). One high backcross and one low backcross queen were selected as sources of the workers for the two mapping populations HBC and LBC, respectively. The foraging behavior of these workers was monitored as they initiated foraging. Additionally, we studied the foraging behavior of worker offspring of one intraline cross in the high ("Highs") and the low pollen hoarding line ("Lows").

Experiment
All four queens were simultaneously confined on empty comb space in their respective colonies to induce simultaneous, maximal egg laying. Directly before emergence (May 22, 2000), the brood combs were placed in individual cages and transferred to a temperature-controlled (34°C) and moisture-controlled (50% relative humidity) incubator. Newly emerged workers were collected regularly, marked with a source-specific enamel color and introduced in a common unrelated hive.

We observed the entrance of this hive for at least 2 h per day and collected all marked bees that returned from foraging trips. Generally, 2 h provide ample opportunity to collect most bees that

have initiated foraging on any given day (Pankiw and Page 2001). Collection date, pollen-load size, nectar-load size, and nectar concentration (percentage sucrose) were recorded for each bee, following established protocols (Page et al. 2000). After the information on foraging loads was extracted, bees were frozen at –80°C for subsequent DNA extraction.

Genetic Analysis

DNA was extracted from individual bees using CTAB-lysis and a single phenol-chloroform extraction (Hunt and Page 1995). Final DNA concentration was adjusted to 100ng/ml, and specific primers were used to amplify sequence-tagged sites (sts markers) that were closely linked to the previously identified QTL (*pln1*, *pln2*, and *pln3*) and to the candidate gene *Amfor*. We used sts-D8-.33f (Hunt et al. 1995) for *pln1*, sts-tyr (Rüppell et al., 2004) for *pln2*, sts-Q4-ecap (Page RE et al., in preparation), RAPD Y15 (Hunt et al. 1995) for *pln3*, and sts-pkg-ecap (Rüppell O et al., submitted) for *Amfor*.

For QTL mapping, we generated AFLP® markers for 182 individuals from the HBC population and 94 individuals from the LBC population, using the AFLP® Core Reagent Kit (Invitrogen Life Technologies). We proceeded according to the manufacturer's recommendations, except for reducing the reaction volumes to 50% and proceeding with custom-made primers in the preamplification and selective amplification. In each of the secondary polymerase chain reactions (PCRs; selective amplifications), one of the primers was labeled with γ-^{33}P-ATP (ICN Radiochemicals), and the reaction products were separated on a 45-cm standard, denaturing sequencing gel (for 2.5 h at 1600 V). Gels were dried on filter paper and exposed to Biomax MR film (Kodak) for 24–96 h. Preliminary studies showed that this procedure resulted in more reliable polymorphic bands than separation on an ABI 377 sequencer. We ran the worker samples of each reaction together with the 10-base-pair Sequamark^{TM} size standard (Research Genetics) and the grand-paternal samples to determine the linkage phase. All polymorphic bands were scored twice for each individual to minimize scoring error, and each marker (polymorphic band) was assigned a unique name based on the primer combination and its size (Rüppell et al., 2004).

Statistical Analysis

Four foraging variables were analyzed (pollen-load size, nectar-load size, pollen proportion of load, and nectar concentration). Pollen weight and nectar concentration demonstrated a significant time trend (Figure 1). For these two we evaluated raw values (uncorrected) and residuals from a regression of the raw values on time (time corrected).
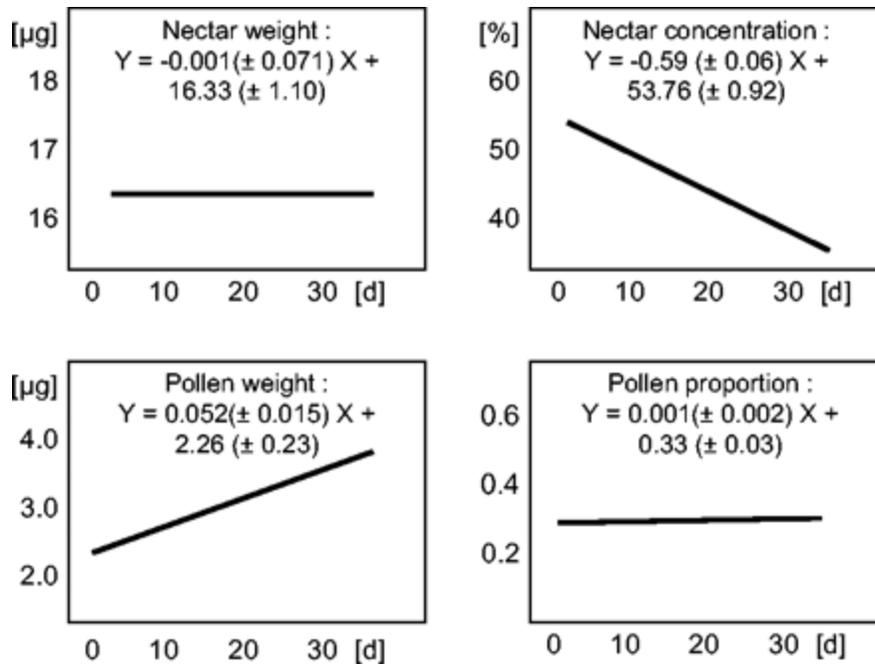
Figure 1. Of the four foraging parameters (on the *y*-axis) measured, nectar concentration and pollen weight collected by bees demonstrated a significant trend over time (on the *x*-axis) during the experimental period; only regression lines with the corresponding equation (and SE of parameters) are shown.

All variables differed significantly from normality (pollen-load size: Kolmogorov-Smirnov $Z = 10.824$, $P < .001$; nectarload size: Kolmogorov-Smirnov $Z = 8.543$, $P < .001$; pollen proportion: Kolmogorov-Smirnov $Z = 8.381$, $P < .001$; nectar concentration: Kolmogorov-Smirnov $Z = 1.842$, $P = .002$), and no standard transformation improved their fit to normality. However, the large sample sizes allowed us to evaluate the effect of the candidate genomic regions with ANOVAs, for this method is generally robust to such deviations because of the central limit theorem (Kallenberg 1997). Thus, we assessed differences between the four experimental groups with simple one-way ANOVAs including Tukey's HSD post hoc tests. We employed two-way ANOVAs (fixed effects, type III) to evaluate the effect of sts markers in both backcross populations, and within each backcross we tested for epistatic interactions with multiway ANOVAs (fixed effects, type III). Throughout this study, we employed standard probability thresholds even though we performed multiple tests. The justification for this is that we tested multiple hypotheses (each candidate locus constituted an independent hypothesis) instead of performing multiple tests on a single hypothesis.

All AFLP® markers were initially screened for an association with any of the foraging parameters, with simple one-way ANOVAs. This initial screen allowed us to order markers according to the strength of their association with a particular foraging variable. Additionally, we combined the AFLP® markers with the sts markers and constructed two linkage maps (HBC and LBC maps) for subsequent QTL analyses. We used Mapmaker 3.0b (Lander and Botstein 1989), employing Kosambi's 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).

We ordered the HBC and LBC marker sets according to the results of the linkage mapping and analyzed both data sets with the programs MapQTL 4.0 (Van Ooijen et al. 2002) and QTL Cartographer (Basten et al. 1994, 2002). When interval mapping was initially used, both programs resulted in almost identical likelihood profiles, and we show the results of only QTL Cartographer here. The genome-wide significance thresholds of LOD 3.0 were adapted from Hunt et al. (1995) according to Lander and Botstein (1989). For comparison, we also determined significance thresholds for interval mapping empirically by bootstrapping (Churchill and Doerge 1994). These values clustered around the theoretical LOD threshold of 3.0: nectar-load size 3.3, nectar concentration 3.1, nectar concentration (time corrected) 3.0, pollen-load size (time corrected) 3.0, pollen-load size 2.9, and pollen proportion 2.9.

As the foraging variables investigated in our study are related, we used multiple-trait mapping (Jiang and Zeng 1995) to evaluate the following two pairs of variables jointly: (1) nectar load size/nectar concentration, and (2) nectar load size/pollen load size. Furthermore, we compared the genomic mapping results from MapQTL's MQM algorithm (Jansen 1993) and QTL Cartographer's CIM function (Zeng 1993, 1994). For cofactor selection in QTL Cartographer, we used forward and backward regression (model 6 with default parameters: Basten et al. 2002). In MapQTL we used a manual forward regression strategy: we determined iteratively a stable set of cofactors with LOD scores above 2.0, starting with markers that had LOD scores above 2.0 in interval mapping.

RESULTS

Group Differences

The three variables nectar-load size, pollen-load size and nectar concentration (and consequently the compound variable, pollen proportion) varied among groups in accordance with each other (Figure 2), and the foraging variables were significantly correlated with each other (Table 1).
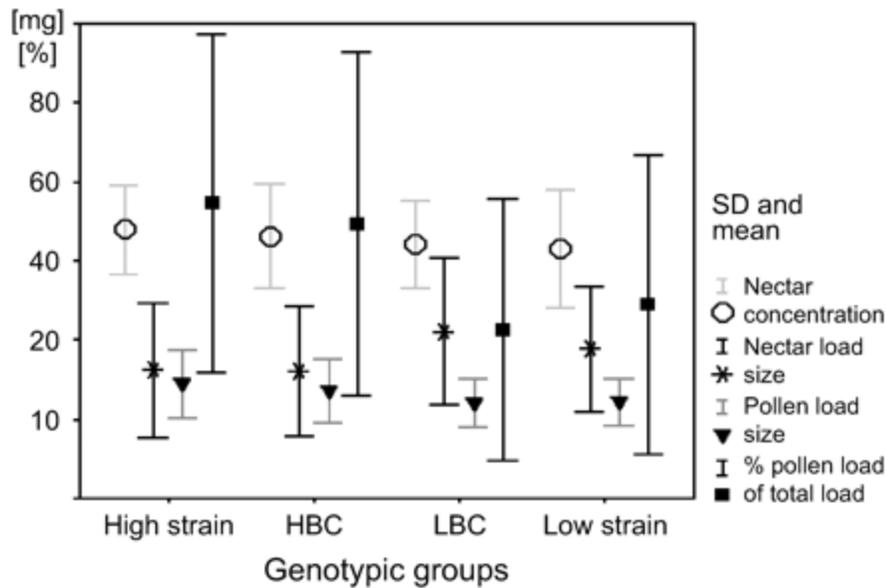
Figure 2. Differences among experimental groups were significant for all foraging variables considered, except the concentration of nectar collected. The units are (mg) for nectar and pollen-load size, and (%) for nectar concentration and pollen proportion.

Table 1. Correlations (Pearson's *r* and its significance, assuming normality) of foraging variables

	Pollen weight	Nectar concentration	Pollen proportion
Nectar weight	$r = -.353$	$r = .173$	$r = -.745$
	$n = 1,742$	$n = 1,100$	$n = 1,482$
	$P < .001$	$P < .001$	$P < .001$
Nectar concentration	$r = -.050$		$r = -.069$
	$n = 1,100$	—	$n = 1,100$
	$P = .095$		$P = .023$
Pollen proportion	$r = .807$	$r = -.069$	
	$n = 1,482$	$n = 1,100$	—
	$P < .001$	$P = .023$	

Nectar-load size exhibited a significant overall differentiation [$F_{(3,1741)} = 41.61$, $P < .001$], and all groups except HBC and High were significantly different from each other [High: 12.33 mg ± 17.03 (mean ± SD); HBC: 12.10 mg ± 16.49; LBC: 22.15 mg ± 18.57; Low: 17.77 mg ± 15.90]. The concentration of nectar that bees returned to the hive was also significantly different among

groups [$F_{(3,1099)} = 5.01$, $P = .002$]. High bees (47.88% ± 11.16) collected significantly higher concentrations than LBC (44.22% ± 11.03) and Low bees (42.99% ± 14.79). The HBC group was intermediate (46.18% ± 13.23) and significantly different only from the Low group. Corrected for time, however, this parameter did not show any significant group differences [$F_{(3,1099)} = 0.72$, $P = .538$].

The quantity of pollen also differed significantly among groups [$F_{(3,1744)} = 34.96$, $P < .001$], and post hoc tests showed a significant difference between each pair of groups, except LBC and Low (High: 8.87 mg ± 8.62; HBC: 7.15 mg ± 7.88; LBC: 4.11 mg ± 6.01; Low: 4.29 mg ± 5.92). In this case, time correction increased the group differences [$F_{(3,1744)} = 45.84$, $P < .001$]. The proportion of food collected that was pollen demonstrated the strongest overall differences among groups [$F_{(3,1483)} = 57.05$, $P < .001$], but the High (54.57% ± 42.67) and HBC group (49.33% ± 43.23) were not significantly different from each other (LBC: 22.58% ± 33.18; Low: 29.06 ± 37.86).
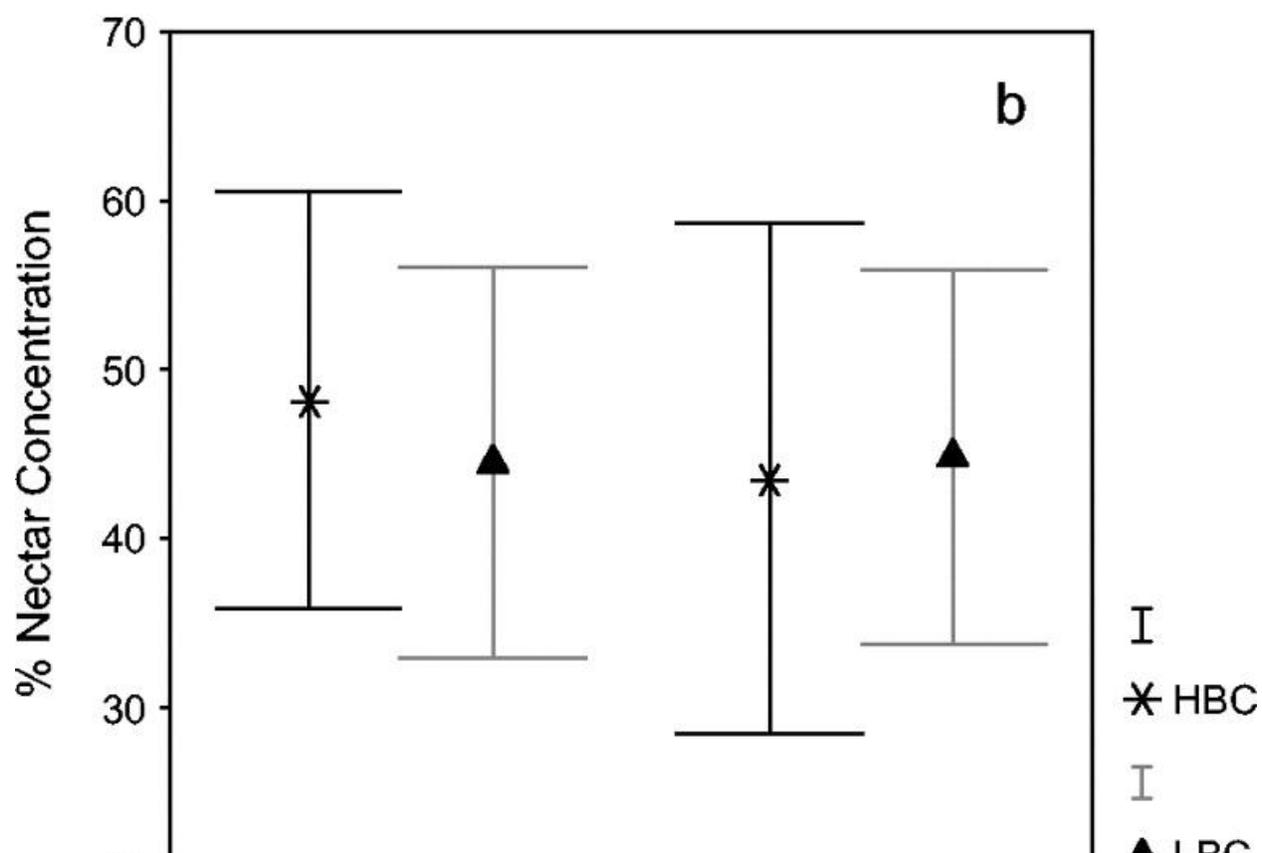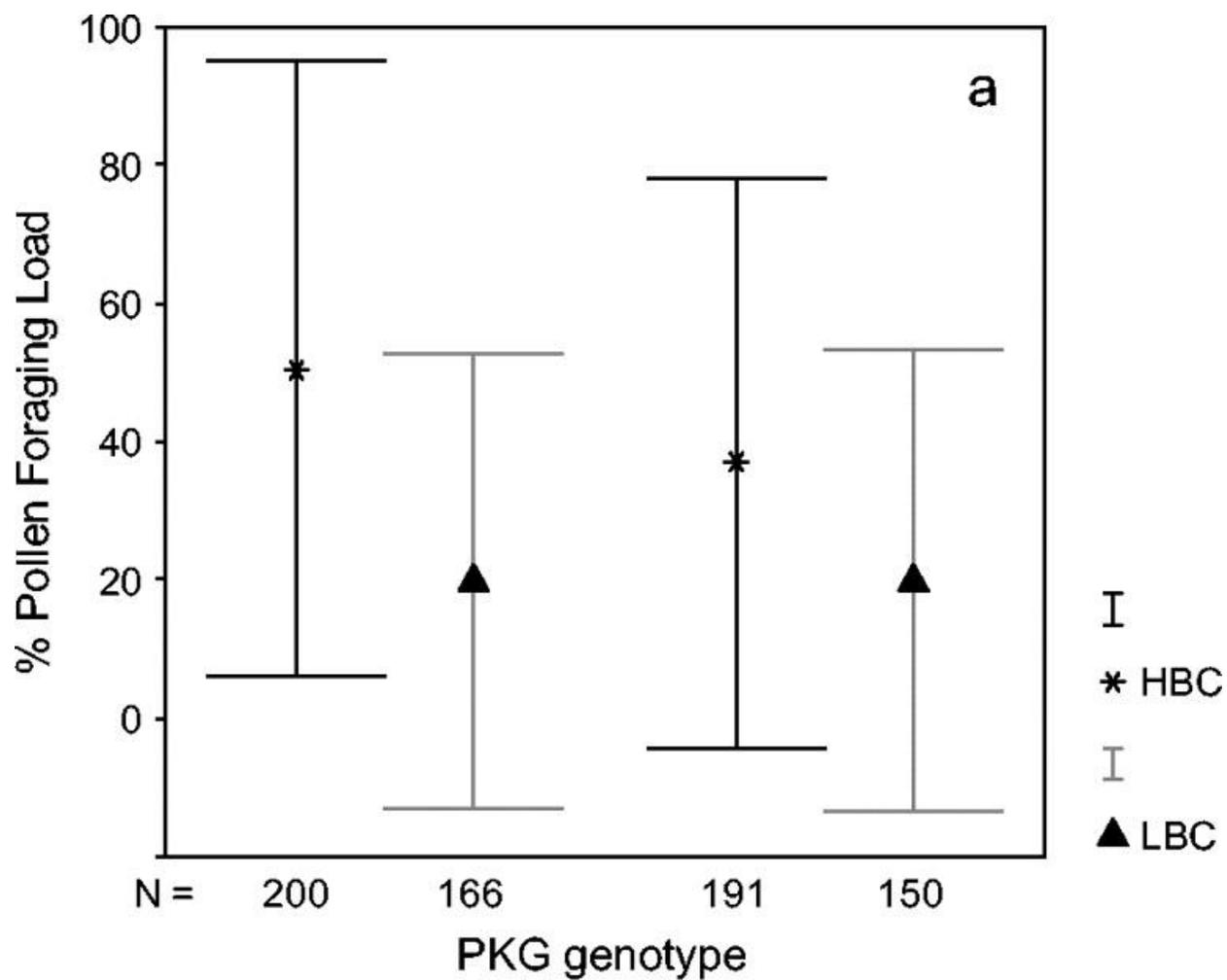
Candidate Loci

Although the four investigated foraging variables correlated with each other (Table 1), the markers of the four investigated candidate loci (*pln1, pln2, pln3*, and *Amfor*) showed different effects. While the markers for *pln2* and *Amfor* influenced all variables, the exact nature of their effect varied, and the *pln1* and *pln3* markers were found to be involved in only a few interaction effects. Time correction did not alter any results significantly; consequently, only the results on uncorrected data are reported (Table 2).

Table 2.. Summary of significant *sts*-marker effects on the four different foraging variables investigated

Genetic marker(s)	Source	Significant effects
Pln2	HBC	Nc
Amfor	HBC	Pp
pln1 x *pln3*	LBC	Pp
pln2 x *Amfor*	HBC	Nl, Pl, Pp
pln3 x *Amfor*	HBC	Nc
pln1 x *pln2* x *pln3*	HBC	Pl
pln2 x *pln3* x *Amfor*	HBC	Pl
pln1 x *pln2* x *pln3* x *Amfor*	LBC	Pp, Nc

Nc = nectar concentration; Nl = nectar-load size; Pl = pollen-load size; Pp = pollen proportion.

In all two-way ANOVAs (evaluating simultaneously the effects of genotype and single-candidate loci), genotype had a highly significant effect ($F > 32$, $P < .001$) on all foraging variables, except for the concentration of nectar collected ($F < 1.2$, $P > .2$). For pollen-load size and nectar-load size, no single marker effects were detected. The *Amfor* marker significantly affected the proportion of pollen collected [$F_{(1,703)} = 5.37$, $P = .021$]. However, there was a significant interaction between overall genotype and *Amfor*-marker genotype [$F_{(1,703)} = 5.37$, $P = .021$], which occurred because the *Amfor* marker influenced the proportion of pollen collected in the HBC [$F_{(1,389)} = 9.85$, $P = .002$], and not in the LBC [$F_{(1,314)} = 0.00$, $P = .999$; Figure 3a].

Figure 3.. Plots of the interaction effects between genotype and marker alleles near candidate loci (*Amfor* and *pln2*) on the proportion of pollen collected (**a**) and the sugar concentration of the nectar (**b**), respectively. In both cases the marker has a significant effect in the high backcross but shows no effect in the low backcross.

Similar results were obtained for the *pln2* marker with respect to the concentration of nectar collected: *Pln2*-marker genotype exhibited a significant overall effect [$F_{(1,553)} = 4.02$, $P = .045$] and a significant interaction between overall genotype and *pln2*-marker genotype [$F_{(1,553)} = 5.39$, $P = .021$]. This interaction was based on a *pln2* effect in the HBC [$F_{(1,255)} = 7.25$, $P = .008$) but not in the LBC [$F_{(1,298)} = 0.07$, $P = .799$; Figure 3b].

The multiway ANOVAs, evaluating the effects of all four candidate markers simultaneously within the HBC and the LBC, revealed some further effects. Nectar-load size was found to be significantly affected in the HBC by an interaction of *pln2* x *Amfor* markers [$F_{(1,269)} = 8.01$, $P = .005$]. The interaction between these two markers was also found to affect the pollen-load size in the HBC [$F_{(1,269)} = 4.77$, $P = .030$], but two three-way interactions were also identified [*pln1* x *pln2* x *pln3*: $F_{(1,269)} = 4.13$, $P = .043$; *pln2* x *pln3* x *Amfor*: $F_{(1,269)} = 6.09$, $P = .014$]. Nectar- and pollen-load sizes were not affected by any marker genotype in the LBC.

Pollen proportion was affected in the HBC by the interaction of *pln2* x *Amfor* markers [$F_{(1,269)} = 7.62$, $P = .006$]. In the LBC pollen proportion showed a significant interaction of *pln1* x *pln3* markers [$F_{(1,228)} = 4.24$, $P = .041$] and among all four candidate markers [*pln1* x *pln2* x *pln3* x *Amfor*: $F_{(1,228)} = 4.92$, $P = .028$]. The data on nectar concentration in the LBC showed only this highest order interaction [*pln1* x *pln2* x *pln3* x *Amfor*: $F_{(1,197)} = 7.23$, $P = .008$]. In the HBC, the interaction *pln3* x *Amfor* [$F_{(1,187)} = 4.90$, $P = .028$] influenced the concentration of nectar collected by foragers.

QTL Analyses

The QTL mapping was based on the two genomic maps (HBC and LBC). The HBC map covered roughly 3900 cM of the honey bee genome with 387 ALFP markers. The LBC map was slightly smaller overall (3700 cM) but contained more (396) markers. When the HBC and the LBC maps were compared according to colinearity of related markers, 79% and 75% of the maps were identified as homologous, respectively (Rüppell et al., 2004).

The three AFLP markers that demonstrated the strongest effects on any foraging variable in the initial ANOVA screen on the HBC and LBC data are listed in Table 3. However, only one of the tabulated effects is significant after simple Bonferroni correction for the number of markers tested (409 in the HBC, 459 in the LBC): *E8M6355* has a significant effect on the concentration of nectar collected [$F_{(1,79)} = 19.7$, $P_{corrected} = .013$]. Correcting the foraging data for linear time trends changed the most notable markers in the LBC for nectar concentration and pollen-load size, in the HBC for nectar concentration only. Overall, four markers with strong single effects (Table 3, designated with a superscript *a*) were not included in any linkage group and thus are not reflected in the subsequent QTL mapping.

Table 3.. The three AFLP markers with the strongest effect for each foraging variable and backcross (BC)

	Nectar concentration	Time-corrected nectar concentration	Nectar-load size	Pollen-load size	Time-corrected pollen-load size	Pollen proportion
HBC	E5M3445[a]	E6M5210	E7M1495	E7M1495	E7M1495	E7M1495
	$F_{(1,127)} = 10.45$	$F_{(1,120)} = 11.73$	$F_{(1,175)} = 6.32$	$F_{(1,175)} = 13.89$	$F_{(1,175)} = 13.89$	$F_{(1,175)} = 9.07$
	$P = .0016$	$P = .00084$	$P = .013$	$P = .00026$	$P = .00026$	$P = .0030$
	E6M5210	E6M4173	E6M6427	E2M2163	E2M2163	E5M6249
	$F_{(1,120)} = 9.66$	$F_{(1,128)} = 8.55$	$F_{(1,171)} = 5.45$	$F_{(1,169)} = 10.79$	$F_{(1,169)} = 10.79$	$F_{(1,79)} = 8.63$
	$P = .0023$	$P = .0041$	$P = .021$	$P = .0012$	$P = .0012$	$P = .0043$
	E4M5406	E4M1530	E2M8138	E4M8387	E4M8387	E4M4134
	$F_{(1,104)} = 8.07$	$F_{(1,106)} = 7.68$	$F_{(1,111)} = 5.22$	$F_{(1,163)} = 10.72$	$F_{(1,163)} = 10.72$	$F_{(1,167)} = 7.92$
	$P = .0054$	$P = .0066$	$P = .024$	$P = .0013$	$P = .0013$	$P = .0055$
LBC	E8M6355[a]	E8M6066	E2M2128	E6M6307	E4M4410[a]	E5M2530
	$F_{(1,92)} = 19.71$	$F_{(1,86)} = 14.03$	$F_{(1,84)} = 8.73$	$F_{(1,92)} = 6.78$	$F_{(1,78)} = 7.03$	$F_{(1,78)} = 7.58$
	$P = .000029$	$P = .00033$	$P = .0041$	$P = .011$	$P = .0097$	$P = .0074$
	E1M5118	E1M5118	E3M1098	E5M6364[a]	E6M6307	E3M8166
	$F_{(1,90)} = 13.03$	$F_{(1,90)} = 11.72$	$F_{(1,86)} = 7.64$	$F_{(1,90)} = 6.41$	$F_{(1,91)} = 6.74$	$F_{(1,82)} = 5.62$
	$P = .00052$	$P = .00093$	$P = .0070$	$P = .013$	$P = .011$	$P = .020$
	E2M2128	E6M6426	E2M7320	E5M8086	E5M8086	E2M9415
	$F_{(1,77)} = 10.95$	$F_{(1,91)} = 9.21$	$F_{(1,91)} = 7.25$	$F_{(1,90)} = 6.23$	$F_{(1,89)} = 6.07$	$F_{(1,83)} = 5.14$
	$P = .0014$	$P = .0031$	$P = .0084$	$P = .014$	$P = .016$	$P = .026$

P values are given before statistical correction for multiple comparisons.
[a] Markers that are not part of any linkage map and thus are not reflected in the QTL mapping.

Simple interval mapping revealed no significant QTL in the HBC and only one for the concentration of nectar collected (raw data: LOD 4.1; time corrected: LOD 3.0) in the LBC (Figure 4). Another suggestive QTL for the concentration of nectar collected was found in the LBC on a different linkage group (marker *E1M5118*) with a LOD score of 2.4 (raw data) or 2.6 (time corrected). A suggestive QTL for nectar concentration was also found in the HBC (marker *E6M5210*) with a LOD score of 2.0 (raw data) or 2.5 (time corrected). In the LBC no further suggestive QTL were identified, but in the HBC interval mapping suggested at least three more QTL for pollen-load size (*E5M6151*: LOD 2.9; *E4M8387*: LOD 2.2; and *E3M3297*: LOD 2.1). Furthermore, three QTL were suggested for the proportion of pollen collected. One of them (*E3M3297*: LOD 2.4) was already identified as influencing the pollen-load size, but the other two were not (*E2M8540*: LOD 2.2; and *E5M8093*: LOD 2.0).
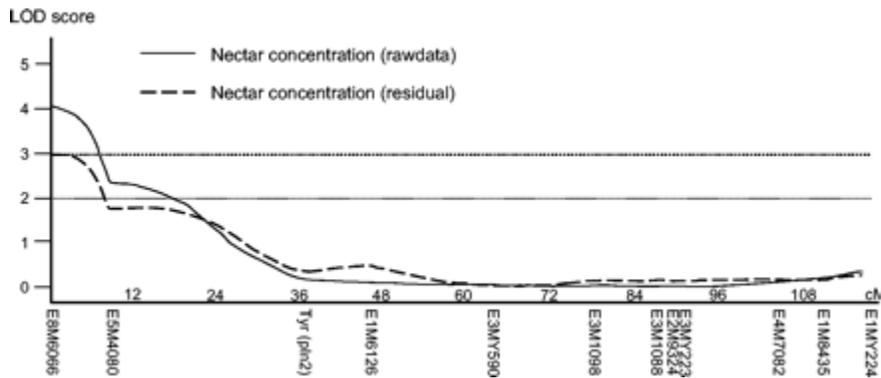

Figure 4.. The LOD score peak of the new QTL for the concentration of nectar brought home by workers that was found 36 cM away from the marker for QTL *pln2* in the LBC.

Multiple-trait mapping of the nectar concentration and the amount of nectar collected revealed one significant QTL (LOD 3.1: Figure 5a) for the LBC joint trait that was not reflected in any of the single foraging variables. This genomic region showed an even stronger effect in the joint analysis of nectar-load size and pollen-load size (LOD 3.9: Figure 5b). The joint analysis of all three variables in this genomic region resulted in an increase in LOD score to 4.2. No other new significant QTL were detected by multiple-trait mapping in the HBC or LBC.
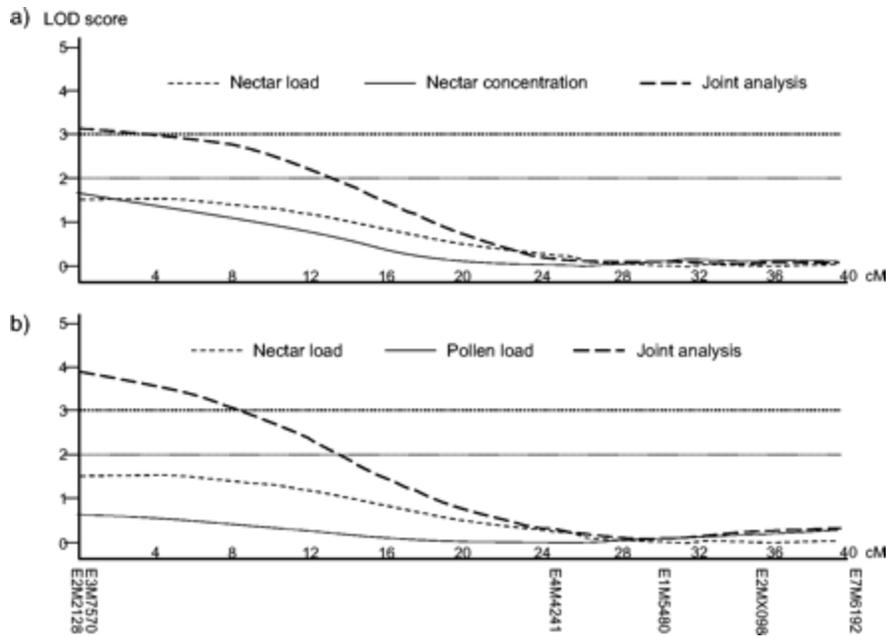
Figure 5.. Joint analysis of two individual foraging variables revealed a genomic region, in the LBC mapping population, that influences foraging behavior significantly. Both joint analyses, nectar load size/nectar concentration, and nectar load size/pollen load size, indicated a significant LOD score in the same region.

Two further QTL were detected in the LBC with QTL Cartographer's CIM function. A LOD score of 4.5 was recorded for the concentration of nectar collected (time-corrected LOD = 2.1) in a previously disregarded genomic region (Figure 6a). On yet another linkage group, a QTL for nectar-load size with a LOD score of 3.1 was revealed (Figure 6b). Our MQM mapping strategy resulted in a set of stable cofactors for all variables. However, only in the HBC for pollen-load size (raw data) and for nectar concentration (time corrected) did this set involve a new QTL region with a LOD-score of 3.0 or larger (Figure 7).
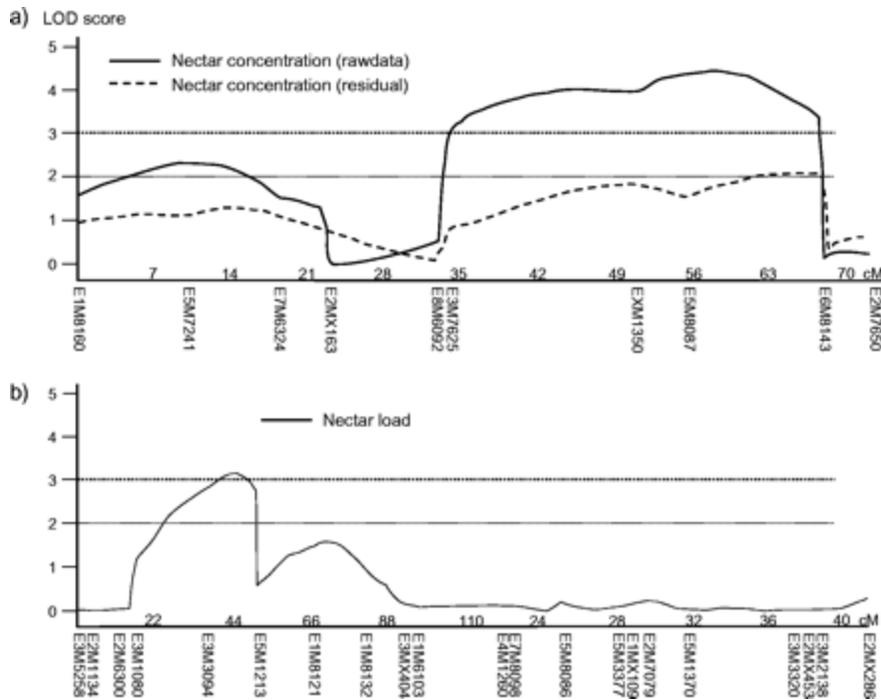
Figure 6.. Two further genomic regions identified by the QTL Cartographers CIM mapping function exceeded LOD 3.0 in the LBC for nectar concentration collected **(a)** and nectar-load size **(b)**.
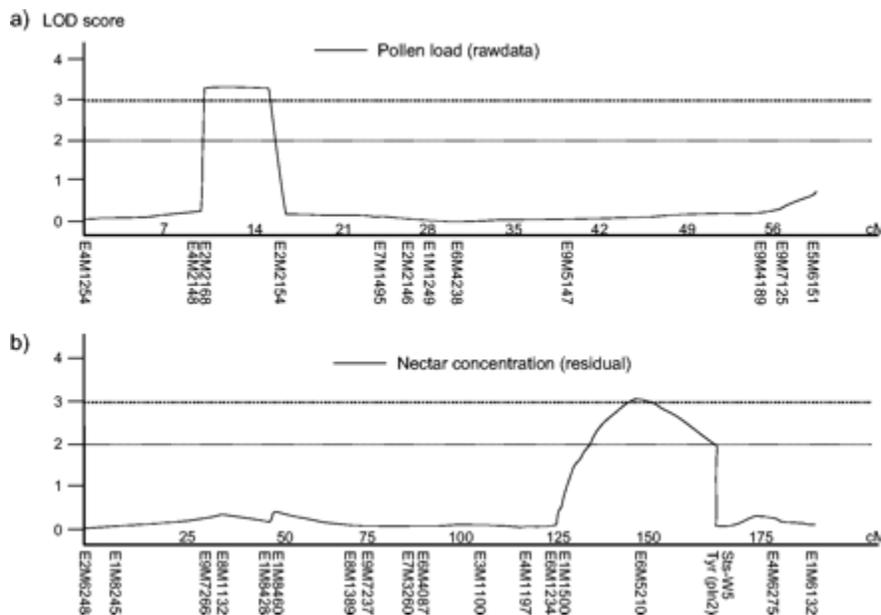
Figure 7.. MQM analysis using MapQTL resulted in two additional QTL with significant LOD scores in the HBC—**(a)** for pollen-load size, and **(b)** for the concentration of nectar collected— under the assumption that significance thresholds for interval mapping also hold for the corresponding MQM mapping.

DISCUSSION

This study draws a complex picture of the genetic architecture of honey bee foraging behavior. Our results suggest that the genetic differentiation among our experimental groups can be translated into direct and interaction effects of previously identified QTL, as well as a number of new QTL. Genetic complexity is expected for this type of behavioral trait (Greenspan 2001), which combines decision making with input from several sensory modalities, physiological individual- and colony-level state variables, and environmental fluctuations.

We found significant genetic differentiation among our experimental groups of all investigated traits. The amount of differentiation was variable, but it did not predict our success in identifying individual genetic factors as influential, because the complexity of their genetic architecture (or their dimensionality—the number of loci and their interactions) differed, presumably. For example, the overall differentiation among groups was weakest for the sucrose concentration of nectar collected, yet we were able to directly confirm a genetic effect in the genomic region of *pln2* (as well as several interaction effects of candidate markers), and this trait was the only trait with a significant QTL detected by simple interval mapping.

The investigated foraging variables were correlated with each other, which might be because nectar and pollen resources both depend on flower availability (Shuel 1992). In addition, the total load size of individual workers is constrained and leads, together with foraging specialization (Robinson 1992), to a negative correlation between nectar- and pollen-load size (Page et al. 2000), which was reconfirmed in this study. Additionally, across individuals (particularly within genotypic groups) nectar concentration and weight were positively correlated, which makes multiple-trait mapping a powerful addition to the QTL mapping approach (see below).

While this individual correlation may be brought about by environmental covariation between nectar availability and quality, the reverse trend (apparent across groups, Figure 2) might be more indicative of genetic correlations mediated by sucrose responsiveness (Page et al. 1998; Pankiw and Page 2000).

A priori, all phenotypic correlations make the same genetic loci potentially influential on the correlated traits (pleiotropy). Our tests revealed ubiquitous genotypic effects of the markers for *Amfor* and *pln2*. However, the pleiotropic effects of the markers mainly involved interactions with other markers. These sets of influential markers were overlapping but not identical among foraging variables (partially overlapping genetic architecture based on different interactions among several genetic factors). This suggests that different systems are determining the investigated foraging variables that involve some common (molecular) components.

The complex interactions found among markers for our candidate genes suggest interacting factors in a multilayered organization, a pattern that is recurring in colony organization, neurophysiological functioning, and intracellular signaling (Page and Erber 2002). Our study confirms genetic effects on foraging behavior near the previously mapped QTL *pln1, pln2*, and *pln3* (Hunt et al. 1995; Page et al. 2000). These effects persisted through two outcrossings and 10 generations of continued selection, and in spite of a different sampling protocol.

The collection of new foragers over 1 month allowed us to explore the interaction between environmental changes over time and the foraging decisions that bees make on their first

foraging trip. However, we found a significant linear time trend for only two variables, nectar concentration and pollen proportion collected, and even for these variables most analyses yielded similar results, with or without time correction. Nevertheless, the consistency of the behavioral QTL *pln1, pln2*, and *pln3* is remarkable. We could also show a clear effect of the marker for *Amfor*, which we would like to dub *pln4* because there is currently no data to suggest whether the genetic effect is indeed caused by the *Amfor* gene or another closely linked locus.

Given the different experimental conditions and our more sophisticated QTL mapping techniques, the identification of additional QTL influencing foraging behavior is not surprising. Also, this study is the first to use HBC and LBC simultaneously, whereas previous studies relied exclusively on the HBC (Hunt et al. 1995; Page et al. 2000). Many of the reported new QTL have a larger effect size than the candidate loci that we tested, but their significance is less compelling because of smaller sample sizes and the fact that we had no a priori expectation for any of the AFLP® markers. Our QTL results with the various mapping techniques employed also demonstrate the sensitivity of this kind of study to the statistical methodology used. We adhere to the view that QTL mapping represents an exploratory stage of scientific inquiry with accordingly relaxed statistical threshold levels (Weller et al. 1998) but requiring confirmation. For this reason, we report suggestive QTL with a LOD score of ≥2.0 for our interval mapping. However, we are disinclined to name any of the newly reported QTL (*pln5*, etc.) before confirmation of their effects. The poor correspondence of effects between HBC and LBC cannot be regarded as evidence against the new individual QTL, because dominance and epistatic effects play a major role, as indicated by our study of candidate loci.

In general, the statistical methods used provided similar results, but more sophisticated methods resulted in the detection of more QTL. Initial screening of the effects of all markers proved to be important, because four of the markers with strongest effects did not map to any linkage group and thus were not analyzed in any of the QTL mapping. Although genomic linkage is an important criterion to establish the validity of genetic markers, these isolated markers might represent areas of high recombination rate, which are often of particular functional interest (e.g., Beye et al. 1999; Rice and Chippindale 2001).

Interval mapping provides several advantages over single-marker mapping (Lander and Botstein 1989), while its statistical methodology is considerably less complex than multiple-QTL mapping (MQM) techniques. Both computer packages (MapQTL and QTL Cartographer) gave very similar results. Several genetic effects that were suggested by single-marker analysis were reconfirmed with higher statistical confidence. However, there was only one statistically significant QTL (in the LBC, for the concentration of nectar collected). This QTL was 36 cM distal to the previously reported *pln2*. We could not resolve the question whether the two QTL are identical, because our confidence on the QTL position is low because of the relative paucity of markers in that genomic area and our limited sample size. However, there are several lines of evidence that suggest the existence of multiple QTL in this genomic area: First, MQM and CIM locate the LOD peak in this area distal to the *tyr* marker in the LBC and proximal in the HBC. Second, the original description of *pln2* involves a bimodal LOD peak (Hunt et al. 1995, figure 2), and a follow-up study found two small but clearly distinct LOD peaks in this genomic region (Page et al. 2000, figure 1). Additionally, it is interesting that a QTL identified for learning in the honey bees maps close to this area (Chandra et al. 2001) because of the demonstrated link

between sucrose responsiveness, learning, and foraging behavior (Pankiw and Page 2000; Scheiner et al. 2001).

While composite interval mapping (CIM; Zeng 1993, 1994) and MQM (Jansen 1993; Jansen and Stam 1994) are conceptually similar, their implementation in MapQTL (MQM) and QTL Cartographer (CIM) is different. We chose to screen our data with different procedures, and the outcome differed significantly. Both methods reflected similarly the factors identified by simple interval mapping. However, only in one case (*E6M5210*, Figure 7) was a suggestive QTL found as significant upon our reducing background variability in the more sophisticated mapping procedures.

We were moderately conservative selecting cofactors in both, MQM and CIM, because too many cofactors decrease the power of subsequent tests (Zeng 1994). However, the two approaches had different success in detecting new QTL in the two different data sets (HBC and LBC). Our analyses with MapQTL's MQM indicated two additional QTL in the HBC, while QTL Cartographer's CIM suggested two unrelated QTL in the LBC. This difference may be due to data structure or the difference in sample size of HBC and LBC.

QTL Cartographer offered the possibility of multiple-trait mapping (Jiang and Zeng 1995), which we believed suitable in our analysis because the investigated foraging variables are interrelated. The rationale for selecting our two combinations of factors was that "nectar-load size / nectar concentration" investigates the quantity and quality of the nectar collection simultaneously, and "nectar-load size / pollen-load size" indicates the total foraging effort or loading propensity of a foraging bee. Both of these composite analyses revealed a significant QTL in a genomic region that displayed only weak effects on any single foraging variable (LODs between 0.7 and 1.7). The comparison of the two joint analyses (Figure 5) also demonstrates that the LOD score of the joint analysis is not simply an addition of the two independent LOD scores. However, for most of the genome, the LOD score of the joint analysis was determined by the higher LOD score of either variable.

As mentioned, we regard the new QTL detected in this study as new hypotheses that should be tested in additional studies. The results presented here on our candidate loci show that such confirmatory studies of behavioral QTL are profitable and possible, provided that the genetic stocks are maintained. Confirmation experiments of the reported QTL, together with functional analyses (e.g., Beye et al. 2003) and sequence analyses aided by the imminent publication of the honey bee genome sequence (http://www.hgsc.bcm.tmc.edu/projects/honeybee/) will be crucial next steps for a comprehensive understanding of the genetic architecture of honey bee foraging.

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