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ORIGINAL RESEARCH

A Genetic Analysis of the Stinging and Guarding Behaviors of the Honey Bee

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Abstract In order to identify genes that are influencing defensive behaviors, we have taken a new approach by dissecting colony-level defensive behavior into individual behavioral measurements using two families containing backcross workers from matings involving European and Africanized bees. We removed the social context from stinging behavior by using a laboratory assay to measure the stinging response of individual bees. A mild shock was given to bees using a constant-current stimulator. The time it took bees to sting in response to this stimulus was recorded. In addition, bees that were seen performing guard behaviors at the hive entrance were collected. We performed QTL mapping in two backcross families with SNP probes within genes and identified two new QTL regions for stinging behavior and another QTL region for guarding behavior. We also identified several candidate genes involved in neural signaling, neural development and muscle development that may be influencing stinging and guarding behaviors. The lack of overlap between these regions and previous defensive behavior QTL underscores the complexity of this behavior and increases our understanding of its genetic architecture.

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Introduction

The division of worker labor in a honey bee colony is generally directed by temporal polyethism, with environmental and genetic factors contributing to the age of onset and tendency to perform specific behaviors. In general, the youngest workers (4–12 days after eclosure) will act as the "nurse bees", and provide care for the larvae (Johnson 2008). Middle aged workers (12–20 days after eclosure) focus on nectar processing, nest maintenance and guarding behaviors (Johnson 2008; Moore et al., 1987). The oldest workers (>20 days after eclosure) forage for nectar and pollen. Task specialization of an individual can also be influenced by its genotype. For example, hygienic behavior, which consists of removing corpses or diseased brood from the nest, has a significant heritable component, as does defensive behaviors such as nest guarding and stinging (Breed and Rogers 1991; Robinson and Page 1988; Arechavaleta-Velasco et al. 2003; Arechavaleta-Velasco and Hunt 2004; Breed et al. 1990).

Defensive behavior in honey bee colonies is a complex system influenced by many interacting environmental and genetic factors (Shorter and Rueppell 2012). Workers at the colony entrance perform guarding behaviors by antennating incoming foragers, removing non-nestmates and other arthropods and emitting alarm pheromone when disturbed (Moore et al. 1987). Guarding behavior appears to be a specialized task because at most 10–15% of worker bees will be seen to guard the nest entrance (Hunt et al. 2003; Moore et al. 1987). Workers inside the colony will respond to alarm pheromone and fly out towards moving targets. These

defenders will fly out to pursue, harass and sting a target (Breed et al. 2004). The distinction between workers that perform guarding behaviors and workers that respond by stinging is unclear. Researchers have typically measured the colony-level stinging response to estimate the level of defensiveness. By creating a disturbance to the colony and waving a leather flag at the entrance, it is possible to quantify the number of stings a colony will deposit over several minutes (Guzman-Novoa et al. 1999; Guzman-Novoa et al. 2004; Hunt et al. 1998). In colony-level stinging tests, guards represented very few of the individuals that stung, but the level of defensive response had a positive correlation with the number of guards at the entrance and the number of guards that stung (Arechavaleta-Velasco and Hunt 2004; Arechavaleta-Velasco et al. 2003).

Previous research has tried to identify the genetic determinants that underlie the increased defensive behavior of Africanized honey bees (AHB). Several different genetic factors influence the defensive behavior of honey bees. Three regions of the genome, *sting-1*, *sting-2* and *sting-3,* have been identified as influencing colony-level defensive behavior (Hunt et al. 1998). Those three regions also influence guarding behavior to different extents (Arechavaleta-Velasco et al. 2003; Guzman-Novoa et al. 2002; Arechavaleta-Velasco and Hunt 2004). Guarding behavior can be thought of as a binary trait because bees are either performing the behavior or not. Using binary trait locus (BTL) mapping and amplified fragment length polymorphic markers, seven putative BTLs were found to influence guarding behavior, but only one was mapped to a previously identified defensive behavior QTL at *sting-2* (Arechavaleta-Velasco and Hunt 2004). For now, evidence seems to suggest that there is at least some overlap in the genes that influence guarding and stinging behaviors.

Epigenetic processes may also influence defensive behavior. Guzman-Novoa et al. (2005) showed that defensive behavior is strongly influenced by the paternity of a colony. Colonies of F_1 workers with African paternity consistently stung at rates typical of fully Africanized colonies; whereas the phenotypes of F_1 colonies with European paternity were intermediate between parental types. In crosses using an Africanized drone, the genetic contribution from the queen might be masked by gene expression from the paternal allele. The large paternal effect on colony-level stinging behavior requires us to rethink previous results that suggested high defensive behavior is a purely dominant trait.

In order to identify genes that influence defensive behavior, we used an individual-level assay that can be conducted in a laboratory setting. A laboratory assay may allow us to genetically dissect defensive behavior into manageable components by removing effects of social interactions. If we can understand the genetic architecture of defensive acts performed by individual bees, such as sting response and guarding behaviors, we may be able to better understand how genes and individuals interact to influence colony defensive responses. We quantified the individual stinging response of worker bees using an electric shock assay in two unrelated backcross families. For one of these families, we also collected workers at the nest entrance that were observed performing guarding behaviors. Here we describe a new strategy for QTL mapping of genes influencing individual defensive behaviors using a large set of single-nucleotide polymorphic markers (SNPs) within the coding sequences of genes.

Methods

Colony origin and backcrosses

Colonies were reared near Ixtapan de la Sal, Mexico (18°50′N, 99°40′W), during the winter of 2007–2008 and selected on the basis of their stinging response in March of 2008. Individual worker bees from five European and six Africanized colonies were tested for their rapidity of stinging in response to an electrical stimulus (see below). Two highly defensive colonies from Africanized sources were crossed to two low defensive European colonies to create two unrelated F_1 hybrid crosses. The first cross used a European queen and an Africanized drone to produce an EA F_1 queen, which was backcrossed to an Africanized drone from the parental colony (EAA backcross). A second cross using a European queen and a Africanized drone produced another EA F_1 queen, which was backrossed to a European drone from an unrelated source (EAE backcross).

The behavioral assay: sting response to electrical stimulation

To test sting response, worker bees were subjected to mild electrical pulses (see below for assay parameters) from a constant-current stimulator apparatus (Isostim 360, model A 320R-E, World Precision Instruments). The constant-current stimulator was connected to electrodes comprised of 21, 1.5 mm diameter bars on a wooden frame with 5.0 mm spacing. The bars were wired so that adjacent bars were of opposite polarity. The constant-current stimulator corrects for variation in resistance caused by the bees' connection to the electrodes to transmit a specified current during the test. Bees responded to shocks by stinging a suede leather patch under the metal bars. The stimulus was presented to each bee and the time between initial stimulus and stinging was recorded (Uribe-Rubio et al. 2008). Individual worker bees were placed on paper underneath clear plastic covers. One experimenter transferred the bee

to the apparatus and applied the stimulus while another observer measured time. The stinger was removed from the patch with forceps after each bee was tested. After two stings had been embedded in each side of a patch, a new patch was used. The metal bars were wiped off with distilled water after each bee to minimize the odor of alarm pheromone near the test subject. This assay is a refinement of methods used in other studies that simply varied the voltage to change stimulus levels (Kolmes and Fergusson-Kolmes 1989). However, it should be noted that even though attempts were made to minimize the presence of alarm pheromone the tests were not performed in a fume hood and bees were probably exposed to some olfactory stimulation in addition to the electrical current.

Workers from the two backcrosses were permitted to emerge as adults from combs containing pupating brood that had been placed in an incubator for 24 h at 32°C. Adult bees were placed in a cage inside a colony until the assay was conducted. Caged bees were allowed to be fed by nestmates through the holes in the cage, which also contained sugar "candy" mixed with pollen as a protein source. Assay parameters were slightly different for the two backcrosses. For the EAA backcross, 674 5- to 8-day-old worker bees were tested. For both families, the stimulus parameters for the electrical pulses were 300 ms intervals; 3 ms pulse width; 0.25 or 0.5 mA amplitude. For the EAA workers tested, the current used was 0.25 mA. For the EAE backcross, all workers received a 0.5 mA shock. The first 287 bees tested from the EAE backross were 5–6 days old. After all of the workers of known age were tested, some bees of unknown age were also tested to increase the sample size. These were collected from the exterior portion of the nest inside the hive because young bees are found primarily in the brood nest and bees in the exterior of the nest are likely to be more than 5 days old. There was no significant difference in response between these two groups ($p > 0.05$; t test on log-transformed data), and they were treated as a single group. Previous studies have shown that the sting reflex is "mature" between 5 and 7 days after emergence (Burrell and Smith 1994). The tendency to sting of individual workers tested with assays similar to the one we employed showed little variation with age, but the highest stinging tendency was reported at the approximate age when bees are most likely to guard the nest entrance (10–20 days after eclosure) (Kolmes and Fergusson-Kolmes 1989; Paxton et al. 1994).

Sample selection and DNA extraction

Workers performing guarding behavior were captured and immediately frozen in liquid nitrogen. Guards were recognized as those workers that patrolled the nest entrance and repeatedly approached and inspected other bees with their antennae (Moore et al. 1987). Eighty-eight guard bees from the Africanized backcross colony (EAA) were collected over a period of 5 days.

Individuals with the most extreme sting-times were also selected for analysis. Abdomens or thoraces were taken from 96 bees that stung between 0 and 0.8 s (fast), and 96 bees that stung between 2.2 and 30 s (slow) totaling to 192 bees from the Africanized backcross. From the European backcross, 96 bees that stung from 0 to 0.8 s (fast) and 96 bees that stung from between 2.5 and 30 s (slow) were sampled. For the bees seen performing guarding behaviors, abdomens from 88 individuals were taken from the Africanized backcross. DNA extraction and quantification was performed as in Ammons and Hunt (2008).

SNP probe design and selection

To design the DNA probes, genomic DNA from a sister of the F1 queen used in the EAA backcross and a daughter from the corresponding Africanized parental colony were sequenced using an ABI SOLiD sequencing platform and then compared to the sequenced honey bee genome. SNPs were identified with ABI DiBayes software using the honey bee genome assembly version 4 as a reference (http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/bee_genome4_chromo/). SNPs were selected that were homozygous and different between the queens to maximize the number of polymorphic markers in the EAA backcross. A total of 1,536 SNP probes were designed using the Assay Design Tool from Illumina. Genotyping was performed using the Illumina Bead Station. There were 473 informative SNPs in the EAA backcross and 4,61 informative SNPs in the EAE backcross.

Linkage and QTL analysis

JoinMap 4 (Van Ooijen 2006) was used for linkage analysis. Linkage groups were identified based upon the test for independence with a minimum LOD score of 3.0. For the Africanized backcross, a total of 464 individuals were used to create a linkage map. This included 190 bees that were tested for stinging response, 88 guard bees and 186 bees for which we did not have defensive behavior data. For the European backcross, 154 individuals tested for stinging response were used in the linkage analysis. The order of markers was compared with their orders in the assembled honey bee genome, assembly 4.0 (http://hymenopteragenome.org/beebase; Weinstock et al. 2006. For both backcrosses, JoinMap 4 software correctly grouped the loci in each linkage group together according to the genome sequence, but several markers within linkage groups had an incorrect phase so their phases were flipped. The within-group order was corrected to correspond to the sequence

assembly. The genotype of the father of the F_1 queen for the EAA backcross was used to determine marker phase. This was not possible for the European backcross because the father of the F_1 queen was not available, so the influence of paternity could not be determined for the EAE cross. The JoinMap Maximum Likelihood mapping algorithm was used to determine the order of loci and to construct maps. Markers that expanded the size of linkage groups by more than 5 cM were removed and excluded from the analysis.

MapQTL 5 (Van Ooijen 2004) was used for QTL analyses. Sting-time data were log transformed [log (sting time + 1)] to approximate a normal distribution. Interval mapping was performed using the least squares regression to estimate the QTL parameters. Our analyses involved selective genotyping because not all shocked individuals were genotyped. Only those individuals with the most extreme phenotypes were genotyped. For the EAA backcross, we had 630 individuals with phenotypic information and obtained genotypic information for 166 individuals. For the EAE backcross, the number of individuals with phenotypic information was 465 and the number with genotypic information was 154. Thirty-eight individuals in the EAE backcross had to be excluded because genotype information showed that they were not from that family. These worker bees were probably "drifters" that had entered the wrong colony but were accepted by nestmates once they passed the guard bees. Significance of QTLs was assessed with 1,000 genome-wide permutations.

Candidate gene identification

Candidate genes were selected by annotating genes within the 95% confidence interval region that lies within the 1.5 LOD value of the QTL peak (Dupuis and Siegmund 1999). In all cases, we were able to approximate the 95% confidence interval regions for the QTL location. We estimated the region using average values for confidence intervals of honey bee behavioral QTL (reviewed in Hunt Hunt 2007). Genes were identified on the Honey Bee Genome Assembly 4 (http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/bee_genome4_chromo/) and peptide sequences were aligned to the non-redundant protein database using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches with the most significant e-values were selected, and information on function, homologs and relatedness to fly orthologs (http://flybase.org/) was collected.

Analysis of candidate genes

Candidate genes in the three putative QTL regions were annotated and likely candidate genes are shown in Tables 1, 2, 3 (See supplementary Tables S1, S2 and S3 for a complete list of all genes). FlyBase (Tweedie et al. 2009) was used as a reference for putative functions of orthologs and other information was obtained from BLASTp hits and literature searches. The DAVID website (Dennis et al. 2003; Huang et al. 2009) was used to determine gene ontology and functions by comparing homologs of *Drosophila melanogaster* genes for over-representation of functional clusters.

Results and discussion

Sting response

The Africanized backcross had a significantly quicker average sting response time than the European backcross. Africanized backcross bees stung at an average of 1.68 s and European backcross bees stung at an average of 2.13 s (log-transformed data, ANOVA, $F_{1,1171} = 6.215$, $p = 0.0128$).

Linkage analysis

The maps constructed for the Africanized backcross (EAA) and European backcross (EAE) were similar in size: 445 markers and 4270 cM versus 441 markers and 4105 cM, respectively. A map of 445 SNP markers on 23 linkage groups was created using JoinMap 4. The 4270 cM EAA map spanned all 16 chromosomes with an average spacing of 9.6 cM. The 4105 cM EAE map contained 32 linkage groups, spanning all 16 chromosomes with an average spacing of 9.3 cM. The physical size of the honey bee genome is approximately 262 Mb (Beye et al. 2006; Weinstock et al. 2006). The estimated recombination rate for the EAA map (16.3 cM/Mb) and EAE map (15.7 cM/Mb) are similar to other mapping studies in honey bees (Lapidge et al. 2002; Ammons and Hunt 2008; Beye et al. 2006; Solignac et al. 2007).

QTL analyses for stinging behavior

Analysis of individual stinging behavior using the European (EAE) backcross revealed one suggestive QTL influencing stinging behavior on chromosome 1 with a LOD score of 2.10 (Fig. 1). This QTL accounts for 2.6% of the variance of the trait. The genome-wide permutation test for significance at the 5% level was 2.4. The 95% confidence interval region (approximated by the LOD 1.5 interval) for this QTL spans approximately 50 cM and we identified 81 genes within this region.

In the Africanized (EAA) backcross, one suggestive QTL was identified on chromosome 11 with a LOD

Table 1 Annotations of candidate genes associated with a QTL affecting sting-time in the EAE backcross population

Bee gene name	Fly homolog	Annotation
GB13493	CG5279	*Rhodopsin 5* A blue-sensitive opsin
GB15220	CG4795	*Calphotin* Calcium ion binding and rhabdomere development
GB11433	CG42734	*Ankyrin 2* There is experimental evidence that it is involved in the following biological processes: microtubule cytoskeleton organization; neuromuscular junction development; axon extension
GB10510	CG11804	*Ced-6* Mushroom body development
GB10232	CG8581	*Frazzled* Experimental evidence it is involved in: motor axon guidance; salivary gland boundary specification; axon guidance; dendrite morphogenesis; axon midline choice point recognition; dendrite guidance
GB17906	CG10997	Chloride intracellular channel
GB12585	CG1511	*Eph receptor tyrosine kinase* Ephrin receptor activity; mushroom body development
GB14187	CG10737	Diacylglycerol binding, intracellular signaling pathway

Table 2 Annotations of candidate genes associated with a QTL affecting sting-time in the EAA backcross population

Bee gene name	Fly homolog	Annotation
GB15573	CG9695	*Disabled* SH2 domain binding
GB19195		Uncharacterized GPCR
GB10540	CG6410	*Sorting nexin 16* Positive regulation of synaptic growth at neuromuscular junction
GB11309	CG8727	*Cycle* Regulation of circadian rhythm
GB13478		*Rab21* GTPase activity

Table 3 Annotations of candidate genes associated with a QTL affecting guarding in the EAA backcross population

Bee gene name	Fly homolog	Annotation
GB13412	CG33152	*Homeobrain* Predicted transcription factor activity, regulation of DNA-dependent transcription
GB16164	CG4379	*cAMP-dependent protein kinase c2* There is experimental evidence for; anatomical structure development; reproductive process in a multicellular organism; reproductive cellular process; learning or memory; regulation of developmental process; rhythmic process; macromolecule modification; regulation of biological process; memory.
GB19717	CG10052	*Retinal Homeobox* Predicted to have specific RNA polymerase II transcription factor activity, regulation of transcription from RNA polymerase II promoter
GB10285	CG10036	*Orthopedia* DNA binding, predicted to be involved in the regulation of transcription
GB14474	CG13969	*Brain washing* There is experimental evidence that it is involved in brain development
GB10955	CG7207	*Ceramide transfer protein* Predicted to have protein serine/threonine kinase activity
GB11417	CG7771	*Single-minded* Experimental evidence that it is involved in adult walking behavior; axon guidance; determination of genital disc primordium; ventral midline development; brain development; axonogenesis; ventral cord development

score of 2.39 (Fig. 2). This accounts for 3.0% of the variance of the trait. The 95% threshold from the genome-wide permutation test was 2.8. The 95% confidence interval region for this QTL was estimated to be approximately 2 Mb long and we identified 74 genes within this region.

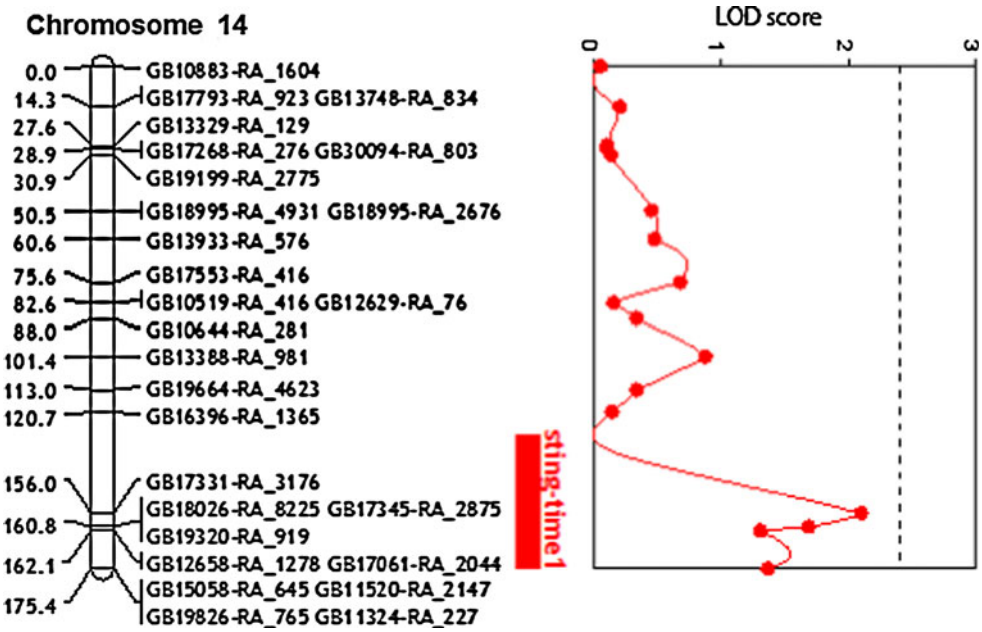

Fig. 1 Putative QTL for stinging behavior on chromosome 14 of the EAE backcross as mapped by JoinMap 4. The markers correspond to SNPs identified in the labeled genes. Distances are represented in cM on the left of the map, the *dotted line* of the *graph represents* the genome-wide significance level at the 5% level (2.4), and the *solid bar* represents the approximate 95% confidence interval for QTL location. One suggestive QTL is present with a LOD score of 2.1

Genetic analysis for individual guarding behavior

The genotypes of guard bees were compared to those used in the stinging assays by treating guarding behavior as a binary trait with MapQTL software, which uses a χ^2 test for goodness-of-fit to determine the significance of deviations from a one-to-one segregation in guards relative to non-guards. Non-guarding workers were designated as "zero" for the behavior and guarding workers were given a "one". There was one suggestive QTL, located on chromosome 1 with a LOD score of 2.03 (Fig. 3). The genome-wide permutation test for significance at the 5% level was 3.2. The 95% confidence interval region for this QTL stretched approximately 75 cM and we identified 63 genes within this region.

Candidate gene identification

We have identified a number of candidate genes likely influencing stinging or guarding behaviors (Tables 1, 2, 3). Analyses conducted using DAVID identified which functional categories were most highly represented in the QTL regions (Table 4). The functional categories of genes in the three QTL regions (sting-time 1, sting-time 2 and guarding) that were the most highly represented included axonogenesis, ion transport, synaptic function, neuron development and neuron differentiation. Among these general functional categories, there were genes involved in several systems relevant to behavior.

Discussion

This study took a unique approach to investigate the genetic basis for the defensive response of honey bees. Our approach was to assay individual bees' stinging response outside the context of colony defense. We also collected workers performing guarding behavior at the colony entrance. We identified two previously unreported QTL for individual stinging behavior and one previously unreported QTL for guarding behavior. The statistical support for these QTL do not reach the level of significance for a genome-wide scan and must be considered putative until confirmed in independent crosses. However, several previous QTL for stinging behavior with lower LOD scores have been confirmed (Hunt 2007), suggesting these may be as well. Interestingly, we identified different QTL for the same trait in our two backcross-families. There are several possible explanations for this result. The two families might have had different alleles segregating due to chance since we do not have inbred lines. Behavioral traits are usually complex, and have many interacting factors, each of which could be lost with each unique cross or affected by genetic background or epistasis (Mackay 2004). In addition, defensive behavior QTL with large paternal effects would be masked in the African backcross (EAA).

The QTLs found using this individual bee stinging response assay appear to be unique from the previous colony-level defensive response assays. Stored drone DNA from the original colony-level study was genotyped using the same GoldenGate assay probes used in this study to

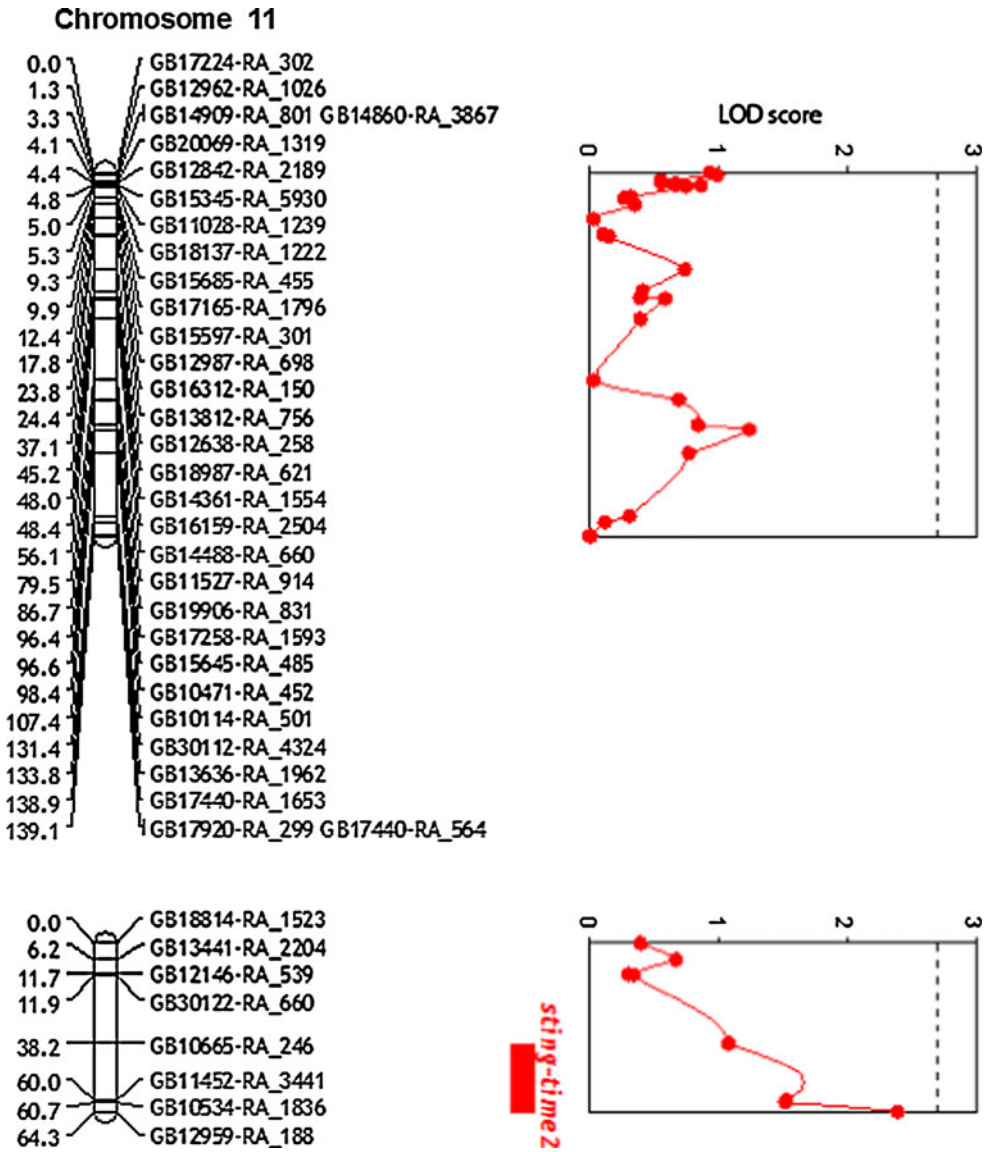

Fig. 2 Putative QTL for stinging behavior on chromosome 11 of the EAA backcross as mapped by JoinMap 4. The markers correspond to SNPs identified in the labeled genes. Distances are represented in cM on the left of the map, the *dotted line* represents the significance level at the 5% level (2.8), and the *solid bar* represents the approximate 95% confidence interval for QTL location. One suggestive QTL is present with a LOD score of 2.39

provide markers that could be better aligned to the sequenced genome (Hunt et al. 1998). A comparison showed that although there appears to be a QTL influencing colony level defensive behavior (LOD 2.42) on chromosome 11 it does not coincide with the QTL in the present study, suggesting no overlap between QTLs for colony level defensive behavior and QTLs for individual stinging response (unpublished data). This is likely due to the different behaviors that each assay was designed to test. Colony-level assays, while quantifying the number of actual stings, may be influenced by the sensory systems of the bees and social interactions. Bees that sting to defend the colony may actually be responding to visual stimuli (waving the flag target) and to the release of alarm pheromone as defenders become aroused (extending their stingers in the process). Africanized workers are known to respond in greater numbers than European workers to application of alarm pheromone to the colony entrance, demonstrating a greater sensitivity to the pheromone and a lower threshold to respond (Collins et al. 1982, 1987, 1988). The assay with the constant-current stimulator tests for a response to an aversive stimulus. Results of this assay may be influenced by genes that affect central nervous system excitability rather than specific sensory modalities, since we strove to minimize any exposure to alarm pheromone and visual stimuli. In the present study there was no overlap between QTLs for stinging response and guarding behaviors, suggesting that perhaps stinging and guarding behaviors are controlled by different sets of genes. However, colony-level stinging QTL were previously shown to influence propensity to guard the nest entrance Guzman-Novoa et al. (2002); Arechavaleta-Velasco et al. 2003).

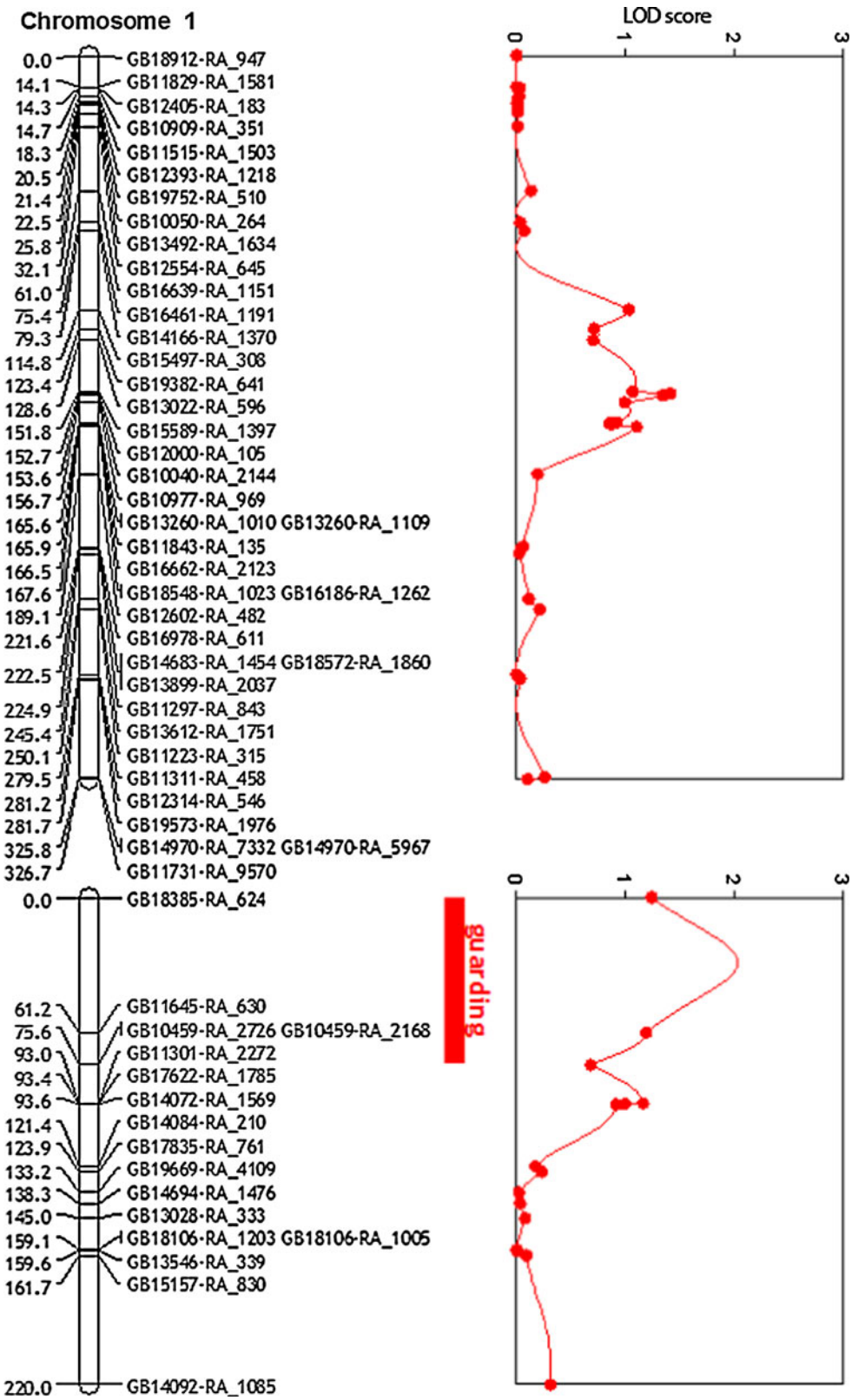

Fig. 3 Putative QTL for guarding behavior on chromosome 1 of the EAA backcross as mapped by JoinMap 4. The markers correspond to SNPs identified in the labeled genes. Distances are represented in cM on the left of the map, the significance level at 5% is 3.2 is indicated by the *dotted line*. One suggestive QTL is present with a LOD score of 2.03

Table 4 The hierarchical clustering analysis generated using DAVID

QTL	Category	Term	Count	%	*P* value
Sting-time 1 (EAE)	GOTERM_BP_FAT	Neuron differentiation	8	11.1	0.0052
Sting-time 1 (EAE)	GOTERM_BP_FAT	Neuron development	7	9.7	0.0093
Sting-time 1 (EAE)	GOTERM_BP_FAT	Axonogenesis	5	6.9	0.02
Sting-time 2 (EAA)	GOTERM_BP_ALL	Anatomical structure development	10	22.2	0.07
Guarding (EAA)	GOTERM_BP_FAT	Ion transport	6	11.5	0.013
Guarding (EAA)	GOTERM_CC_FAT	Synapse	4	7.7	0.024
Guarding (EAA)	SMART	HOX	3	5.8	0.084

Genes involved in central nervous system (CNS) and brain functions

A number of genes in the three QTL regions studied here are orthologs or homologs of fly genes known to influence brain development, brain function and neural signaling. These genes could play an important role in honey bee defensive behaviors, and may explain some of the behavioral differences between Africanized and European populations. In the QTL region for stinging in the EAE backcross, there are seven genes with functions associated with neuron development and eight associated with neuron differentiation. A homolog of *Ced-6*, a gene involved in mushroom body development (GB10510) is in the EAE backcross sting-time QTL. The mushroom body is the primary region of the insect brain responsible for learning and memory. *Ced-6* is required for normal synaptic growth in *Drosophila*, and disruption of this gene results in accumulation of presynaptic debris (Fuentes-Medel et al. 2009). Function of this gene is also required during metamorphosis where mushroom body neurons are pruned (Awasaki et al. 2006). *Eph receptor tyrosine kinase* (GB12585) is important for signaling and guiding mushroom body branches to their correct synaptic targets (Boyle et al. 2006). *Eph receptor tyrosine kinase* is also known to control photoreceptor axon and cortical axon topography during development of the visual system (Dearborn et al. 2002). *Frazzled* (GB10232) encodes a cell surface receptor on embryonic CNS neurons and its protein helps to guide axons across the midline during formation of the anterior and posterior commissures (Dorsten et al. 2007; Hummel et al. 1999). *Ankyrin 2* (GB11433) is also expressed in the nervous system and its protein helps to stabilize the presynaptic membrane cytoskeleton (Koch et al. 2008).

In the QTL identified using the EAA backcross, a homolog of *Disabled* (GB 15573) was found in the QTL region for sting behavior. Proteins from this gene are believed to help in synaptic transmission. Mutations of this gene will result in impaired synaptic function through a reduction of neurotransmitter release and disrupted synaptic vesicle endocytosis (Kawasaki et al. 2011). Another gene, *Nexin 16* (GB10540), has a role in membrane trafficking and synaptic development (Hanson and Hong 2003; Rodal et al. 2011). Nexin-16 helps regulate synaptic growth signaling cascades and is required to down-regulate activated receptors (Rodal et al. 2011). *Rab21* (GB13478) is another interesting candidate gene found within this region. It produces a small GTPase and is a positive regulator of neurite growth (Burgo et al. 2009). *Cycle* (GB11309) is a HLH-PAS transcription factor and a regulator of circadian rhythm. A nearby gene (GB19195) appears to be mis-annotated and likely is two different genes. The first part of this gene is a rhodopsin-type G protein-coupled receptor (GPCR) of unknown function. Most honey bee neuropeptide and biogenic amine GPCRs have been identified (Hauser et al. 2006). Neuropeptide Y and its insect homolog, neuropeptide F, have been associated with aggression in mammals and fruit flies but no neuropeptide F receptors have been identified in hymenoptera (Dierick and Greenspan 2007). The GPCR found here on chromosome 11 appears to have attributes intermediate between neuropeptide and biogenic amine receptors (Frank Hauser, pers. comm.).

Several genes influencing brain function and neural development were identified within the QTL for guarding behavior in the EAA cross: four genes that are involved in synapse formation and five that are involved in ion channel activity. For example, *Single-minded*, a master regulatory gene, encodes a HLH-PAS transcription factor (GB11417) involved in motor axon guidance, brain development and midline cell development of the embryonic ventral nerve cord (Hummel et al. 1999; Pielage et al. 2002). This gene is particularly interesting because its protein forms a dimer with tango. This protein complex controls specification of the CNS midline cell lineage (Sonnenfeld et al. 1997). Another HLH-PAS transcription factor, *Tango*, was identified as a candidate gene on the *sting-1* region influencing colony-level defensive behavior and individual guarding behavior (Hunt 2007). The interaction of *tango* and *single-minded* in brain and midline cell development make them ideal targets for future research on defensive behaviors. The *ceramide transfer protein* encoded by gene

GB10955 regulates oxidative stress and damage response, and lifespan (Rao et al. 2007). The *brain-washing* gene (GB14474), encodes a ceramidase that is involved in brain development (Boquet et al. 2000). Ceramidase cleaves ceramide to produce a second messenger that is involved in synaptic transmission (Stancevic and Kolesnick 2010; Rohrbough et al. 2004). *cAMP-dependent protein kinase c2* (GB16164) is associated with learning and memory in *Drosophila* (Renger et al. 2000; Skoulakis et al. 1993; Gervasi et al. 2010). This gene is especially active in the mushroom body, and works together with several other genes including *dunce* and *rutabaga* to regulate cAMP levels (Gervasi et al. 2010). In honey bees, *cAMP-dependent protein kinase c2* (GB16164) is one of three genes encoding catalytic subunits of cAMP-dependent kinase (PKA), which plays a crucial part in long term memory formation by contributing to long term synaptic plasticity (Eisenhardt et al. 2006). Interestingly, previous research on honey bee defensive behavior identified the first catalytic subunit of cAMP-dependent kinase (PKA) within a QTL region for colony-level defense (Hunt 2007). This subunit is also unique in insects, as it has no counterpart in mammals (Eisenhardt et al. 2006). The guarding QTL also contained a sequence of homeobox genes controlling early development. Three genes, *Retinal Homeobox* (GB19717), *homeobrain* (GB13412) and *orthopedia* (GB10285) are known to work in concert during stages of eye and brain development (Eggert et al. 1998; Walldorf et al. 2000; Simeone et al. 1994). These genes are expressed in the CNS and are evolutionarily conserved between vertebrates and insects.

Conclusions

Our study has opened up two new regions for investigation into stinging behavior and another region for guarding behavior. Our sting response assay on individual bees confirmed behavioral differences between AHB and EHB backcrossed individuals. Involvement of a number of candidate genes controlling brain development, neurological signaling and visual development could explain some of the observed differences in defensive response. Our candidate genes do show an overlap between other defensive behavior candidate genes. A candidate gene for guarding behavior, *cAMP* (*PKA*) catalytic subunit 1 was identified. This subunit forms a complex with *cAMP* (*PKA*) catalytic subunit 2, which was identified as a candidate gene in a previous study. Additionally, we also identified *single-minded* as a candidate gene, and its protein is activated when bound to the transcription factor *tango*, which was also identified as a candidate previously. These results suggest a closer look at these genes is warranted to assess their influence on defensive behavior.

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