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Two important traits that contribute to honey bee (Apis mellifera) colony survival are resistance to varroa and longevity of worker bees. We investigated the relationship between a panel of single nucleotide polymorphism markers and three phenotypic measurements of colonies: (a) percentage of mites in brood (MIB); (b) proportion of fallen injured mites; and (c) longevity of workers. We used single marker analysis to identify genetic intervals that may confer resistance and increased lifespan. One gene related to memory and learning, Ddc was identified for MIB, as was acj6, which functions for olfactory perception. These genes may contribute to elevated levels of mite detection and removal. Three genes were identified with high relevance for mite injury. CYP315A1 and Ptp69D function in motor neuron axon guidance in response to chemical stimuli. RabGAP11 is also involved in sensory function, specifically sensory organ development. Evidence for the longevity quantitative trait locus was also strong and one gene (Orct) is related to improved lifespan in both humans and Drosophila. Together, these genes provide possible avenues to be pursued for further development for eventual marker-assisted selection.

Introduction

Selective breeding of honey bees (Apis mellifera L.) has improved resistance to varroa (Varroa destructor) mites in addition to other economically important traits in honey bees (Afik, Dag, Yeselson, Schaffer, & Shafir, 2010; Büchler, Berg, & Le Conte, 2010; Danka, Harris, Ward, & Ward, 2008; Kasper, 2010; Rinderer et al., 2001; Rinderer, Harris, Hunt, & De Guzman, 2010; Villa & Rinderer, 2008; Witherell, 1976). However, continued selection and the discovery of new relevant traits are important for maintaining the improved traits and also for further stock development. Currently, colony loss is one of the primary concerns among beekeepers. Improving longevity and resistance to V. destructor may help alleviate this problem.

Resistance to V. destructor is a complex trait, probably involving a suite of traits and genes. Expanding our knowledge of the mechanisms involved in resistance will improve breeding efforts, and ultimately colony survival. One strategy thought to be used by honey bees to combat V. destructor is grooming behavior against adult mites, characterized by the mutilation of mite body parts (Guzman-Novoa, Emsen, Unger, Espinosa-Montaño, & Petukhova, 2012; Kirrane et al., 2012; Rinderer, De Guzman, & Frake, 2013a, 2013b; Ruttner & Hänel, 1992; Stanimirovic, Stevanovic, Aleksic, & Stojic, 2010). Conflicting data for this trait suggest that while all honey bees appear to groom mites, the behavior is not clearly related to reductions in mite.
populations, except for perhaps in Africanized honey bees (Rinderer et al. 2013a). Damaged mites are found on the bottom boards of colonies, but the mechanism and timing by which the damage is inflicted is not clear (Arehavaletael-Velasco & Guzman-Novoa, 2001). The predation of ants and wax moths and the damage caused by bees to dead mites in standard colonies can also result in erroneous evaluations of grooming (Bienefeld, Zautke, Pronin, & Mazeed, 1999; Szabo & Walker, 2000). High proportions of living mites drop onto the bottom boards of nests, but the cause of this drop has largely been ignored. Some of the possible mechanisms for the removal of mites from colonies are autogrooming, allogrooming, or by workers removing infested pupae (and their associated mites) (Danka & Villa, 1998). Mite drop may be a worthy trait to pursue for eventual improvement through breeding and marker-assisted selection (MAS), if a connection between the fall of injured mites and mite resistance can be demonstrated.

While many traits currently being pursued with selective breeding are related to pests and diseases, other traits also have relevance to colony survival. Longevity is just such a trait. Improved longevity in honey bees, depending upon season, could improve winter survivorship and lengthen time of foraging, therefore improving the colony’s ability to build rapidly in the spring and increasing the amount of colony resources. Worker age at first foraging is a good predictor of life expectancy in honey bees. Worker bees that transit early from in-hive duties to foraging have shorter lifespans. This is likely due to a decrease in vitellogenin and an increase in susceptibility to pathogens, not early “wear and tear” resulting from foraging activities (Rueppell, Bachelier, Fondrk, & Page, 2007). Two genomic regions have previously been identified for pollen hoarding syndrome and age at first foraging, both of which are indicated in determination of longevity (Hunt et al., 2007; Rueppell, 2009; Ruppell, Pankiw, & Page, 2004).

Selective breeding for any trait is enhanced using simple, but reliable and efficient measures. One method of improving the efficiency of breeding strategies is to employ genetic markers that are predictive of desirable traits, using MAS. Quantitative trait locus (QTL) analysis identifies DNA markers that may potentially be used to select breeder queens with favorable traits (e.g., resistance to V. destructor, grooming behavior, and longevity). From the mid to late 1990s, QTL studies of honey bee behavior identified genomic regions that showed strong association with foraging behavior, hygienic behavior, stinging, and defensive behavior (Hunt, Collins, Rivera, Page, & Guzman-Novoa, 1999; Hunt, Guzman-Novoa, Fondrk, & Page, 1998; Hunt, Page, Fondrk, & Dullum, 1995). More recently, QTL have been identified for the removal behavior associated with Varroa-sensitive hygiene (VSH), larval resistance to chalkbrood, tracheal mite resistance (unpublished data), and markers for grooming behavior (Behrens et al., 2011; Guzman-Novoa, Hunt, Uribe, Smith, & Arechavaleta-Velasco, 2002; Holloway, Sylvester, Bourgeois, & Rinderer, 2012; Tsuruda, Harris, Bourgeois, Danka, & Hunt, 2012). A targeted 44K SNP assay was also recently designed to focus on the analysis of Varroa-specific behavior in A. mellifera carnica (Spoetter, Gupta, Nurnberg, Reinsch, & Bienefeld, 2012).

Here, we tested for the presence of single nucleotide polymorphisms (SNPs) which associate with three traits (proportion of MIB, proportion of fallen injured mites (FIM), and longevity of worker bees) either with known or potential associations to colony survival in a backcrossed Russian honey bee population. Single Marker Analysis (SMA) revealed SNPs associated with each trait and a series of putative candidate genes for MAS.

Materials and methods

Backcross design and phenotyping assays

Colony setup and testing structure
A total of 72 single drone-inseminated backcross F1 queens (Russian-x-Italian x Italian) were produced and established in colonies in September – November 2007. From March to August 2008, worker bees from all colonies were phenotyped for three traits, proportion of MIB, proportion of FIM, and longevity of worker bees. The number of colonies varied among traits tested due to differences in the number of viable colonies at the time of sampling.

Percentage of MIB
Fifty-eight colonies were used to estimate the percentage of MIB. Measurements were conducted in March 2008, four to six months after the colonies were established. This ensured that all the worker bees in test colonies were daughters of their F1 mother at the time of collection. In order to calculate the percentage of MIB, the population of V. destructor for each colony was estimated using a suite of measurements as described by Rinderer et al. (2003) (Table 1). Estimates of colony

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony and infestation parameters (n = 58 colonies)</td>
<td></td>
</tr>
<tr>
<td>Number of capped worker brood</td>
<td>3648.6 ± 344.1</td>
</tr>
<tr>
<td>Number of adult bees</td>
<td>5496.8 ± 414.8</td>
</tr>
<tr>
<td>Number of mites in worker brood</td>
<td>306.8 ± 43.5</td>
</tr>
<tr>
<td>Number of mites in drone brood</td>
<td>8.6 ± 5.1</td>
</tr>
<tr>
<td>Number of mites on adult bees</td>
<td>208.3 ± 31.2</td>
</tr>
<tr>
<td>Total colony mites</td>
<td>315.4 ± 45.0</td>
</tr>
<tr>
<td>Percentage of mites in brood</td>
<td>54.0 ± 3.0</td>
</tr>
<tr>
<td>Mite drop parameters (n = 53 colonies)</td>
<td></td>
</tr>
<tr>
<td>Total trapped mites</td>
<td>28.4 ± 1.7</td>
</tr>
<tr>
<td>Number of uninjured mites</td>
<td>22.8 ± 1.5</td>
</tr>
<tr>
<td>Number of injured mites</td>
<td>5.6 ± 3.3</td>
</tr>
<tr>
<td>Longitude parameters (n = 51 colonies)</td>
<td></td>
</tr>
<tr>
<td>Days to 50% bees remaining</td>
<td>15.4 ± 6.0</td>
</tr>
</tbody>
</table>
mites were derived from: (a) number of mites in 200 worker brood cells (50 cells on each side of two combs); (b) number of mites in drone brood if available; (c) number of mites on approximately 300–500 adult bees determined using the powdered sugar method (Macedo, Wu, & Ellis, 2002); (d) estimates of worker and drone brood size (in²) using a grid, which were then converted to numbers of capped brood cells; and (e) comb by comb estimates of the number of bees present in each test colony. MIB was calculated as total number of MIB/total colony mites.

**Fallen injured mites**

Bottom board traps were coated with vegetable oil and installed into the bottom boards of 55 colonies. Trapped mites were assessed for one week (three consecutive days per week) monthly for three months (April–June 2008). Each day, traps were removed and replaced with fresh traps. Mites were collected from each trap using an insect brush, counted, and examined for injuries using a dissecting microscope. Mites with missing legs or damage to the exoskeleton were considered injured. Proportions of injured mites were calculated as the number of injured mites/total number of mites collected on the trap across the trapping period for each colony.

**Longevity of worker bees**

Emerging worker bees were collected from each of 51 colonies and placed in groups of 50 into hoarding cages (one to five cages/colony, depending upon colony strength at time of sample collection). Bees were fed sucrose: water (50:50 mixture by weight) and dead bees were removed and counted daily. Values used for longevity analysis were mean number of days of 50% survival per colony (i.e., days until 25 live bees remained in the hoarding cage, among all cage replicates per colony).

**DNA extraction from drones**

DNA was extracted from the single drones that sired each of the 72 colonies following the published protocol (Bourgeois & Rinderer, 2009). Briefly, thoraces were removed and cut into four pieces to improve efficiency of homogenization. Tissues were homogenized using resin pestles in 1.5-mL microfuge tubes. Cell lysis was accomplished with lysis buffer (TE, SDS) and proteinase K (10 mg/mL) at 70 °C for 10 min, followed by protein precipitation with 7.5 M NH₄OAc. Genomic DNA was then precipitated from the supernatant with 100% isopropanol and washed with 70% EtOH, dried by passive evaporation, after which 25 μL of Millipore filtered dH₂O was added for rehydration. DNA was quantified on a Nanodrop1000™ (Nanodrop Technologies, Wilmington, DE) and samples were diluted to 50 ng/μL for downstream processing.

**SNP detection and SMA**

SNP genotyping was performed using the Illumina BeadStation for SNP typing (Honey Bee Genome Consortium, 2006), using a SNP panel (Illumina, San Diego, CA, GS0010394-OPA) on which some SNPs were changed to increase the number of polymorphic markers (D. Weaver pers. comm.) as compared to the original, previously published, panel (Whitfield et al., 2006). Statistical associations between SNPs and the three traits were determined using SMA in JMP Genomics 6.0 (SAS). Statistical significances of SNP associations were determined by the output LOD score of markers as well as confirmation of P-values determined for each marker by Student's t-test followed by Bonferroni correction with a False Discovery Rate of .05.

A megaBLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990; Altschul et al., 1997) of the sequence interval defined by each QTL was completed to identify potential candidate genes for each trait. We limited the subsequent candidate gene identification to only those gene sequences with ≥90% similarity to each respective SNP interval (i.e., SNP intervals for MIB, FIM, and longevity).

**Results**

**SNP associations with colony phenotype**

The 72 drones individually siring each of the backcross colonies were genotyped using the SNP panel. Filtering of the 1536 SNP data found 382 polymorphic markers from the backcrossed population. Of those, 373 aligned to chromosomes 1 through 16 (according to the Amel4.5 assembly, NCBI) and were used for the association mapping. The remaining nine markers could not be assigned to a chromosome (i.e., they have not been assigned to a chromosome in the most recent honey bee genome assembly).

**Percentage of MIB**

A genomic region on chromosome 16 was identified that had strong association (LOD = 3.31; F = 14.28; \( p = .0003 \)) to MIB, suggesting that a QTL may exist in this region (Figure 1). This interval contains 258 known and hypothetical genes. Of the 258 genes identified, 215 have a known categorical function or homolog in insects. Forty of these genes have been identified or are homologs in *Apis* (Table 2). Evaluation of those genes revealed genes potentially involved in MIB that are involved in neural and sensory function.

**Percentage of FIM**

A genomic region on chromosome 5 was identified with strong association (LOD = 2.893; F = 11.60; \( p = .0016 \)) to FIM, suggesting that a QTL may exist in this region (Figure 1). This interval contains 162 known and
hypothetical genes, of which 140 have a known categorical function or homolog in insects and 47 of these have been identified or are homologs in *Apis* (Table 2). Evaluation of those genes revealed one gene potentially involved in longevity.

**Discussion**

Resistance of honey bee colonies to *V. destructor* is a complex trait comprised of numerous mechanisms controlled by an unknown number of regions within the genome and more specifically, an unknown number of genes. Multiple putative genomic regions have been identified that are associated with various components of resistance to the varroa mite. QTL analysis of bees exhibiting the removal and uncapping components of the VSH trait identified one strong QTL on chromosome 9 and one more weakly associated region on chromosome 1 (Tsuruda et al., 2012). Three other QTL were shown to have epistatic effects associated with suppressed varroa reproduction (Behrens et al., 2011). In a large-scale SNP study, Spötter et al. (2012) identified 1929 SNPs associated with hygienic behavior of sister worker bees in response to varroa-infested cells. Congruence between studies is difficult to establish due to variation in the traits measured and in populations studied. Here, we tested a backcross population for three measures associated with resistance to varroa. None of the SNPs associated with traits measured here were in the regions identified in the previous studies.

Mapping bees from colonies with differential mite loads in the brood identified a SNP interval containing 258 genes on chromosome 16. Many of these genes were homologs among multiple species of insects, meaning that functionally they were repeats of the same gene. Accounting for gene function, this reduced the effective number of genes to 40. These genes were primarily neurological and regulatory in function, with a small subset potentially involved in resistance to *V. destructor* through sensory pathways.

One of these 40 genes is involved in memory and learning, *dopa decarboxylase* (*Ddc*). This gene encodes the enzyme aromatic L-amino-acid decarboxylase (AADC) which has the primary function of dopamine and serotonin synthesis and is necessary for sclerotization and melanization of the cuticle. This gene, however, has also been indicated as critical to long-term memory function (24 h) in *Drosophila* (Blenau & Baumann, 2001; Chen et al., 2012; De Luca et al., 2003). *Ddc* has pleiotropic effects on mating behavior, fertility, circadian rhythms, endocrine secretion and aggression and direct effects on longevity in *Drosophila* (Lunan & Mitchell, 1969). While this gene was associated with the MIB QTL, it was not associated with our longevity QTL. Increased ability for memory and learning may contribute to worker detection of infected pupae and subsequent reduction of mites within the colony. Similarly, olfactory function is very likely to be significantly

![Figure 1. Map of QTL identified for mites in brood (MIB), fallen injured mites (FIM), and longevity of worker bees with single marker analysis (SMA). Peaks are MIB (chromosome 16): LOD = 3.31; *F* = 14.28; *p* = .0003; FIM (chromosome 5): LOD = 2.80; *F* = 11.19; *p* = .0016; Longevity (chromosome 4): LOD = 2.97; *F* = 12.24; *p* = .0011. Dashed line indicates significance threshold of *p* = .05, LOD = 1.35.](image-url)
involved in mite detection and removal. Two genes involved in sensory perception were also found within the SNP interval. Protein spitz ligand is a component of the epidermal growth factor receptor pathway and is sensory in function (Rahn, Leippe, Roeder, & Fedders, 2013). It is indicated in memory and learning at moderate levels of expression. Over- or under-expression results in attenuation of olfactory learning and memory formation (Rahn et al., 2013). The transcription factor abnormal chemosensory jump 6 (acj6) is perhaps more relevant. It is involved in detection of chemical stimuli, specifically the perception of smell (Langen et al., 2013). An alternative function of this gene is direction of axon connection in the optic lobe of the Drosophila brain during development (Langen et al., 2013). With further study of more specific measures of mite resistance, perhaps these genes will show a direct tie to mechanisms driving resistance.

The SNP interval on chromosome five showed the highest potential for candidate genes for mite removal (as indicated by FIM). Of the 73 genes with high similarity to the specified region, most were neurological, developmental, and regulatory. Two genes were involved in motor function, cytochrome P450 315A1 (sad in Drosophila; CYP315A1), and receptor-type tyrosine protein phosphatase N2 (Ptp69D). These genes are involved in motor axon guidance, but during developmental processes (Marlo & Desai, 2006; Song, Ginger, & Desai, 2008; Warren et al., 2002). Sad is associated with central nervous system development and guides motor neuron placement during early developmental stages. The gene Ptp69D is involved in motor neuron axon guidance and is activated upon detection of chemical stimuli. Perhaps Ptp69D is activated in response to detection of phoretic mites and contributes to the motor function aspect of mite removal. A third gene, RabGTPase activating protein 11 (RabGAP11), in the SNP interval shows high relevance to mite removal, relative to detection. This gene is a component of sensory organ development (Jafar-Nejad et al., 2005). High expression of this gene could contribute to elevated sensory detection of mites.

SNP associations with longevity were strong (LOD = 2.97) and nearly as high as for the MIB phenotype. Of the 47 genes within the SNP interval with known function, one showed potential relevance to the longevity phenotype, organic cation transporter protein-like (Orct in Drosophila) (Taylor, Stanley, & Shirras, 1997). Expression of a human analog to Orct (organic cation transporter, OCTN2) was reduced in elderly patients, indicating a role for increased lifespan (Karlic et al., 2003). Meta-analysis has revealed evidence that both Orct and Orct2 in only male Drosophila have elevated expression and are associated with increased life span (Kapushesky et al., 2012). Characterization and expression studies may show similar trends for Orct in the honey bee.

Investigation of multiple traits in a single QTL study revealed a suite of genes that are potential candidates for further development. These genes, if their relevance remains high after validation studies, could be used in marker development for MAS in honey bees to improve colony survival through improving resistance to varroa mites as well as worker longevity.

### Acknowledgments
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### Table 2. Putative role of genes located within the intervals associated with mites in brood, fallen injured mites, and longevity.

<table>
<thead>
<tr>
<th>Putative cellular function</th>
<th>Mites in brood</th>
<th>Fallen injured mites</th>
<th>Longevity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Biological regulation</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Biosynthesis</td>
<td>4</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Cell metabolism</td>
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<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cell regulation</td>
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</tr>
<tr>
<td>Cell repair</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Developmental</td>
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<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Immunological, cellular response</td>
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<td>3</td>
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<td>1</td>
</tr>
<tr>
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<td>Response to gravity</td>
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</tr>
<tr>
<td>Sensory</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
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</table>

332 A.L. Bourgeois et al.
Disclosure statement
No potential conflict of interest was reported by the authors.

References


