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Manipulation of colony environment modulates honey bee aggression and brain gene expression

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The social environment plays an essential role in shaping behavior for most animals. Social effects on behavior are often linked to changes in brain gene expression. In the honey bee (*Apis mellifera* L.), social modulation of individual aggression allows colonies to adjust the intensity with which they defend their hive in response to predation threat. Previous research has showed social effects on both aggression and aggression-related brain gene expression in honey bees, caused by alarm pheromone and unknown factors related to colony genotype. For example, some bees from less aggressive genetic stock reared in colonies with genetic predispositions toward increased aggression show both increased aggression and more aggressive-like brain gene expression profiles. We tested the hypothesis that exposure to a colony environment influenced by high levels of predation threat results in increased aggression and aggressive-like gene expression patterns in individual bees. We assessed gene expression using four marker genes. Experimentally induced predation threats modified behavior, but the effect was opposite of our predictions: disturbed colonies showed decreased aggression. Disturbed colonies also decreased foraging activity, suggesting that they did not habituate to threats; other explanations for this finding are discussed. Bees in disturbed colonies also showed changes in brain gene expression, some of which paralleled behavioral findings. These results show that bee aggression and associated molecular processes are subject to complex social influences.

Keywords: Africanized honey bee, behavioral maturation, behavioral plasticity, single-cohort colony, stress

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Like many other animals, honey bees (*Apis mellifera* L.) modulate their behavior in response to their social environment. The social environment within the beehive is the result of nestmate responses to external environmental stimuli. Effects of social environment on behavior are increasingly linked to changes in brain gene expression (Robinson *et al.* 2008).

Nest defense highlights the complex relationship between the social environment, individual behavior and brain gene expression. Guard bees that monitor the colony entrance release alarm pheromone in response to a predator. Some bees that receive this cue (called soldiers) undergo rapid induction of aggression and also become sensitized to defensive stimuli, a state that lasts for hours (Alaux & Robinson 2007). Rapid induction of aggression enables a response to the immediate threat, while temporary sensitization allows individuals to adjust their propensity to launch an aggressive response, depending on local threat and colony status (Alaux *et al.* 2009b; Couvillon *et al.* 2008; Rivera-Marchand *et al.* 2008). Changes in aggression levels are reflected in the brain transcriptome: one exposure to alarm pheromone caused expression changes in over 400 genes in the soldier brain (Alaux *et al.* 2009b). However, the high level of agitation and arousal in soldier bees may also impact the aggressive state of other colony members who perceive a threatened social environment. It is unknown how chronic exposure to a threatened social environment affects aggression levels and brain gene expression.

Previous studies examined the effects of social environment on aggression by comparing co- and cross-fostered adult bees originating from subspecies that are naturally either relatively docile or aggressive (European or African subspecies, respectively). The average aggression level within a hive appears to affect aggressive behaviors in cross-fostered nestmates (Guzmán-Novoa & Page 1994; Guzmán-Novoa *et al.* 2004; Hunt *et al.* 2003). Hive social environment and individual bee genotype are nearly equal contributors to variation in brain gene expression. Moreover, acute exposure to alarm pheromone and living in an aggressive colony lead to transcriptional changes in some of the same genes (Alaux *et al.* 2009b), suggesting that similar mechanisms underlie behavioral responses to an acute threat and chronic exposure to a social environment with a high number of aggressive individuals. Here we determine whether chronic exposure to an external threat, which causes repeated induction of aggression and a persistently aggressive social environment, also leads to a stable shift in aggression and brain gene expression.

We hypothesized that bees reared in a social environment under the influence of chronic predation threat would show lasting increases in aggressive behaviors and aggressive-like brain gene expression patterns. We subjected small colonies to a precisely controlled chronic disturbance. We used a combination of approaches to agitate the bees,

some of which match stimuli experienced during a predator disturbance and others that artificially induce an agitated state. We used standard assays to measure aggressive behavior. For the brain gene expression analysis, we drew on prior transcriptomic analyses to develop a small set of aggression 'biomarker' genes.

Materials and methods

Colony construction

Behavioral experiments were conducted from 1 June to 31 July 2012. Aggression levels in honey bees vary strongly as a function of colony size and age demography (Giray *et al.* 2000; Hunt *et al.* 2003), and so we controlled these variables in our experiment by using small, single-cohort colonies of known population, constructed using bees of known age (initially 1-day-old bees; Huang & Robinson 1992; Schulz *et al.* 2002). In order to control for any other environmental conditions that may affect behavior, we established colonies in pairs, one pair at a time, arbitrarily designating one colony to be disturbed and the other as the control. Once constructed, we kept pairs together in similar microenvironments (Winston 1987) in one of the three apiaries within 8 km of each other in and around Urbana, IL, USA.

We also controlled for the effects of genotypic variation on aggression (Collins *et al.* 1982; Giray *et al.* 2000; Guzmán-Novoa *et al.* 2004; Hunt *et al.* 2003; Uribe-Rubio *et al.* 2008). Brood was obtained from typical source colonies managed according to standard methods and headed by naturally mated queens, representing a mix of European sub-species, primarily of the Italian strain of the Western honey bee, *A. mellifera ligustica*. We controlled for genotype by splitting brood obtained from a given source colony evenly across disturbed and control colonies within an experimental pair.

We collected honeycomb frames with capped brood from source colonies and housed them in an incubator at 33°C until adult emergence (1–10 days). Each day, we pooled newly emerged adults, counted them and assigned them to a colony. Each 1-day-old bee was marked on the thorax with Testors paint (Rockford, IL, USA) to ensure colony identity and age and to allow us to identify and remove foreign bees once colonies were established in the field. Because the number of newly emerged 1-day-old bees was variable from day to day, starting colony size also varied, but both the disturbed and control colonies within a pair started with the same number of bees. We added bees for up to three consecutive days or until we reached 4000 bees per colony. Marked bees were kept in the incubator in small containers and fed honey until we completed marking all bees belonging to both members of a pair. Hereafter, we refer to colony and/or bee age as the age of the oldest bees within the colony.

Once marking was complete, we established each experimental colony in a small beehive (5-frame BeeMax Reinforced Nuc Box; Betterbee Inc, Greenwich, NY, USA) with three or four honeycomb frames and an *ad libitum* food supply (see Appendix S1 for details; Schulz & Robinson 1999). Once all frames and bees were established in the hive box, we introduced a naturally mated queen to complete colony construction.

Disturbance method

One member of each pair of colonies was left completely undisturbed (control) while the other was chronically disturbed to simulate a social environment following a predation event. Because small colonies composed of young bees are only modestly responsive to defensive stimuli (Giray *et al.* 2000; Hunt *et al.* 2003; Kastberger *et al.* 2008), we used several techniques to induce a high level of arousal. First, we subjected 1-day-old bees to a single electric shock in order to artificially induce an aggressive response at an age when bees are largely unresponsive to other aggressive social cues, e.g. alarm pheromone. At the end of each day of bee marking and counting, we exposed the marked, 1-day-old bees from the disturbed colony to a brief electric shock (direct current, 9 V, 3 mA, BK Precision 1696 power source; Fotronic Corp, Melrose, MA, USA), a technique adapted from Uribe-Rubio *et al.* (2008). Bees were lifted by hand in groups of approximately 100–150 bees onto a 13 cm by 13 cm electrified grid with 2 mm wires spaced 3.5 mm apart. Bees received a shock when they made contact with two wires simultaneously. We held bees on the grid for 5 seconds. This procedure was performed in a separate room away from other bees in order to contain any pheromones that may have been emitted during the process. This electric shock clearly disturbed the bees because it caused them to extrude their stingers and to increase their rates of locomotion as they do when their colony is attacked. However, the treatment resulted in no appreciable mortality.

Once colonies were established in apiaries, we performed additional precise periodic disturbances to induce a chronically threatened environment. A disturbance event consisted of the following: we removed the lid to the colony and placed a cloth with 500 μl of isopentyl acetate (IPA; Sigma-Aldrich, St. Louis, MO, USA) inside the hive near the entrance. Isopentyl acetate is the major active compound in honey bee alarm pheromone (Boch *et al.* 1962). We then lifted one honeycomb frame to a height of approximately 6.5 cm above the bottom of the hive and dropped it back into the hive so that it landed in its original position. We did this procedure with each frame in the colony 10 times. In colonies with only three frames (see Appendix S1), we dropped one frame 20 times so that each disturbance resulted in 40 total frame drops. This procedure lasted about 2 min. We then closed the lid. Five min later, we reopened the hive to retrieve the cloth with IPA. This treatment resulted in behaviors resembling those observed in a colony following predation threat (see Appendix S1).

When colonies were 3 days old, we performed the disturbance three times, between 0830 and 0930 h, between 1430 and 1530 h and between 1700 and 1800 h. For days 4–8, we performed the disturbance twice a day, between 0830 and 0930 h and between 1430 and 1530 h. On days 7 and 8 of the experiment, we performed physical disturbances without adding alarm pheromone to the colonies to minimize the chances of habituation (Al-Sa'ad *et al.* 1985).

Behavioral measurements

Aggression assay

Unlike the disturbance method described above, the aggression assay invoked a consistently quantifiable behavioral response. The disturbance method and the aggression assay contain similar components, but they are not identical. This is because small, young colonies are fairly unresponsive to aggressive stimuli, and the aggression assay paradigm is not adequate to produce an appreciable aggressive response when colonies are very young (C. Rittschof and G. Robinson, personal observation).

The aggression assay was modified from previous studies, particularly Giray *et al.* (2000). We assayed aggression when bees were 9 days old, between 0700 and 0900 h in the morning, a time when most bees were in the colony and not actively foraging. Both members of a pair were assayed one after the other (in random order). We grasped a small piece of brown fabric (approximately 4 × 2 cm) in a pair of forceps and applied 25 μl of IPA diluted 1:10 with mineral oil (Sigma-Aldrich). We waved this patch slowly in front of the colony entrance. At the same time, we banged a brick on the roof of the colony 40 times, which took approximately 30 seconds. We then continued to wave the patch for an additional 3 min. Although the components of this assay were similar to those used in other studies (Breed *et al.* 1989; Giray *et al.* 2000), our assay was longer and likely involved relatively more stimulation per bee because we used small colonies (adult population of approximately 4000 bees compared with a typical colony of 40 000 bees), composed of young bees, which are relatively non-aggressive.

We counted the number of bees visible on and around the front of the hive every 30 seconds during the assay. We refer to these bees as soldier bees, because they flew out of the entrance in response to the defensive stimulus (Breed *et al.* 1990), but a few might have been patrolling the entrance as guards prior to the test. We took bee counts from still images from movies recorded on an iPhone 4-S (Apple Inc, Cupertino, CA, USA). The person operating the video

recorder stood at a distance of approximately 1 m from the colony during the assay. We counted all bees visible on the front of the hive, as well as any bees flying in the air within 10 cm of the hive. In addition to counting the number of bees behaving as soldiers, we counted the number of stingers embedded in the cloth at the end of the assay.

Foraging assay

Because aggressive behavior and foraging effort co-vary on both the individual and colony levels (Giray *et al.* 2000; Wray *et al.* 2011), we measured foraging activity to determine whether our treatment affected aggressive behavior alone, or a syndrome of associated behaviors. Young single-cohort colonies lack the older adult bees that typically make up the colony foraging force and as a result, young bees show precocious behavioral maturation and begin to forage as young as 4 or 5 days of age (Huang & Robinson 1992). We monitored foraging behavior twice a day (between 1030 and 1200 h and again between 1300 and 1500 h) for a total of 3.5 h per day. A description of the procedures used to identify foragers can be found in Appendix S1. In order to compare behavioral development across the disturbed and control colonies, we counted the number of unique individuals observed foraging when bees were 5, 6 and 7 days of age. We reported the cumulative number of unique foragers observed by day 7.

Measurement of colony size

After the aggression assay, we removed the queens from both the disturbed and control colonies. We then replaced the lid and waited approximately 5 min for bees to settle back into the hive. Following this, we opened the lid and quickly poured liquid N_2 into the top of the hive to flash-freeze all remaining bees in the colony. This method enabled us to precisely count the bees at the end of the experiment to get an accurate measure of colony size.

Brain gene expression analysis

The two goals of the gene expression analysis were (1) to identify genes that could serve as markers of socially induced changes in aggressive behavior, and (2) to use marker genes to determine whether the behavioral effects of our chronic disturbance treatment are reflected in the brain.

Genes selected for screening

We screened 12 genes for possible use as brain gene expression markers for aggression. We selected candidate genes that were strongly and consistently associated with aggression in previous studies (Alaux *et al.* 2009b; Hunt *et al.* 1998). Six candidate genes came from a list of seven genes that were differentially expressed in the brain in response to acute alarm pheromone exposure (Alaux *et al.* 2009b) and also found in independently derived aggression quantitative trait loci (Hunt *et al.* 1998). The other six genes were selected from a list of 32 genes that were consistently differentially expressed in the brain across three aggressive contexts: (1) bees exposed to alarm pheromone vs. control, (2) Africanized vs. European honey bees (the former are generally more aggressive), and (3) forager bees vs. nurse bees (the former are relatively more aggressive; Alaux *et al.* 2009a,2009b). Two of these six genes, *moody* and *cyp6g1/2*, also show an association with aggression in other species (Drnevich *et al.* 2004; Soma *et al.* 2008). The other four of this second set of six genes showed the highest fold changes in the alarm pheromone experiment as well as high average expression levels (Alaux *et al.* 2009b). The 12 candidate genes are listed in Table 1, using *Drosophila melanogaster* ortholog names if they exist; the gene with a 'GB' number has no known ortholog and thus is of unknown function.

Additional validation of selected genes

We screened the 12 candidate genes by comparing brain expression levels for soldier bees vs. Returning Foragers; these two groups are of similar age and stage of behavioral maturation but soldiers are more responsive to colony threats than foragers (Breed *et al.* 1990). For this validation, we collected bees from a typical colony that was not used in other experiments. This colony was average in size and headed by a naturally mated queen. We collected Returning Foragers ($N = 10$) and then exposed the same colony to alarm pheromone and collected soldiers ($N = 10$). Soldiers were held in a container and then flash-frozen 1 h after collection in order to allow for event-related transcriptional changes. We did not hold Returning Foragers for 1 h because the act of caging bees is known to induce alarm pheromone release, which may confound our results (Alaux *et al.* 2009b). Results for each gene were analyzed gene-by-gene using a two-tailed *t*-test. Of the 12 genes screened, four showed robust differences (Table 1), and thus were used as 'biomarkers' in the main experiment.

Bee collections

We did not perform collections from the 11 pairs of colonies assayed for aggressive behavior out of concern that the collections might affect behavioral outcomes. Instead, we built an additional pair of colonies specifically for sampling bees for brain gene expression analysis. This 12th pair of colonies was treated identically to the preceding 11 pairs except no foragers were marked. The aggression assay showed that the colonies used for gene expression analysis showed similar behavioral patterns to the other 11 pairs of colonies (Table S1).

We collected three behavioral groups of bees from both the disturbed and control colony: two groups of soldier bees and one group of Returning Foragers. Soldiers were collected within 5 min of the end of the aggression assay (day 9) from the front of the hive, and we avoided collecting soldiers that stung the cloth and lost their stingers during the assay. One group of soldiers was immediately flash-frozen in liquid N_2 following collection. This group provides a measure of basal soldier gene expression because we did not allow time for transcriptional changes to occur following the aggressive response (Alaux *et al.* 2009b; hereafter 'Soldiers Immediate'). We kept a second group of soldiers in a container for 1 h before freezing (Alaux *et al.* 2009b; hereafter 'Soldiers 1 h'), which did allow for gene expression changes related to the aggressive response to occur; these bees' expression levels probably reflect both the basal neurogenomic state associated with being a soldier and transcriptional changes associated with their aggressive response. We collected Returning Foragers (identified as described in Appendix S1) from each colony in the afternoon (1400 h) on the day prior to the aggression assay, when the colonies were 8 days old. Returning Foragers were immediately frozen in liquid N_2 at the time of collection. In addition to comparing Soldiers 1 h and Returning Foragers (as in Alaux *et al.* 2009b) to determine brain expression response to alarm cue, we also compared Soldiers Immediate and Soldiers 1 h groups as a more stringent assessment because both groups of soldiers were collected from the same starting pool of bees (see Appendix S1 for details about how we quantified gene expression levels).

Statistical analysis

We performed statistical analyses using JMP PRO 9.0.2 (SAS Institute, Cary, NC, USA). In all analyses, we accounted for colony pair to control for pair to pair variation in environmental conditions, colony size and genotypic make-up. For the aggression assay, we performed a repeated measures analysis of variance (ANOVA) to assess treatment differences in behavior. The response variable was the number of soldier bees divided by the final colony size. Treatment, time and their interaction were included as fixed factors, and colony pair (1–10) was included as a random factor. Only 10 pairs of colonies were included in this analysis because we lost the middle portion of the video footage for one colony during the assay, and so it and its partner colony were excluded.

In typical colonies, defensive response is correlated with colony size. We checked for this relationship in our experiment using the maximum proportion of the colony acting as soldiers (maximum soldiers observed/final colony size) as the dependent variable in a linear mixed model analysis (using Restricted maximum likelihood,

Table 1: Normalized brain expression values for candidate aggression marker genes

	Soldiers 1 h		Returning Foragers		Two-tailed *t*-test	
	Mean	SE	Mean	SE	*t*-ratio	*P*-value
Regulated by alarm pheromone and found in aggression QTL						
pros25	0.70	0.019	0.66	0.025	−1.55	0.14
l(1)g0232	2.03	0.15	2.73	0.45	1.48	0.16
ckIIβ	1.28	0.026	1.24	0.031	−0.84	0.41
hsc70-3	1.86	0.077	1.77	0.088	−0.74	0.47
med22	0.19	0.007	0.18	0.008	−0.69	0.50
psf2	0.51	0.021	0.51	0.022	0.20	0.84
Up- or downregulated across three aggressive contexts						
GB53860	**60.0**	**4.12**	**44.4**	**2.63**	**−3.19**	**0.005**
inos	**4.8**	**0.265**	**5.8**	**0.340**	**2.5**	**0.022**
chord	0.99	0.056	0.96	0.051	−0.34	0.73
drat	**11.6**	**0.73**	**9.3**	**0.330**	**−2.95**	**0.009**
moody	0.65	0.035	0.55	0.049	−1.51	0.15
cyp6g1/2	**0.73**	**0.053**	**0.53**	**0.015**	**−3.61**	**0.002**

Bold values are significant at $P < 0.05$. Genes were selected based on previous studies (Alaux *et al.* 2009b; Hunt *et al.* 1998). Genes 'Regulated by alarm pheromone and found in aggression QTL' were differentially expressed as a function of alarm pheromone exposure by Alaux *et al.* (2009b) and were found in independently derived aggression quantitative trait loci (Hunt *et al.* 1998). Genes 'Up- or downregulated across three aggressive contexts' were differentially expressed comparing bees exposed to alarm pheromone vs. control, old vs. young bees and bees originating from Africanized vs. European genetic lines. Following Alaux *et al.* (2009b), Soldiers 1 h bees were flash-frozen 1 h after exposure to alarm pheromone; Returning Foragers were collected prior to alarm pheromone stimulus and immediately flash-frozen to serve as a non-defensive control ($N = 10$ per group). Patterns of expression for significant genes are summarized in Table S4.

REML). Colony size, treatment and their interaction were fixed effects and colony pair was a random effect (all 22 colonies were included in the analysis).

To compare the number of stings across treatments, we calculated the proportion of bees stinging (number of stings/final colony size, square-root transformed for normalization), and performed a two-tailed *t*-test, blocking for colony pair (1–11). Finally, we used a two-tailed *t*-test to compare the total proportion of the colony observed foraging on or before day 7 (number of unique foragers/final colony size, square-root transformed for normalization). We again blocked for colony pair. The foraging analysis included data from 10 pairs of colonies only; we moved 1 pair of colonies from one apiary to another during the course of the experiment, and this pair was excluded because hive movement is known to affect foraging behavior (Seeley 1983).

For the four aggression biomarker genes (Table 1), we analyzed brain expression data using a two-way ANOVA, with behavioral group (Returning Foragers, Soldiers Immediate and Soldiers 1 h), treatment and their interaction as factors. To achieve normal distributions, we transformed the normalized expression data for *drat* and *cyp6g2* (log transformation) and *inos* (inverse transformation). Final sample sizes included in the two-way ANOVA are listed in Table S2. The two-way ANOVA showed no behavioral group by treatment interactions for any of the genes, and so we pooled disturbed and control data and used a one-way ANOVA (Table S3) followed by *post hoc* Student's *t*-tests to more closely examine behaviorally related differences in brain gene expression.

Results

Behavior

Effect of chronic disturbance

The disturbance treatment caused behavioral effects similar to a typical predator disturbance (see Appendix S1). However, although bees took off into the air during the disturbance, no bees stung the experimenters nor did they sting the cloth containing IPA, and the disturbance treatment did not cause appreciable bee mortality. Mortality rate from the start of the experiment (day 1 of marking) to the end did not significantly differ between disturbed and control ($t = -1.8$, $P = 0.10$, $N = 11$ pairs). Disturbed and control colonies also did not significantly differ in final colony size ($t = 1.7$, $P = 0.11$, $N = 11$ pairs).

Chronic disturbance had significant effects on aggression, assayed on day 9. Chronically disturbed colonies had fewer soldiers during the aggression assay compared with control colonies ($F_{1,9} = 46.6$, $P < 0.0001$, $N = 10$ pairs; Fig. 1). Bees from chronically disturbed colonies also stung significantly less relative to control colonies ($t = 3.4$, $P = 0.007$, $N = 11$ pairs). Finally, chronically disturbed colonies had fewer foragers, measured as the cumulative proportion of bees observed foraging by day 7 ($t = 2.7$, $P = 0.027$, $N = 10$ pairs; Fig. 2).

Parallels with typical colonies

Because our experimental colonies were small and composed of young bees, we assessed some behavioral characteristics to provide evidence that experimental colonies behaved in a way comparable to typical colonies. One common characteristic of typical defensive response is an increase in soldier activity over time during a defensive event (Collins *et al.* 1982). In keeping with this expectation, in our experiment, the number of soldiers we detected increased over time during the aggression assay regardless of treatment ($F_{5,45} = 6.71$, $P < 0.0001$, $N = 10$ pairs; Fig. 1).

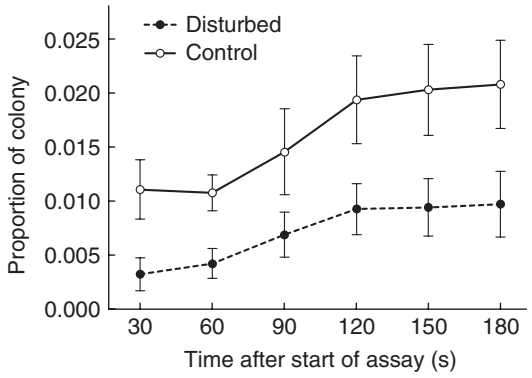

Figure 1: The proportion of a honey bee colony's adult population acting as soldiers over time during the aggression assay. Data points represent mean ± SE. The number of defensive bees increased over time ($F_{5,45} = 6.71$, $P < 0.0001$), and chronically disturbed colonies had fewer responding bees ($F_{1,9} = 46.6$, $P < 0.0001$). There is no significant interaction between treatment and time.

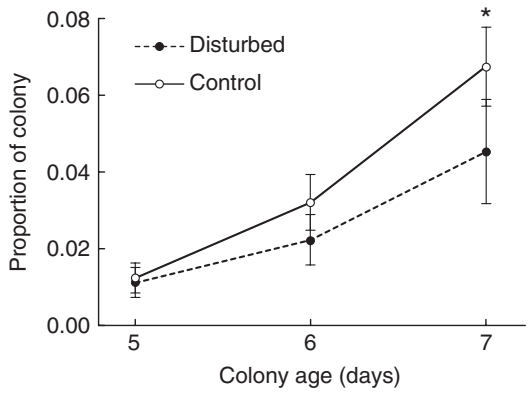

Figure 2: The proportion of a honey bee colony's adult population observed foraging. Counts represent unique foragers and are cumulative across days 5, 6 and 7 of colony age. By day 7, a significantly higher proportion of bees foraging in the control vs. chronically disturbed colonies was observed ($t = 2.7$, $P = 0.027$). *Significant difference at $P < 0.05$.

Another common pattern among typical colonies is a positive correlation between colony size and the number of bees acting as soldiers (Giray *et al.* 2000; Hunt *et al.* 2003), and we observed this correlation overall ($R^2 = 0.21$, $P = 0.033$, $N = 22$). An analysis of the effects of treatment, colony size and their interaction ($N = 11$ pairs) showed a significant effect of disturbance treatment ($t_{8.7} = -2.34$, $P = 0.045$) and a treatment by colony size interaction ($t_{11.4} = -2.2$, $P = 0.049$), but no main effect of colony size ($t_{13.7} = 0.98$, $P = 0.34$). Figure 3 suggests that, similar to typical colonies, undisturbed control colonies showed a positive relationship between the proportion of responsive bees and colony size, while disturbed colonies did not.

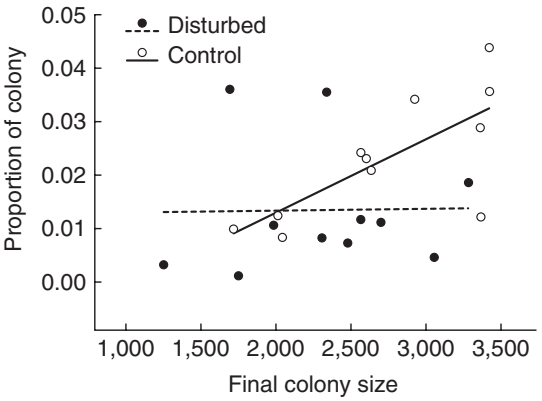

Figure 3: The maximum proportion of a honey bee colony's adult population observed acting as soldiers vs. colony size. Overall, there is a significant correlation between colony size and the percent of the colony acting as soldiers during the aggression assay ($N = 22$, $R^2 = 0.21$, $P < 0.033$). However, a linear mixed model analysis showed a significant treatment by colony size interaction ($t_{11.4} = -2.2$, $P = 0.049$) in addition to a main effect of treatment ($t_{8.7} = -2.34$, $P = 0.045$).

Brain gene expression

Aggression marker genes

The screening procedure resulted in identification of four aggression marker genes (Table 1) with robust aggression-related patterns of brain expression. All four of these genes were among the six candidates that were differentially expressed across three contexts of aggression described in the study of Alaux *et al.* (2009b). Three of the four genes have orthologs with known functions in *D. melanogaster* (Mcquilton *et al.* 2012). *cyp6g1/2* (which encodes a cytochrome P450 protein) is associated with aggression in *D. melanogaster* (Drnevich *et al.* 2004) and has been reported to be involved in the biological process oxidation–reduction (Gene Ontology, GO: 0055114). *drat* also encodes a protein associated with oxidation–reduction (GO: 0055114), but has additional experimental evidence for involvement in response to hypoxia (GO: 0001666; Azad *et al.* 2009) and cellular response to ethanol exposure (GO: 0071361; Chen *et al.* 2012). *inos* encodes myo-inositol-1-phosphate synthase and is associated with inositol biosynthesis (GO: 0006021) and phospholipid biosynthetic process (GO: 0008654). *GB53860* has been identified as a protein-coding gene in the honey bee genome (Honey Bee Genome Sequencing Consortium), but with no known function. It was nevertheless chosen as a biomarker because it showed the highest normalized expression levels and the second highest aggression-related fold change of all tested genes.

Experimental colony analysis

Two of the four aggression biomarker genes showed effects due to chronic disturbance ('treatment effects') (Table 2, Fig. 4). *drat* was downregulated in bees from the chronically disturbed colony relative to control, while *GB53860* was upregulated in bees from the chronically disturbed colony.

Table 2: Two-way ANOVA results for effects of chronic colony disturbance on brain gene expression

	Behavioral group		Treatment		Interaction	
	F-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value
drat	5.86	**0.005**	28.3	**0.0001**	1.7	0.19
GB53860	5.52	**0.006**	13.9	**0.0004**	0.65	0.52
inos	3.51	**0.036**	3.36	0.07	0.72	0.49
cyp6g1/2	1.34	0.27	0.05	0.82	0.10	0.91

Behavioral group: Returning Foragers, Soldiers Immediate and Soldiers 1 h. Bold numbers indicate significance at $P < 0.05$.

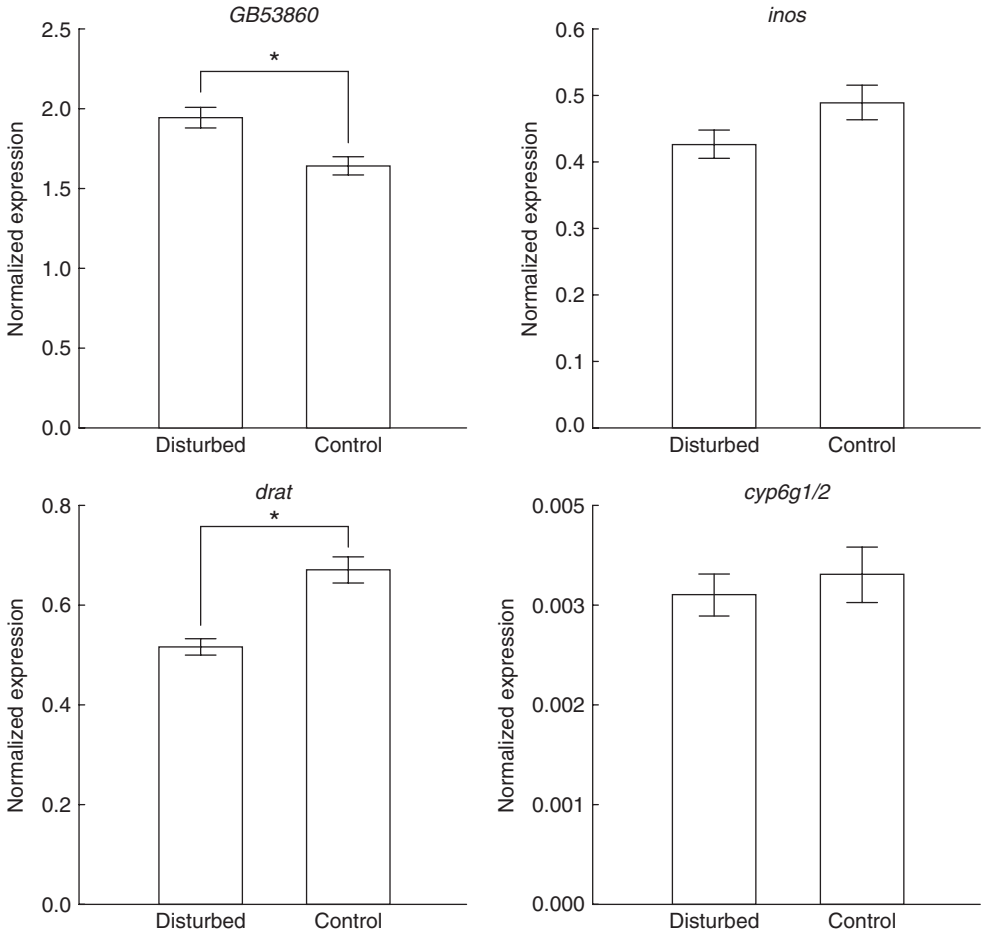

Figure 4: Effects of chronic colony disturbance on brain gene expression. Normalized, non-transformed gene expression values (mean ± SE), pooled across behavioral groups, for the four genes measured in the current study. The two-way ANOVA analysis showed significant treatment differences for *GB53860* and *drat* (Table 2). *Significant difference at $P < 0.05$.

In addition to the above treatment effects, we found differences in expression levels across behavioral groups (Returning Foragers, Soldiers Immediate and Soldiers 1 h) for three of the four genes (Table 2), with no behavioral group by treatment interactions. These genes were *drat*, *GB53860* and *inos*. However, only *drat* and *GB53860* showed a signature of the aggressive event, i.e. a change in expression level comparing Soldiers 1 h to Soldiers Immediate (Fig. 5). In both disturbed and control colonies, these genes were upregulated in the brain as a result of the aggression assay.

The Returning Foragers and Soldiers Immediate groups showed differences in brain expression for two genes (*drat* and *inos*), suggesting that soldiers and foragers have distinct gene expression patterns that are not a function of the response to alarm pheromone. *drat* was upregulated in the Soldiers Immediate group, while *inos* was downregulated. Finally, a comparison of Returning Foragers to Soldiers

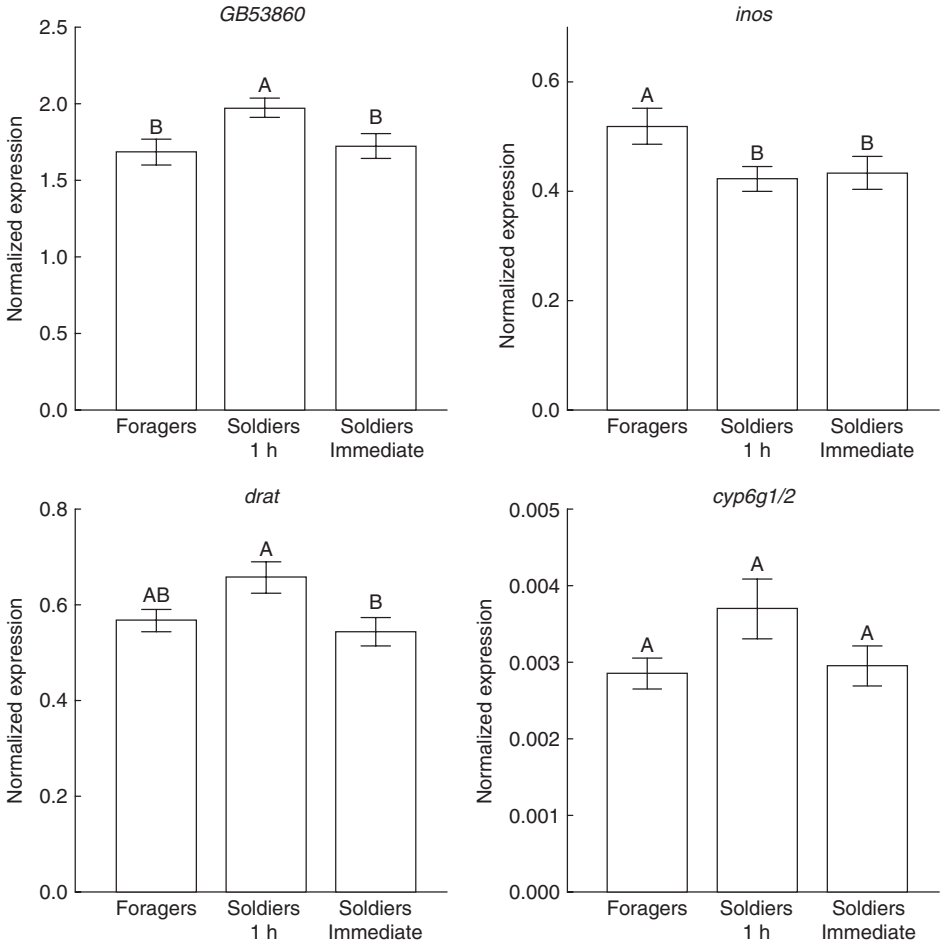

Figure 5: Behaviorally related differences in brain gene expression independent of chronic colony disturbance. Normalized, non-transformed gene expression values (mean ± SE), pooled across disturbed and control colonies, for the four genes measured in this study. Letters above each bar represent the results of a one-way ANOVA pooling across treatments (Table S3) followed by post-hoc student's *t*-tests. Groups with the same letters did not differ in the *post hoc* test.

1 h suggests that single-cohort colony bees exhibit similar patterns of aggression marker gene expression across behavioral groups compared with typical colonies (Table S4, Fig. 5). *GB53860* and *drat* were upregulated in Soldiers 1 h, while *inos* was down-regulated, relative to Returning Foragers.

Discussion

Our findings suggest that chronic disturbance, which modifies the social environment by repeatedly agitating bees within the beehive, has lasting effects on individual and colony aggression levels (Table 2, Fig. 1). Surprisingly, the effect of disturbance was decreased, rather than increased aggression.

Prior studies with honey bees manipulated social environment using cross-fostering techniques and found that adult European bees kept in Africanized colonies were more likely to respond aggressively to an acute disturbance compared with European bees kept in European colonies (Guzmán-Novoa & Page 1994; Guzmán-Novoa *et al.* 2004; Hunt *et al.* 2003). However, from these studies alone, it was difficult to assess whether the social environment had transient or lasting effects on aggressive behavior (Guzmán-Novoa & Page 1994; Guzmán-Novoa *et al.* 2004). Our method of disturbing an entire colony resulted in fewer soldiers responding to an acute stimulus, indicating that disturbance resulted in decreased colony aggression (Fig. 1). Furthermore, we assayed colonies 13–15 h after the final disturbance event, indicating that the effect of disturbance was fairly stable. In addition, treatment differences in aggression-related brain gene expression suggest that chronic exposure to a threatened social environment alters individual brain state (Table 2, Fig. 4).

The gene expression analysis showed that the behavioral changes associated with chronic disturbance are reflected in markers of aggression in the brain. However, the results

are complex (Tables 1,2, S3–S5, Figs. 4,5). *drat* and *GB53860* showed treatment effects, as well as an effect of brief exposure to alarm cues (Soldiers 1 h vs. Soldiers Immediate), supporting previous studies associating these genes with socially induced changes in aggression. *drat* is consistently positively correlated with aggression: in both Alaux *et al.* (2009b) and this study, it is upregulated in bees exposed to aggressive social cues relative to control. Furthermore, *drat* is upregulated in bees reared in more aggressive Africanized colonies, and downregulated in bees exposed to chronic disturbance. In contrast, *GB53860* was upregulated as a result of acute exposure to aggressive cues, but also upregulated in bees from the less aggressive disturbed colony relative to control. *inos* was differentially expressed comparing Soldier groups to Returning Foragers, but it showed no social effects either as a result of the aggression assay or chronic disturbance. *cyp6g1/2* showed no association with aggression in the experiment, although it was associated with aggression in a typical colony.

The inconsistency of *GB53860* and the absence of social effects for *inos* and *cyp6g1/2* are surprising because all four tested genes were strongly associated with aggression in previous experiments (Alaux *et al.* 2009b). Furthermore, for each gene, we validated a correlation with aggression in a typical colony, and expression patterns in typical and experimental colonies are generally similar (Table S4). Perhaps only some aggression-related genes are consistent indicators of socially induced aggression (e.g. *drat* appears to be the most consistent indicator of the four). Alternatively, the variable gene expression patterns may reflect the surprising complexity of the treatment effects in our experiment. For example, the extreme nature of our chronic disturbance paradigm imposed a number of stressors on the bees, and some of the brain gene expression patterns may reflect a more generalized response to stressors in addition to a change in aggression.

Overall, our gene expression results emphasize an association between changes in brain metabolic gene activity and aggressive behavior. The two metabolic biological processes significantly associated with aggression in previous honey bee studies, oxidation–reduction and inositol biosynthesis (Alaux *et al.* 2009b), are represented in our marker genes (*drat* and *inos*, respectively). The *drat* protein product is involved in ethanol-induced cellular apoptosis (Chen *et al.* 2012), a possible mechanistic connection between ethanol sensitivity and aggression in bees (Ammons & Hunt 2008). *inos* is the rate-limiting enzyme in inositol biosynthesis (Park *et al.* 2000), a pathway associated with both aggression and depression in mammals (Coupland *et al.* 2005; Taha *et al.* 2009). Differential expression of *drat* and *inos* may be related to other known aggression-induced metabolic adaptations in bees, e.g. decreased activity of enzymes involved in oxidative phosphorylation (Alaux *et al.* 2009b).

Our study is the first to measure soldier basal brain gene expression levels (Soldiers Immediate), rather than measuring soldiers only after they have reacted to a disturbance (Soldiers 1 h). Foragers and Soldiers Immediate groups showed differences in brain gene expression (Fig. 5), supporting the idea that forager and soldier bees are distinct behavioral groups despite similarities in age. This result supports previous genetic and morphological evidence distinguishing soldiers and foragers (Breed *et al.* 1990), and is consistent with previous studies showing that different behavioral groups of bees exhibit neuromolecular predispositions (Brockmann *et al.* 2009).

Surprisingly, long-term chronic disturbance resulted in decreased aggression, while short-term exposure to aggressive cues tends to have the opposite effect (Alaux & Robinson 2007; Couvillon *et al.* 2008). One explanation for our results is that disturbed colonies habituated to aggressive stimuli, resulting in decreased aggression. Bees habituate to alarm pheromone if it is repeatedly applied (Alaux & Robinson 2007; Al-Sa'ad *et al.* 1985; Free 1988), and no study has assessed whether bees habituate to physical disturbance. However, approximately 13–15 h lapsed between the final disturbance and the aggression assay. Prior studies suggest that this amount of time is adequate to allow colonies to recover from habituation (Al-Sa'ad *et al.* 1985; Free 1988). Also, our disturbance treatment resulted in decreased foraging behavior, while studies of alarm pheromone habituation have documented normal colony foraging activity (Al-Sa'ad *et al.* 1985). It appears that our treatment resulted in a syndrome of behavioral effects that cannot be adequately explained by habituation to aggressive stimuli: decreased foraging activity and decreased aggressive response. However, we cannot rule out that our results reflect other types of habituation, e.g. habituation to chronic stressors.

To our knowledge, this is the first study to experimentally address the effects of chronic (simulated) predator disturbance on aggression levels. We imposed very high levels of stressors to small colonies of young bees, while previous studies used minor disturbances on large, typical colonies; these variables may alter colony defensive strategy. Low levels of predation can make a colony more defensive (Couvillon *et al.* 2008; Winston 1987), but with increased intensity, the colony may change its response, e.g. abandoning the nest in cases of extreme threat (Seeley & Seeley 1982; Spiewok *et al.* 2006). Nest abandonment is a risky strategy, rare in European honey bees (Hepburn *et al.* 1999). It is possible that, instead, disturbed colonies responded to extreme threat by decreasing outside activities such as foraging and nest defense, reminiscent of a fear response in vertebrates (Johansen *et al.* 2011). In our experiment, the role of fear is difficult to evaluate because little is known about the underlying mechanisms in honey bees. The small size of our experimental colonies may have also influenced colony defensive strategy, which is correlated with colony size in other social insects, particularly ants (Holldobler & Lumsden 1980; Holldobler & Wilson 1994). Undisturbed control colonies, although also small, showed behavioral patterns resembling typical large colonies, which raises the possibility that a size by disturbance interaction is driving the observed behavioral changes.

Another possibility is that young bees may be particularly prone to respond to stressors, including predator threat, with decreased activity. Juvenile hormone and biogenic amine levels influence aggression and foraging behaviors (Bateson *et al.* 2011; Robinson 1987; Sullivan *et al.* 2000). Titers of these compounds are affected by stress, and in some cases the effects are age-dependent (Lin *et al.* 2004). Furthermore,

there are performance differences comparing precocious (i.e. young) and normal-aged foragers (Ben-Shahar & Robinson 2001; Vance *et al.* 2009). Future studies could address how disturbance level, colony size and bee age affect the nature of the response to chronic disturbance.

In other social insects, aggression and foraging activity are negatively correlated (Passera *et al.* 1996), resulting in a clear cost to a highly aggressive strategy. Although similar trade-offs sometimes occur for honey bees (Giray *et al.* 2000; Rivera-Marchand *et al.* 2008), studies of colonies with normal age demographics show that aggression and foraging activity are consistently positively correlated (Winston & Katz 1982; Wray *et al.* 2011). Because our disturbance seems to have shifted the colony-level strategy, our experimental method may be a useful means of studying the mechanisms leading to colony 'personality' differences and the relative stability of these differences (Wray *et al.* 2011).

It is unclear whether the colony response to disturbance we measured is specific to the context of predation. Future studies should measure changes in aggression, foraging behavior and brain gene expression with the application of stressors that are not associated with predation. The surprising results presented here underscore the need to develop a better understanding of the relationship between social environment, colony behavioral response and individual brain states.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Details of behavioral and molecular methods.

Table S1: Similarities in aggressive behavior between the 11 pairs of colonies used for behavioral analysis and the 1 pair of colonies used for brain gene expression analysis.

Table S2: Sample sizes for disturbance experiment gene expression analysis (Table 2).

Table S3: The results of the *post hoc* one-way ANOVA to determine the effects of caste on gene expression.

Table S4: Patterns of brain expression and inferred Gene Ontology biological processes for four marker genes assayed in both the typical colony and the disturbance experiment colonies.

Table S5: For genes showing an effect of the chronically disturbed social environment, known relationships between brain gene expression and socially induced changes in aggression across short- and long-term time scales.