Honey bee aggression supports a link between gene regulation and behavioral evolution

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A prominent theory states that animal phenotypes arise by evolutionary changes in gene regulation, but the extent to which this theory holds true for behavioral evolution is not known. Because "nature and nurture" are now understood to involve hereditary and environmental influences on gene expression, we studied whether environmental influences on a behavioral phenotype, i.e., aggression, could have evolved into inherited differences via changes in gene expression. Here, with microarray analysis of honey bees, we show that aggression-related genes with inherited patterns of brain expression are also environmentally regulated. There were expression differences in the brain for hundreds of genes between the highly aggressive Africanized honey bee compared with European honey bee (EHB) subspecies. Similar results were obtained for EHB in response to exposure to alarm pheromone (which provokes aggression) and when comparing old and young bees (aggressive tendencies increase with age). There was significant overlap of the gene lists generated from these three microarray experiments. Moreover, there was statistical enrichment of several of the same *cis* regulatory motifs in promoters of genes on all three gene lists. Aggression shows a remarkably robust brain molecular signature regardless of whether it occurs because of inherited, age-related, or environmental (social) factors. It appears that one element in the evolution of different degrees of aggressive behavior in honey bees involved changes in regulation of genes that mediate the response to alarm pheromone.

Changes in gene regulation are believed to underlie the evolution of novel animal phenotypes (1–4). Emerging from studies of animal development, evidence for this theory consists largely of differences in temporal or spatial patterns of gene expression related to morphological evolution (see ref. 5 for a review). The extent to which this theory holds true for the evolution of other complex traits such as behavior is largely unknown (6). However, the underlying process is consistent with the idea that phenotypic evolution proceeds by Waddington's "genetic assimilation" of plastic responses to the environment (7, 8), which should hold widely true for behavior.

We explored this theory for behavior by taking advantage of a new perspective on the effects of "nature and nurture." With the advent of routine transcriptomic profiling, it is now possible to study nature and nurture in terms of hereditary and environmental effects on brain gene expression, respectively (9). We used this perspective to determine whether environmental influences on a behavioral phenotype could have evolved into inherited differences via changes in gene regulation. One indication of this would be hereditary and environmental influences on the expression of common genes. For that purpose, we studied whether changes in gene regulation could be involved in the evolution of differences in aggressive behavior in the honey bee (*Apis mellifera*).

We used honey bee aggression because extensive prior analysis has shown that it is a rich and intricate behavioral system complete with well-defined environmental, maturational, and inherited components (10, 11). All honey bee colonies respond aggressively when their colony is disturbed or attacked, but there is striking variation in the intensity of their response. In docile colonies only a few bees may respond, whereas in more aggressive colonies, the response may involve hundreds or even thousands of stinging individuals. Colony defense begins when "guard" bees detect a disturbance at the hive entrance and release alarm pheromone, which alerts the entire colony. Older bees (who mostly forage for nectar and pollen) are more likely to respond aggressively than younger bees, but a subset of the colony's older bees, "soldiers," are the first to seek out and attack an intruder. The most prominent example of inherited differences in honey bee aggression is the extremely aggressive disposition of Africanized honey bees (AHB) compared with European honey bees (EHB). AHB have spread through most of the New World after the introduction in 1957 of the African subspecies, *A. m. scutellata* (12), causing deaths of humans and animals in some parts of their newly inhabited range due to massive stinging responses. AHB derive from hybridization between *A. m. scutellata* and EHB (predominantly *A. m. ligustica* in the New World). AHB mostly have *A. m. scutellata*-like behavioral traits, especially a highly aggressive colony defense (13).

Aggression is a complex, polygenic trait (14). An appropriate test of the idea that there is a common molecular basis for differences in aggression due to environmental, maturational, or inherited factors thus requires analysis of multiple genes in different pathways. We therefore obtained transcriptional profiles with microarray analysis.

Results and Discussion

Hereditary Influences. There were hundreds of differences in brain gene expression between three groups of co- and cross-fostered AHB and EHB: guards, soldiers and foragers, all under typical field conditions (Table 1). The number of genes differentially expressed between AHB and EHB increased with increased involvement in colony defense (foragers < guards < soldiers), suggesting that some of these genes are involved in the regulation of aggressive behavior even though AHB and EHB differ in several behavioral traits (10, 12). Several genes differentially expressed in the brains of AHB and EHB ("AHB list") are implicated in the regulation of aggression in

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Table 1. Aggression-related brain gene regulation in honey bees as a function of heredity, alarm pheromone exposure, and age

Experiment	No. of genes	Raw *P* value
AHB/EHB Guard		
Individual genotype (I)	249	<0.0015
Colony genotype (C)	494	<0.0025
I × C	64	<0.0005
AHB/EHB Soldier		
Individual genotype (I)	538	<0.0025
Colony genotype (C)	830	<0.005
I × C	114	<0.0055
AHB/EHB Forager		
Individual genotype (I)	58	<0.00025
Colony genotype (C)	346	<0.0015
I × C	190	<0.001
Alarm pheromone	437	<0.0025
Old/Young	1396	<0.0055

Number of genes differentially expressed in each experiment. A false discovery rate (FDR) adjusted *P* value <0.05 was used as the threshold to determine statistical significance. I, differences due to individual genotype (AHB/EHB); C, differences due to colony genotype. Gene lists are in Dataset S1. Results based on individual brain gene expression profiles for 230 bees, involving a total of 332 microarrays (178, 64, and 90 for AHB/EHB, alarm pheromone and young/old experiments, respectively). For AHB/EHB, $n = 5$ bees per behavioral group per colony, except in one AHB and one EHB colony where 4 soldiers were analyzed, $n = 2$ colonies each of AHB and EHB; for alarm pheromone, $n = 10$ bees per group per colony, $n = 2$ colonies; for young/old bees, $n = 12$ bees per group per colony, $n = 3$ colonies.

other organisms, e.g., *NMDA receptor 2* and *metabotropic glutamate receptor B* (15).

Bees were cross-fostered to explore the effects of both individual worker and colony genotype on brain gene expression. Principal component analysis (PCA) revealed clear effects of both (Fig. 1). Differences in individual genotype (AHB or EHB) accounted for ≈30% of the variation in brain gene expression (PC2), whereas differences in host colony genotype accounted for ≈25% (PC3). The colony effects agree with previous findings showing that EHB reared in AHB colonies are more aggressive than when reared in EHB colonies, whereas AHB reared in EHB become less aggressive (16). These results also emphasize the close relationship between brain gene expression and aggression. Similar findings of genotype–environment interactions (17) results reflect an emerging appreciation of the potent influences of social environment on both brain gene expression and naturally occurring behavior (9).

Alarm Pheromone Influences. Prior research has shown that the alarm pheromone isopentyl acetate induces both an instantaneous aggressive response and a longer-term sensitization, which is associated with an up-regulation of the immediate early gene and transcription factor *c-Jun* in the antennal lobes (18). Consistent with these results, exposing EHB to alarm pheromone at the hive entrance for 1 min affected brain expression of hundreds of genes, measured 1 h later (Table 1). Among the genes significantly up-regulated were several involved in biogenic amine signaling (*Dopa decarboxylase*, *Tyramine receptor*, and *Octopamine receptor beta-2*), which have been implicated in the regulation of aggression in both vertebrates (19) and invertebrates (20, 21). *c-Jun* was also up-regulated again [false discovery rate (FDR) adjusted *P* value = 0.1]. These results demonstrate strong effects of a very brief environmental stimulus on brain gene expression, which might be related to behavioral sensitization.

Common Brain Transcriptional Networks Associated with Inherited and Environmentally Induced Differences in Aggression. Aggression shows a robust brain molecular signature regardless of whether it occurs because of inherited or environmental factors. Three results demonstrate similarities in the genes differentially regulated between AHB and EHB (AHB list) and in response to alarm pheromone ("alarm pheromone list"). First, ≈5–10% of the genes on each AHB list (guard, soldier, and forager) were also regulated by alarm pheromone, ≈2–3× higher than predicted by chance ($P < 0.01$, hypergeometric test; See Table 1, Table 2, and Table S1). Second, AHB and alarm pheromone brain gene expression profiles were significantly positively correlated (guard: $r = 0.14$, $P = 0.004$; soldier: $r = 0.23$, $P < 0.001$; forager: $r = 0.16$, $P < 0.001$; Table S2). Alarm pheromone thus causes AHB-like changes in brain gene expression in EHB. Third, seven of the genes on the alarm pheromone list are located within previously identified quantitative trait loci for AHB aggression, as are 12 from the AHB list (21) (Table S3). Among them, *14-3-3ε*, a gene up-regulated in the brains of human suicide victims (22), was up-regulated in AHB soldier brains in this study and also in a relatively aggressive EHB strain (21). These and other genes that appear on one or more of our gene lists are good candidates for effectors of honey bee aggression. Their number and diversity underscore the complexity and polygenic nature of this behavioral trait.

We also compared the AHB and alarm pheromone lists with a previously published list of genes differentially expressed in the brain between young and old EHB (23) (Table 1). Aggressive tendencies increase with age in honey bees, because old individuals are more easily aroused by alarm pheromone (24). We again detected significant overlap of gene lists; 3–7× higher than was predicted by chance for AHB, alarm pheromone, and genes that were differentially expressed in old and young bees (Table 3). Among the genes regulated in all three contexts were *moody* and *Cyp6Q1* (Table S4), which have been implicated in aggression in rodents (25) (the mammalian ortholog of *moody* is the *Melatonin receptor 1A*, whose ligand increases aggressiveness) and in reproductive competition in *Drosophila* (26), respectively. There were also significant positive correlations among the AHB, alarm pheromone, and old bee brain gene expression profiles, with the strongest results for AHB soldiers (Table S2).

Bioinformatic analysis of *cis*-regulatory motifs provided addi-

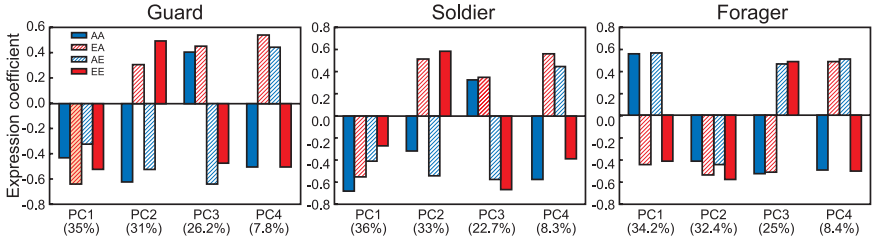

Fig. 1. PCA reveals effects of individual and colony genotype on aggression-related brain gene expression. Co- and cross-fostering produced the following four groups of soldiers, guards, and foragers: AA, AHB reared in AHB colony; EA, EHB in AHB colony; AE, AHB in EHB colony; and EE, EHB in EHB colony. Groups with similar expression coefficients are more similar to each other in terms of brain gene expression. The first pattern (PC1) reflects variation in brain gene expression that is similar across the four groups; PC2 is associated with differences in individual (AHB or EHB) genotype; PC3, with differences in colony genotype; and PC4, with differences in cross-fostered vs. non-cross-fostered bees.

Table 2. Aggression-related brain gene regulation in honey bees: Number of genes overlapping between gene sets and regulated in the same direction

	Old bee				AHB guard				AHB soldier				AHB forager			
Experiment	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value
Alarm pheromone	55.3	139	2.5	<0.0001	10	14	1.4	0.13	21.8	41	1.9	<0.0001	2.3	4	1.7	0.16
Old bee					28.8	27	0.9	0.41	63.7	105	1.6	<0.0001	6.8	14	2.1	0.006
AHB guard (I)									11.6	75	6.5	<0.0001	1.2	24	19.5	<0.0001
AHB soldier (I)													2.7	29	10.7	<0.0001
Alarm pheromone	55.3	139	2.5	<0.0001	19.8	18	0.9	0.4	33.6	47	1.4	0.011	13.9	19	1.4	0.1
Old bee					57.1	70	1.2	0.04	98.3	162	1.5	<0.0001	40.3	57	1.4	0.004
AHB guard (C)									35.4	151	4.3	<0.0001	14.6	87	6.0	<0.0001
AHB soldier (C)													24.9	97	3.9	<0.0001

Data are from the following: Experiment 1, AHB vs. EHB (guards, soldiers, and foragers); Experiment 2, alarm pheromone induced; and Experiment 3, old vs. young bees. Expected, the number of genes expected to overlap in all three experiments by chance alone; *RF*, representation factor; I, individual genotype; C, colony genotype. The statistical significance of the overlap between two lists was determined using an exact hypergeometric test and an extended version of the hypergeometric test for the overlap between three lists (see *SI Text*).

tional support for the notion that hereditary and environmental influences on aggression share a common molecular basis. Several motifs were found in the promoter regions of up-regulated genes on all three gene lists (AHB, alarm pheromone, and old bee; Table 4). In addition, the extent to which the three gene lists share the same motif association(s) is statistically significant ($P < 0.001$, see *SI Text*, *Extreme Value Distribution*). The motifs included *Deaf1*, which targets a transcription factor associated with biogenic amine signaling (27) that has been implicated in aggression in vertebrates (19) and invertebrates (20, 21), and *Trl* (GAGA-binding factor), a motif previously found to be associated with differences in brain gene expression between young and old bees (28). Remarkably, these same results occurred even when all genes common to the three gene lists were removed (Table 4). These findings suggest that genes associated with hereditary and environmental influences on aggression share a common brain transcriptional network.

Functional Insights for Genes Associated with Inherited and Environmentally Induced Differences in Aggression. Gene Ontology (GO) analysis was used to explore what some of the functional components of a brain transcriptional network for aggression might be. The number of common GO molecular process and biological function categories enriched for genes on all three gene lists (AHB, alarm pheromone, and old bee) was significantly higher than predicted by chance ($P < 0.05$) for AHB soldiers and guards, but not for foragers (Table 3). These included "response to stimulus" and "visual perception," which makes sense because bees that are aroused by alarm pheromone visually search for intruders in the vicinity of the hive, and perception of movement stimulates stinging (10, 29) (Fig. 2). *Cyp6a20* has been reported to be involved in both hereditary and environmental regulation of aggression in *Drosophila melanogaster* (30); its expression in olfactory sensory organs also suggests a function in response to aggression-related stimuli.

The most striking functional component of the brain transcriptional network for aggression suggested by GO analysis relates to brain metabolism. Genes involved in metabolism-related GO categories were significantly overrepresented on the AHB soldier, alarm pheromone, and old bee gene lists, all down-regulated. Down-regulation of brain metabolism genes, previously reported for old bees with a different microarray (31), was confirmed by enzyme assays performed on bee brain mitochondrial preparations (Fig. 3). Aggressive bees had lower brain mitochondrial activity in specific assays for Complex I, IV, and V of the oxidative phosphorylation pathway, which were the complexes represented most prominently on the gene lists (Table S5). Because oxidative phosphorylation is a major pathway in brain metabolism, mRNA abundance and enzyme activity in this pathway likely provide a good indication of brain metabolic activity. These whole-brain results may mask increased brain metabolism in some brain regions, but because the integration of information from multiple sensory modalities required for colony defense involves some of the largest

Table 3. Aggression-related brain gene regulation in honey bees: Number of genes and GO categories overlapping in all three experiments

	Genes				GO categories			
Genotype	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value
Indivdual								
Alarm pheromone × old bee × AHB guard	1.3	4	3.1	0.04	0.4	3	8.2	0.006
Alarm pheromone × old bee × AHB soldier	2.8	13	4.6	<0.0001	0.6	8	13.4	<0.0001
Alarm pheromone × old bee × AHB forager	0.3	2	6.7	0.036	0.2	0	0	1
Colony								
Alarm pheromone × old bee × AHB guard	2.5	7	2.7	0.015	0.6	3	4.6	0.023
Alarm pheromone × old bee × AHB soldier	4.3	19	4.4	<0.0001	0.9	16	17	<0.0001
Alarm pheromone × old bee × AHB forager	1.8	11	6.1	<0.0001	0.6	6	9.9	<0.0001

Data are from the following: Experiment 1, AHB vs. EHB (guards, soldiers, and foragers); Experiment 2, alarm pheromone induced; and Experiment 3, old vs. young bees. Expected, the number of genes expected to overlap in all three experiments by chance alone; *RF*, representation factor. The statistical significance of the overlap between two lists was determined using an exact hypergeometric test and an extended version of the hypergeometric test for the overlap between three lists (see *SI Text*).

Table 4. *cis*-regulatory motifs (and corresponding transcription factors) consistently associated with up-regulated gene sets from all three experiments in Table 1

Motif	Transcription factor	Combined *P* value (π_c)	AHB soldier *P* value	Alarm pheromone *P* value	Old bee *P* value
All differentially expressed genes					
CACGCG	*Hairy* (Dmel)	1.35E-05	0.00012	0.00016	0.007
Trl	*Trl* (Dmel)	4.49E-05	0.00058	0.004	0.0088
WHWWWWWW	unknown	8.69E-05	0.00035	0.0063	0.0119
V_AHR_Q5	*AHR* (Hs)	0.00015	0.00035	0.0138	0.011
V_ETF_Q6	*ETF* (Hs)	0.00035	0.0066	0.0037	0.027
Deaf1	*Deaf1* (Dmel)	0.00048	0.0149	0.028	0.00067
MGAAD	*Hsf* (Dmel)	0.00074	0.0107	9.21E-05	0.043
As above, minus those common to two or more gene lists					
CACGCG	*Hairy* (Dmel)	0.00043	0.00025	0.0085	0.032
MGAAD	*Hsf* (Dmel)	0.00058	0.0099	0.00027	0.038
V_CDPCR1_01	*CDP* (Hs)	0.00062	0.038	0.0085	0.0028
Ap	*ap* (Dmel)	0.0009	0.0019	0.0073	0.05
Trl	*Trl* (Dmel)	0.00097	0.0017	0.04	0.02
Klf4	*Klf4* (Hs)	0.0012	0.042	4.5E-05	0.026
CACNNG	unknown	0.0017	0.0064	0.045	0.031
Deaf1	*Deaf1* (Dmel)	0.0018	0.0098	0.072	0.00055

Shown are the *P* values of association for each experiment (Fisher exact test, see *SI Text*). The significance of each motif association across all experiments is shown in Column 3 (π_c), based on the combined measure P_c from Columns 4–6. The corresponding empirical *P* value $\pi_c(P_c^{(o)})$ and its FDR for multiple hypothesis correction was calculated (see *SI Text*). Only motifs with FDR <0.001 are reported. The bottom half of the table shows eight most-significant motifs discovered from the same procedure if the gene sets were purged of any genes that belonged to two or more gene sets. Only soldiers were analyzed because their brain gene expression profiles were most similar to those of alarm-pheromone-exposed and old bees. Transcription factor motifs from: Dmel, *Drosophila melanogaster*; Hs, *Homo sapiens*.

regions in the bee brain (mushroom bodies and optic lobes), the results likely accurately reflect the global neurogenomic state of the aroused bee brain.

We did not expect to find an association between increased behavioral arousal and decreased brain metabolism. Behavioral arousal is associated with increased whole body metabolic activity in honey bees (32).

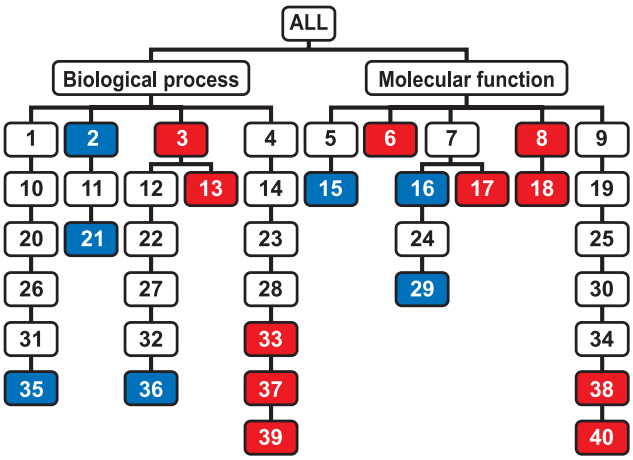

Fig. 2. GO functional analysis of genes associated with aggression: GO biological process and molecular function categories that were significantly enriched among the genes associated with aggression in all three experiments, i.e., as a function of heredity, alarm pheromone, and age (Table 4). Diagram represents GO categories hierarchically from top to bottom. Each box represents a GO category. Blue, up-regulation; red, down-regulation. Significantly enriched categories: (2) response to stimulus, (3) metabolic process, (6) electron carrier activity, (8) structural molecule activity, (13) oxidation reduction, (15) protein binding, (16) isomerase activity, (17) oxidoreductase activity, (18) structural constituent of ribosome, (21) detection of external stimulus, (29) inositol-3-phosphate synthase activity, (33) regulation of S phase, (35) visual perception, (36) inositol biosynthetic process, (37) regulation of S phase of mitotic cell cycle, (38) monovalent inorganic cation transmembrane transporter activity, (39) positive regulation of S phase of mitotic cell cycle, and (40) hydrogen ion transmembrane transporter activity. Other GO terms given in *SI Text*.

The association was all the more surprising considering that the alarm pheromone experiment demonstrated a causal relationship: Exposure to alarm pheromone increases behavioral arousal (18) and we showed that it decreased brain metabolism. Alarm pheromone exposure also is known to increase metabolic activity in honey bees (33) and higher cytochrome *c* oxidase activity is associated with increased aggressive behavior in rodents and lizards (34). However, a correlation between reduced metabolism and aggression has been reported for some regions of the human brain (35).

We speculate that decreased brain metabolism in aroused bees, if reflective of reduced background neural activity, could function as a "contrast enhancement" mechanism to enhance the bees' ability to locate or respond to a threat. Decreased brain metabolism reflects decreased neural activity in rats (36) and monkeys (37), and decreased neuronal activity in the human visual cortex has been reported to lead to improved visual representations of salient shapes (38). Another possibility is that our findings relate to some sort of energy tradeoff between the brain and the rest of the body. This notion is based on the (untested) assumption that, as in vertebrates (39), the insect brain consumes a disproportionately large share of the body's total glucose utilization. According to this idea, arousal-related increases in metabolic rate require a decrease in brain metabolism, at least for a "fight or flight" type of innate behavior. This tradeoff might not exist for a behavior that must be learned; learning is associated with increased brain metabolism (40–42). The relationship between brain metabolism and behavioral performance deserves more study.

Conclusion

Previous research has shown that there are striking differences in aggression between honey bee colonies, and our research has shown that aggression-related brain gene expression in honey bees is subject to strong inherited and environmental influences. AHB and EHB differed in the expression of hundreds of genes, with the strongest differences seen in those bees most-strongly engaged in colony defense. Environmental effects were seen both in terms of effects of alarm pheromone and colony environment on brain gene expression. Based on these findings we find support for the idea that changes in gene regulation underlie the evolution of behavioral diversity.

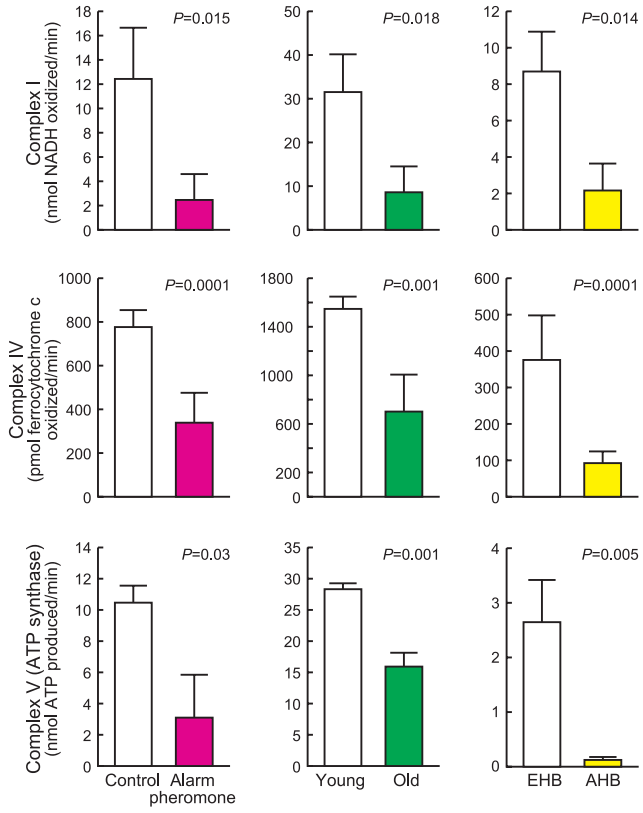

Fig. 3. Aggression-related decrease in brain metabolism as a function of heredity, alarm pheromone exposure, and age. Results of assays of enzyme activity for Complex I (NADH dehydrogenase), IV (cytochrome *c* oxidase), and V (ATP synthase) performed on mitochondrial preparations from bee brains. $n = 4$ biological replicates for each of the six groups labeled on the *x* axes of the three graphs; two pools of five brains per colony per behavioral group from two colonies. $n = 3$ technical replicates per sample. Statistical analysis: 1-way ANOVA with Tukey HSD posthoc. EHB, EHB bees in EHB colonies; AHB, AHB bees in AHB colonies. See *SI Text* for methods. Means ± SD are shown.

We suggest that environmental influences on aggression, i.e., responsiveness to alarm pheromone, could have evolved into the inherited differences in aggression exhibited by AHB and EHB—nurture begets nature. Evolutionary changes in brain gene expression may have resulted in an increase in arousal for AHB, a decrease in arousal for EHB, or both. Alarm pheromone has two behavioral effects: A rapid response, quicker than any transcriptionally based mechanism could possibly generate, and a slower, more long-term, sensitization (18). Given known differences in aggressive behavior between AHB and EHB (10, 11), our proposed scenario would have to involve molecular components of alarm pheromone's slower effects that then result in heritable changes in both the quick and slow responses.

Relating changes in the regulation of gene expression to morphological trait evolution usually involves targeted manipulation of single genes (1, 5). Because the regulation of complex behavioral phenotypes involves many genes and pathways, the transcriptomic and informatic approaches presented here provide an accessible entrée for exploring the relationship between gene regulation and behavioral evolution in other species as well.

Materials and Methods

AHB Experiment. This experiment was performed near Ixtapan de la Sal, Mexico (19° N, 99° W), which has been part of the range of the AHB for 20 years. Bees (n ≈1,000–1,300), paint-marked for genotype upon adult emergence, were co- and cross-fostered and placed in two EHB and two AHB colonies, each composed of ≈5,000 bees with typical age structures. Foragers, guards, and soldiers were identified according to established methods. Briefly, guards were identified as bees at the hive entrance either patrolling or standing with an alert posture (43). Foragers were identified as bees returning to the hive with a pollen load. Soldiers were collected in a specially designed trap after waving a leather patch with 10 μL isopentyl acetate over the top of an opened hive at a rate of 1 circuit/s for 10 s (44). Bees were collected into liquid nitrogen upon identification in the field and transferred to a −80 °C freezer. Brain dissection was as described in ref. 45. Some of our results (up-regulation of the visual perception GO category) may represent the contribution of small amounts of peripheral visual tissue adhering to the brains after dissection, but all samples were handled identically and there is evidence for brain expression of some of the genes in this GO category in insects (opsins and arrestins) (46).

Alarm Pheromone Experiment. This experiment was performed at the University of Illinois Bee Research Facility, Urbana, IL; the bees in this region are a mixture of European subspecies, predominantly *Apis mellifera ligustica*. After presenting a piece of filter paper with 2.5 μL isopentyl acetate diluted in mineral oil (1:10) (Sigma–Aldrich) for 60 s at the hive entrance (18), the most aroused-looking bees at the entrance were caged for 60 min and frozen; the response to alarm pheromone increases for 60 min and then levels off (10). Control bees were collected at the hive entrance before alarm pheromone presentation and immediately frozen. Bees were not caged because this can cause alarm pheromone release. This necessary confound in experimental design was considered tolerable because our main goal was to compare gene expression across the different experiments. Levels of *c-Jun* mRNA in the antennal lobes 30 min after exposure to alarm pheromone were used to identify the most responsive colonies (18); bees from Colonies 24 and 27 were chosen (Fig. S1). Unlike in ref. 18, not all colonies showed significant *c-Jun* induction (Fig. S1).

Microarray Procedures. The microarray has been characterized in previous studies (23, 47). It contains 28,800 oligos (including 2,000 control sequences) based largely on annotation from the honey bee genome sequencing project (48). Loop designs were used, with a total of 332 microarrays used to profile 230 individual brains in the three studies (AHB, alarm pheromone, old bee). Microarray procedures were performed as in ref. 23 (Fig. S2). To quantify gene expression from individual brains, RNA was amplified according to manufacturer instruction with the Amino Allyl MessageAmp II aRNA Amplifcation kit (Ambion) starting with 500 ng RNA. Each microarray hybridization used 2.5 μg RNA. Dye coupling and labeled aRNA cleanup was performed with the Amino Allyl MessageAmp II aRNA Amplifcation kit as in ref. 23. Slides were scanned with an Axon 4000B scanner, and images were analyzed with the GENEPIX software (Agilent Technologies).

Microarray Data Analysis. Analysis was performed as in ref. 23. Genes abundantly expressed in hypopharyngeal glands (a potential source of tissue contamination in brain samples) were filtered as in ref. 23. A Loess transformation was performed using Beehive (http://stagbeetle.animal.uiuc.edu/Beehive) to normalize expression intensities. A linear mixed-effects model implemented by using restricted maximum likelihood was used to describe the normalized $\log_2$ transformed gene intensities values, including the effects of dye, treatment (alarm pheromone or behavioral group), bee, and microarray. Effects were evaluated with an *F*-test statistic and the *P* values were adjusted for multiple testing by using a FDR criterion. Results from four genes from AHB/EHB lists (*Uvop*, *Gβe*, *trp*, and *tpnCl*) were validated with quantitative RT-PCR (Fig. S3; for a list of primers used, see Table S6). PCA was performed on the results from the AHB experiment with GeneSpring (Agilent) by using zero-centered, $\log_2$-transformed normalized values of expression data for all genes expressed on the oligoarrays. PCA was performed on guards, soldiers, and foragers independently, with individual bees from all four groups (AHB and EHB bees in AHB and EHB colonies).

Functional Analysis. GO enrichment analysis was performed with *Drosophila* orthologs to bee genes (Dataset S1). Enrichment was determined by using GOToolBox (49) with a hypergeometric test followed by FDR correction for multiple testing (GO categories at $P < 0.05$ shown). For each experiment the reference gene set corresponds to the total number of genes analyzed on the microarray. Lists of GO functions are provided in Dataset S2.

Statistical Analysis to Determine Overlap on Gene Lists. To determine whether the number of genes that overlapped on two gene lists (AHB, alarm pheromone, old bee) was statistically significant, a "representation factor" was calculated. This factor is the number of observed overlapping genes divided by the expected number of overlapping genes. The denominator is calculated as the product of the number of oligos differentially expressed in each experiment divided by the total number of oligos analyzed (50). Overlap for three experiments and for GO categories from the three gene lists was calculated similarly. We tested statistical significance by using an exact hypergeometric test (1-tailed) for the overlap

between two gene lists and an extended version of the hypergeometric test for the overlap between three gene lists. For a universe of N items and three randomly sampled subsets of sizes n_1, n_2, and n_3, respectively, we calculated the probability of an overlap of size l as per the formula below, by summing over all possible values of the overlap k ($\geq l$) between the first two sets. This probability was then used to calculate a P value for the test, by summing over values of l that are greater than or equal to the observed overlap of the three sets.

$$\sum_{k=1}^{\min(n_1,n_2)} \frac{\binom{n_1}{k}\binom{N-n_1}{n_2-k}\binom{k}{l}\binom{N-k}{n_3-l}}{\binom{N}{n_2}\binom{N}{n_3}}$$

***cis*-Regulatory Analaysis: Motif Collection.** Motifs representing experimentally characterized binding specificities were collected from FlyREG (*D. melanogaster*), Transfac (*D. melanogaster*, *Homo sapiens*), and Jaspar (*H. sapiens*). Computationally predicted motifs (*D. melanogaster*) from Xie et al. (51) were also included. We had a compendium of 602 motifs.

***cis*-Regulatory Analaysis: Determining Genes Targeted by a Transcription Factor Motif.** To determine whether a gene has a motif in its promoter, we considered the 5 Kbp upstream region of the gene, by using the *A. melliferra* genome (Amel_v2.0) and Release 1 of the official gene set (48). Methods were performed as in ref. 23 (see *SI Text*).

Measurements of Brain Mitochondrial Enzyme Activities. Each sample consisted of mitochondrial preparations from a pool of five brains, with bees from two colonies per behavioral group per experiment. Mitochondrial activities were measured 60 min after alarm pheromone exposure. Brain metabolism of AHB soldiers from AHB colonies was compared with EHB soldiers from EHB colonies. Brains were homogenized at 4 °C in ice-cold SET buffer (0.25M sucrose, 0.5 mM potassium EGTA, 10 mM Tris, pH 7.4) by using a Dounce homogenizer, then centrifuged for 3 min at 2,000 × *g* at 4 °C. The pellet was then centrifuged again for 3 min at 2,000 × *g* at 4 °C, and the resulting supernatant was centrifuged at 12,000 × *g* at 4 °C for 15 min to obtain a crude mitochondrial fraction. The mitochondrial pellet was subsequently resuspended in 200 μL ice-cold 3% Ficoll solution and layered onto 800 μL ice-cold 6% Ficoll solution, then centrifuged for 10 min at 11,500 × *g* at 4 °C. The final pellet was resuspended to a final volume of 1 mL in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) or a hypotonic buffer containing 25 mM KH_2PO_4, pH 7.2 and 5 mM $MgCl_2$, then stored on ice until use. Samples were used within 4 h of isolation. Total protein concentration was determined by using a Pierce Micro BCA Protein Assay Kit (ThermoFisher Scientific). See *SI Text* for measurement of mitochondrial enzyme activities.

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1. Carroll SB (2005) Evolution at two levels: On genes and form. *PLoS Biol* 3:e245.
2. Carroll SB, Jennifer K, Grenier JK, Weatherbee SD (2001) *From DNA to Diversity. Molecular Genetics and the Evolution of Animal Design* (Blackwell Science, Oxford).
3. Davidson EH (2001) *Genomic Regulatory Systems: Development and Evolution*, (Academic, San Diego).
4. Stern DL (2000) Evolutionary developmental biology and the problem of variation. *Evolution* 54:1079–1091.
5. Carroll SB (2008) Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell* 134:25–36.
6. Robinson GE, Ben-Shahar Y (2002) Social behavior and comparative genomics: New genes or new gene regulation? *Genes Brain Behav* 1:197–203.
7. West-Eberhard MJ (2003) *Developmental Plasticity and Evolution* (Oxford Univ Press, New York).
8. West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. *Proc Natl Acad Sci USA* 102(Suppl 1):6543–6549.
9. Robinson GE, Fernald RD, Clayton DF (2008) Genes and social behavior. *Science* 322:896–900.
10. Breed MD, Guzman-Novoa E, Hunt GJ (2004) Defensive behavior of honey bees: Organization, genetics, and comparisons with other bees. *Annu Rev Entomol* 49:271–298.
11. Hunt GJ (2007) Flight and fight: A comparative view of the neurophysiology and genetics of honey bee defensive behavior. *J Insect Physiol* 53:399–410.
12. Winston ML (1987) *The Biology of the Honey Bee* (Harvard Univ Press, Cambridge, MA).
13. Schneider SS, DeGrandi-Hoffman G, Smith DR (2004) The African honey bee: Factors contributing to a successful biological invasion. *Annu Rev Entomol* 49:351–376.
14. Nelson RJ (2005) *Biology of Aggression* (Oxford Univ Press, New York).
15. Vekovischeva OY, Aitta-aho T, Verbitskaya E, Sandnabba K, Korpi ER (2007) Acute effects of AMPA-type glutamate receptor antagonists on intermale social behavior in two mouse lines bidirectionally selected for offensive aggression. *Pharmacol Biochem Behav* 87:241–249.
16. Hunt GJ, Guzmán-Novoa E, Uribe-Rubio JL, Prieto-Merlo D (2003) Genotype–environment interactions in honeybee guarding behaviour. *Anim Behav* 66:459–467.
17. Wang J, Ross KG, Keller L (2008) Genome-wide expression patterns and the genetic architecture of a fundamental social trait. *PLoS Genet* 4:e1000127.
18. Alaux C, Robinson GE (2007) Alarm pheromone induces immediate-early gene expression and slow behavioral response in honey bees. *J Chem Ecol* 33:1346–1350.
19. Nelson RJ, Chiavegatto S (2001) Molecular basis of aggression. *Trends Neurosci* 24:713–719.
20. Dierick HA, Greenspan RJ (2007) Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat Genet* 39:678–682.
21. Hunt GJ, et al. (2007) Behavioral genomics of honeybee foraging and nest defense. *Naturwissenschaften* 94:247–267.
22. Yanagi M, et al. (2005) Association of 14-3-3 epsilon gene haplotype with completed suicide in Japanese. *J Hum Genet* 50:210–216.
23. Alaux C, et al. (2009) Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav* 8:309–319.
24. Robinson GE (1987) Modulation of alarm pheromone perception in the honey bee: Evidence for division of labor based on hormonally regulated response thresholds. *J Comp Physiol A* 160:613–619.
25. Soma KK, Scotti MA, Newman AE, Charlier TD, Demas GE (2008) Novel mechanisms for neuroendocrine regulation of aggression. *Front Neuroendocrinol* 29:476–489.
26. Drnevich JM, Reedy MM, Ruedi EA, Rodriguez-Zas S, Hughes KA (2004) Quantitative evolutionary genomics: Differential gene expression and male reproductive success in *Drosophila melanogaster*. *Proc Biol Sci* 271:2267–2273.
27. Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. *J Neurosci* 26:1864–1871.
28. Sinha S, Ling X, Whitfield CW, Zhai C, Robinson GE (2006) Genome scan for cis-regulatory DNA motifs associated with social behavior in honey bees. *Proc Natl Acad Sci USA* 103:16352–16357.
29. Wager BR, Breed MD (2000) Does honey bee sting alarm pheromone give orientation information to defensive bees? *Ann Entomol Soc Am* 93:1329–1332.
30. Wang L, Dankert H, Perona P, Anderson DJ (2008) A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc Natl Acad Sci USA* 105:5657–5663.
31. Ament SA, Corona M, Pollock HS, Robinson GE (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc Natl Acad Sci USA* 105:4226–4231.
32. Harrison JF, Hall HG (1993) African–European honeybee hybrids have low nonintermediate metabolic capacities. *Nature* 363:258–260.
33. Southwick EE, Moritz RFA (1985) Metabolic response to alarm pheromone in honey bees. *J Insect Physiol* 31:389–392.
34. Sakata JT, Crews D, Gonzalez-Lima F (2005) Behavioral correlates of differences in neural metabolic capacity. *Brain Res Brain Res Rev* 48:1–15.
35. Anckarsäter H (2006) Central nervous changes in social dysfunction: Autism, aggression, and psychopathy. *Brain Res Bull* 69:259–265.
36. Du F, et al. (2008) Tightly coupled brain activity and cerebral ATP metabolic rate. *Proc Natl Acad Sci USA* 105:6409–6414.
37. Shmuel A, Augath M, Oeltermann A, Logothetis NK (2006) Negative functional MRI response correlates with decreases in neuronal activity in monkey visual area V1. *Nat Neurosci* 9:569–577.
38. Kourtzi Z, Betts LR, Sarkheil P, Welchman AE (2005) Distributed neural plasticity for shape learning in the human visual cortex. *PLoS Biol* 3:e204, doi: 10.1371/journal.pbio.0030204.
39. Sokoloff L (1960) Section I, Neurophysiology. *Handbook of Physiology*, eds Field J, Magoun HW, Hall VE (Am Physiol Soc, Washington DC), Vol 3, pp 1843–1864.
40. Conejo NM, Gonzalez-Pardo H, Vallejo G, Arias JL (2007) Changes in brain oxidative metabolism induced by water maze training. *Neuroscience* 145:403–412.
41. Deglise P, Dacher M, Dion E, Gauthier M, Armengaud C (2003) Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). *Behav Neurosci* 117:540–547.
42. Poremba A, Jones D, Gonzalez-Lima F (1998) Classical conditioning modifies cytochrome oxidase activity in the auditory system. *Eur J Neurosci* 10:3035–3043.
43. Arechavaleta-Velasco ME, Hunt GJ, Emore C (2003) Quantitative trait loci that influence the expression of guarding and stinging behaviors of individual honey bees. *Behav Genet* 33:357–364.
44. Guzmán-Novoa E, Page RE, Spangler HG, Erickson EH (1999) A comparison of two assays to test the defensive behaviour of honeybees (*Apis mellifera*). *J Apicult Res* 38:205–209.
45. Schulz DJ, Robinson GE (1999) Biogenic amines and division of labor in honey bee colonies: Behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J Comp Physiol* 184:481–488.
46. Lampel J, Briscoe AD, Wasserthal LT (2005) Expression of UV-, blue-, long-wavelength-sensitive opsins and melatonin in extraretinal photoreceptors of the optic lobes of hawk moths. *Cell Tissue Res* 321:443–458.
47. Kocher SD, Richard FJ, Tarpy DR, Grozinger CM (2008) Genomic analysis of post-mating changes in the honey bee queen (*Apis mellifera*). *BMC Genomics* 9:232.
48. Honey Bee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931–949.
49. Martin D, et al. (2004) GOToolBox: Functional analysis of gene datasets based on Gene Ontology. *Genome Biol* 5:R101.
50. Kim SK, et al. (2001) A gene expression map for *Caenorhabditis elegans*. *Science* 293:2087–2092.
51. Xie X, et al. (2005) Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. *Nature* 434:338–345.

Supporting Information

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SI Text

Soldier Trap. To obtain soldier bees, a net trap fitting the dimensions of the beehive with 4 triangular pieces of white wood (two 55.5 × 90 cm and two 45.5 × 90 cm) was placed on top of the opened hive. After stimulation, the trap was removed and the hive-top closed to prevent the soldiers inside it from escaping. The trap was then placed inside a freezer at −18 °C for 5 min.

cis*-Regulatory Analysis.** ***Determining genes targeted by a transcription factor motif. We masked exons (of adjacent genes) and short tandem repeats of period ≤5 bp [Tandem Repeat Finder (1), command-line arguments 2 3 5 80 10 25 5 –m –d] present in the promoter, and removed all masked positions, thus obtaining promoters of variable lengths (≤5 Kbp). We "scanned" each resulting promoter with a sliding window of 500-bp length, in shifts of 250 bp. Each window was scored for presence of the motif by using the Hidden Markov Model-based approach used in ref. 2. This raw score was converted to an empirical P value based on raw scores of randomly generated windows of the same length and guanine-cytosine content. Each promoter was thus associated with a list of "score P values" of varying sizes because the masked promoters are of varying lengths and are covered by varying number of 500-bp windows. Let k be the number of window P values associated with a gene's promoter, and let β be a significance threshold (e.g., 0.05) at which we wish to associate the gene with the motif. We computed $\alpha = 1 - (1 - \beta)^{1/k}$ and used this as a threshold on the window-score P value. If any window has score P value below this threshold α, then the corresponding promoter (and gene) is designated as a target of the motif.

Associating motifs with gene sets. Each motif was tested for association with differentially regulated gene sets in each experiment, as follows: Let U_T and D_T be the sets of genes up- and down-regulated in experiment T (i.e., alarm pheromone, old, or soldier). Each set was partitioned into genes that are targeted by the motif (as determined above, with $\beta = 0.01$) and genes that are not. A Fisher exact test was performed on the 2 × 2 contingency table (up vs. down, motif target vs. nontarget), and the 1-tailed P value was the statistical measure of association between the gene set U_T and the motif. By repeating the same test for the 2 × 2 contingency table: (down vs. up, motif target vs. nontarget), we obtained a P value for the association between the gene set D_T and the motif.

Motifs associated consistently with genes influenced by heredity and environment. A motif was tested for consistent association with up-regulated gene sets from all three experiments as follows. (The same procedure was repeated with down-regulated gene sets.) Let P_1, P_2, and P_3 be the 1-tailed P values of association of the motif with the up-regulated gene sets in the three experiments, respectively. We considered the combined measure

$$P_c = 1 - (1 - P_1)\,(1 - P_2)\,(1 - P_3).$$

We denote this random variable as P_c and its observed value as $P_c^{(o)}$ Under the null hypothesis that each of P_1, P_2, P_3 is uniformly distributed, we computed the probability π_c that the combined measure P_c has a value less than or equal to the observed value $P_c^{(o)}$, i.e., a 1-tailed P value for the combined measure P_c, as follows:

$$\pi_c(x) = \Pr(P_c \le x) = \Pr\left(\prod_j (1 - P_j) \ge 1 - x\right)$$

$$= 1 - \Pr\left(\prod_j (1 - P_j) \le 1 - x\right)$$

$$= 1 - (1 - x)\sum_{i=0}^{2} (-1)^i \frac{(\ln(1 - x))^i}{i!},$$

where the last step is due to the fact that $1 - P_j$ is uniformly distributed in [0,1] under the null hypothesis.

The random variable P_c has the desirable property that it is low only if each of P_1, P_2, P_3 is low, and thus captures consistent motif association (low P value) in all three experiments. (Contrast this with the product of the three P_j's, which may be low even if one or two of the P_j's is close to 1.) However, the P value π_c computed above corresponds to the strong null hypothesis that every individual P_j is uniformly distributed. We therefore empirically estimated the appropriate thresholds to use for this P value, as described below.

Multiple hypothesis correction. We simulated random gene sets of the same size as the original gene sets in each experiment, obtained the π_c for each motif, calculated how many motifs have a π_c below a specific threshold τ, and computed an average of this number over 1,000 independent simulations. This is the empirical expectation of the number of tests with π_c below τ, and was used to estimate a false discovery rate (FDR) corresponding to each $\pi_c(P_c^{(o)})$ in the tests with the original gene sets. Only motifs with estimated FDR < 0.001 are reported in Table 5.

Extreme value distribution. We considered the minimum $\pi_c(P_c^{(o)})$ over all motifs, representing the extent of the strongest consistent motif association. We then computed the corresponding minimum value from each of the 1,000 random simulations described above, and the histogram thus obtained provides us with an estimate of the Extreme Value Distribution of $\pi_c(P_c^{(o)})$. This allowed us to estimate an empirical P value for the minimum $\pi_c(P_c^{(o)})$.

mRNA Quantification by Real-Time Quantitative RT-PCR (qRT-PCR). We determined whether alarm pheromone exposure induces an up-regulation of the transcription factor *c-Jun* in antennal lobes (ALs) (Fig. S1). Seven control and pheromone-exposed workers per colony were analyzed. We also verified the expression levels of 4 genes (*Uvop*, *Gβe*, *trp*, and *TpnCI*) in whole brains of bees from two Africanized honey bee (AHB) colonies compared with bees from two European honey bee (EHB) colonies (Fig. S3). Five to seven workers (mainly AHB workers) per colony were analyzed. Because the expression levels of the tested genes did not differ significantly between the two AHB colonies and between the two EHB colonies (1-tailed permutation test, $P = 0.18$ for each test), results were pooled for analysis.

Whole brains and ALs were dissected, frozen, and then homogenized in TRIzol (Invitrogen Life Technologies) before extracting RNA with the Qiagen RNeasy kit for total RNA with on-column DNase I treatment (Qiagen). Forty nanograms from the two ALs and 200 ng from whole brains were reverse-transcribed with random hexamers by using the RETROscript kit (Ambion). mRNA quantification was performed using an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems). *c-Jun* mRNA levels were normalized to the *rp49* "housekeeping" gene as control (expression level did not vary: $P = 0.64$ for each test) and those of *Uvop*,

$G\beta_e$, *trp*, and *TpnCI* were normalized to the external control *rcp1* (expression level did not vary; $P = 0.11$ for each test). A list of primers is given in Table S5.

Measurements of Mitochondrial Enzyme Activities. Activity of NADH:ubiquinone oxidoreductase was assayed as previously described (3, 4). Briefly, brain mitochondria were lysed by freeze-thawing 3 times in hypotonic buffer (25 mM KH_2PO_4, pH 7.2, 5 mM $MgCl_2$) and added to an assay buffer consisting of 65 μM ubiquinone$_1$, 130 μM NADH, 2 μg/mL antimycin A, and 2.5 mg/mL BSA in the presence or absence of rotenone. Oxidation of NADH was measured at 340 nm for 5 min at 30 °C by using a SpectraMax M2 microplate reader (Molecular Devices), after which 5 μg/mL rotenone was added to the reaction. Activity was then measured for another 5 min and the difference in rate before and after treatment with rotenone was used to determine complex I activity. The molar absorbance coefficient used for NADH was 6.22 $mM^{-1}cm^{-1}$.

Complex IV activity was measured by using a Cytochrome *c* Oxidase Assay kit (Sigma) as per manufacturer instructions. The decrease in absorption at 550 nm, representing oxidation of ferrocytochrome *c*, was monitored over time on a SpectroMax M2 microplate reader (Molecular Devices) at room temperature. Activity was then calculated by using the following formula: $(\Delta absorbance_{550\ nm}/min \times 28.8)/40 \times 21.84$, where 21.84 represents the $\Delta\varepsilon^{mM}$ between ferrocytochrome *c* and ferricytochrome *c* at 550 nm, and 28.8 represents the dilution factor multiplied by the total reaction volume.

Complex V (ATP synthase) activity was determined by measuring the decrease in NADH absorption at 340 nm in the presence or absence of 2 μg/mL oligomycin, a specific inhibitor of the F_1F_0 complex of ATPase, at 30 °C as previously described (5). Mitochondria were freeze-thawed as before and added to an assay medium consisting of 4 mM ATP, 25 mM Tris-Cl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 200 μM NADH, 1.5 mM phosphoenolpyruvate, 5 units lactate dehydrogenase, 3 units pyruvate kinase, and 2.5 μM rotenone.

In addition, assays of ATP production in the presence or absence of 5 μg/mL rotenone (complex I inhibitor), 0.25 mM KCl (complex IV inhibitor), or 2 μg/mL oligomycin (complex V inhibitor) were also performed by using an ATPlite Luminescence Assay System (Perkin-Elmer). Freeze-thawed mitochondria were prepared as above and the lysates incubated at 30 °C in a reaction buffer containing 65 μM ubiquinone$_1$, 130 μM NADH, and 2.5 mg/mL BSA. Aliquots were removed every minute and processed according to manufacturer instructions, and luminescence was detected by using a SpectroMax M2 microplate reader.

All measurements were corrected for background and crude protein concentration and analyzed by using 1-way ANOVA with Tukey posthoc testing.

Functional analysis. The list of GO categories from Fig. 2 is shown below:

1. Multicellular organismal process.
2. Response to stimulus.
3. Metabolic process.
4. Biological regulation.
5. Binding.
6. Electron carrier activity.
7. Catalytic activity.
8. Structural molecule activity.
9. Transmembrane transporter activity.
10. System process.
11. Detection of stimulus.
12. Cellular metabolic process.
13. Oxidation reduction.
14. Regulation of biological process.
15. Protein binding.
16. Isomerase activity.
17. Oxidoreductase activity.
18. Structural constituent of ribosome.
19. Substrate-specific transmembrane transporter activity.
20. Neurological system process.
21. Detection of external stimulus.
22. Alcohol metabolic process.
23. Regulation of cellular process.
24. Intramolecular lyase activity.
25. Ion transmembrane transporter activity.
26. Sensory perception.
27. Alcohol biosynthetic process.
28. Regulation of cell cycle.
29. Inositol-3-phosphate synthase activity.
30. Cation transmembrane transporter activity.
31. Sensory perception of light stimulus.
32. Polyol biosynthetic process.
33. Regulation of S phase.
34. Inorganic cation transmembrane transporter activity.
35. Visual perception.
36. Inositol biosynthetic process.
37. Regulation of S phase of mitotic cell cycle.
38. Monovalent inorganic cation transmembrane transporter activity.
39. Positive regulation of S phase of mitotic cell cycle.
40. Hydrogen ion transmembrane transporter activity.

1. Benson G (1999) Tandem repeats finder: A program to analyze DNA sequences. *Nucleic Acids Res* 27:573–580.
2. Alaux C, et al. (2009) Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav* 8:309–319.
3. Birch-Machin MA, Turnbull DM (2001) Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods Cell Biol* 65:97–117.
4. Tieu K, et al. (2003) D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. *J Clin Invest* 112:892–901.
5. Bosetti F, Yu G, Zucchi R, Ronca-Testoni S, Solaini G (2000) Myocardial ischemic preconditioning and mitochondrial F1F0-ATPase activity. *Mol Cell Biochem* 215:31–37.
6. Hunt GJ, et al. (2007) Behavioral genomics of honeybee foraging and nest defense. *Naturwissenschaften* 94:247–267.
7. Kanehisa M, Goto S (2000) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28:27–30.

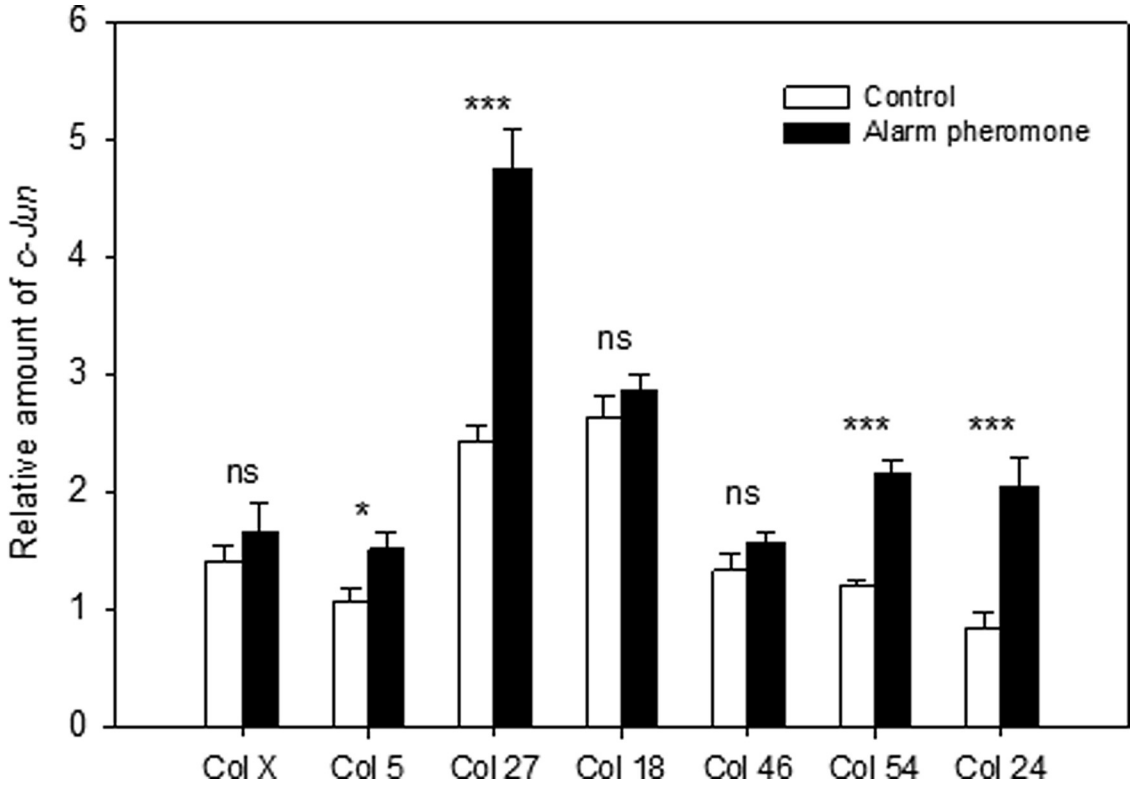


Fig. S1. Effect of alarm pheromone exposure on *c-Jun* responses. Alarm pheromone exposure caused up-regulation of *c-Jun* in the antennal lobes of bees in 4 of 7 colonies. Data normalized to expression levels of *rp49*. Means ± SE shown. Significant differences were determined by using a 1-tailed permutation test (*, $P < 0.05$; ***, $P < 0.001$).

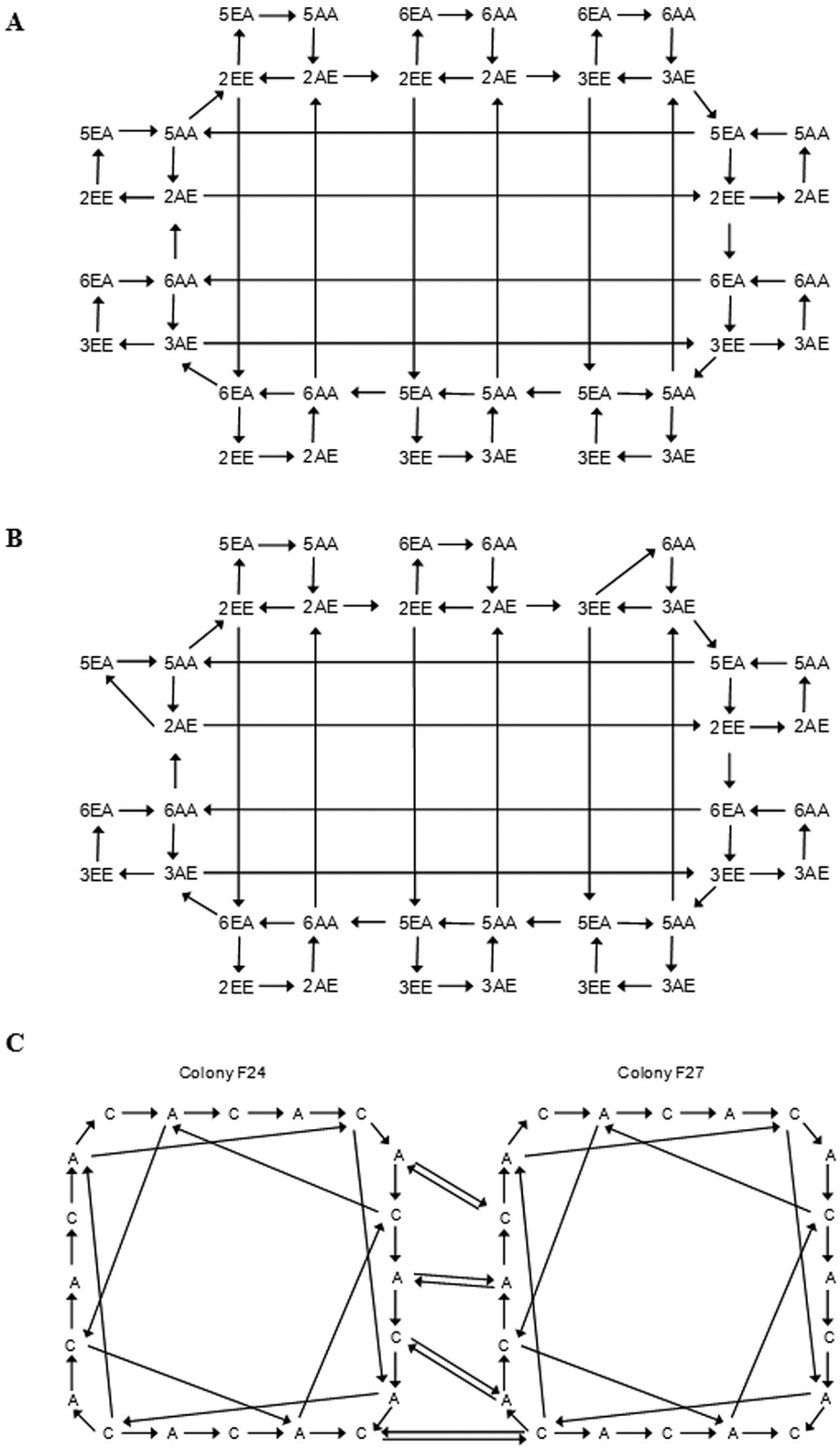
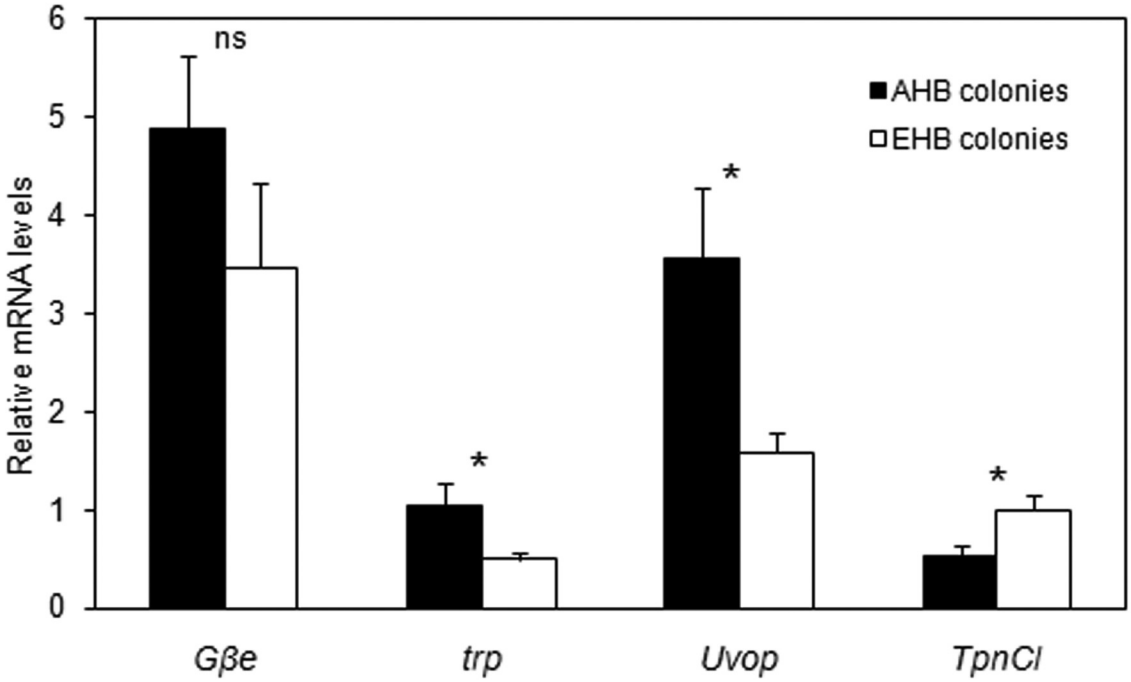


Fig. S2. Microarray experimental designs. Arrow tail indicates Cy3-labeled sample and arrow head indicates Cy5-labeled sample. Each sample was labeled an equal number of times with Cy5 and Cy3. (*A*) AHB/EHB experimental design for guards and foragers. 2E and 3E, EHB colonies; 5A and 6A, AHB colonies; last letter indicates worker genotype; 60 arrays for each behavioral group. (*B*) AHB/EHB experimental design for soldiers. Same as in *A* except that in colonies 2E and 6A, four EHB soldiers were analyzed, 58 arrays. (*C*) Alarm pheromone experimental design. C, control bees; A, bees exposed to alarm pheromone; bees from 2 colonies, 64 arrays.

A



B

Honeybee annotations	Gene name	Microarrays (AHB/EHB)	qRT-(AHB/EHB)
GB10545	*Gβe*	1.5	1.4
GB16264	*trp*	1.4	2.1
GB18171	*Uvop*	1.4	2.2
GB10545	*TpnCl*	0.8	0.5

Fig. S3. Validation of microarray results with real-time qRT-PCR. (*A*) Brain expression levels of four genes identified from microarray analysis as showing different expression levels between AHB vs. EHB. Data normalized to expression levels of *rcp1*. (*B*) Ratio of mean expression levels (AHB/EHB) from array and qRT-PCR results are shown. qRT-PCR samples were from independent biological replicates, not used for microarray analysis. Means ± SE shown. Significant differences were determined by using a 1-tailed permutation test (∗, $P < 0.05$).

Table S1. Aggression-related brain gene regulation in honey bees: Number of genes overlapping between gene sets regardless of direction

	Old bee				AHB guard				AHB soldier				AHB forager			
Experiment	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value	Expected	Observed no.	*RF*	*P* value
Alarm pheromone	55.3	151	2.7	<0.0001	10	24	2.4	<0.0001	21.8	55	2.5	<0.0001	2.3	7	3	<0.008
Old bee					28.8	54	1.9	<0.0001	63.7	147	2.3	<0.0001	6.8	21	8.1	<0.0001
AHB guard (I)									11.6	81	7	<0.0001	1.2	24	19.5	<0.0001
AHB soldier (I)													2.7	29	10.7	<0.0001
Alarm pheromone	55.3	151	2.7	<0.0001	19.8	63	3.2	<0.0001	33.6	64	1.9	<0.0001	13.9	27	1.9	<0.0001
Old bee					57.1	128	2.2	<0.0001	98.3	195	2	<0.0001	40.3	85	2.1	<0.0001
AHB guard (C)									35.4	155	4.4	<0.0001	14.6	91	6.2	<0.0001
AHB soldier (C)													24.9	106	4.3	<0.0001

Data are from the following: Experiment 1, AHB vs. EHB (guards, soldiers, and foragers); Experiment 2, alarm pheromone induced; and Experiment 3, old vs. young bees. Expected, the number of genes expected to overlap in all three experiments by chance alone; *RF*, representation factor; I, individual genotype; C, colony genotype. The statistical significance of the overlap between two lists was determined using an exact hypergeometric test and an extended version of the hypergeometric test for the overlap between three lists (see *SI Text*).

Table S2. Correlation analysis for genes differentially expressed as a function of genotype, alarm pheromone exposure, and age

	Alarm pheromone		Old bee	
Experiment	*r*	*P* value	*r*	*P* value
AHB Guard (I)	0.139	0.004	0.062	0.02
AHB Soldier (I)	0.229	<0.001	0.277	<0.001
AHB Forager (I)	0.161	<0.001	0.302	<0.001
AHB Guard (C)	0.207	<0.001	0.059	0.026
AHB Soldier (C)	0.189	<0.001	0.349	<0.001
AHB Forager (C)	0.357	<0.001	0.15	<0.001
Alarm pheromone	—	—	0.429	<0.001
Old bee	0.65	<0.001	—	—

Correlations were performed on expression values for genes regulated by alarm pheromone or differentially expressed between old and young individuals and the expression values of the same genes from the other lists that are listed. Log_2-transformed ratios were used. I, individual genotype; C, colony genotype.

Table S3. Genes regulated by alarm pheromone and/or differentially expressed between AHB/EHB, and found in independently derived aggression quantitative trait loci (6)

Bee genes	Fly orthologs	Molecular function	Biological process
Alarm pheromone			
GB10390 ↓	*Pros25*	Endopeptidase activity	ATP-dependent proteolysis; cell proliferation
GB18971 ↑	*l(1)G0232*	Rhodopsin-like receptor activity	G-protein-coupled receptor protein signaling pathway
GB12020 ↓	*ubl*	Unknown	Protein modification process
GB12504 ↑	*CkIIβ*	Casein kinase activity	Mushroom body development; circadian rhythm; signal transduction
GB15016 ↑	*Hsc70-3*	ATPase activity	Sleep; response to heat; defense response; protein folding; RNA interference
GB14502 ↑	*MED22*	RNA polymerase II transcription mediator activity	Regulation of transcription from RNA polymerase II promoter
GB13698 ↓	*Psf2*	Transcription elongation regulator activity	Positive regulation of S phase of mitotic cell cycle
AHB/EHB, individual genotype			
Guard			
GB19009 ↓	—	—	—
GB16258 ↑	—	—	—
Soldier			
GB19009 ↓	—	—	—
GB19676 ↑	*CG8165*	Regulation of transcription from RNA polymerase II promoter	Transcription from RNA polymerase II promoter
GB15582 ↑	*14-3-3ε*	Regulation of transcription, protein complexes, protein trafficking	Response to external stimulus; nonassociative learning
AHB/EHB, colony genotype			
Guard			
GB14502 ↓	*MED22*	RNA polymerase II transcription mediator activity	Regulation of transcription from RNA polymerase II promoter
GB11198 ↓	*CG34347*	Actin binding; structural constituent of cytoskeleton	Cytoskeleton organization and biogenesis
Soldier			
GB14824 ↑	*CG7675*	Oxidoreductase activity, acting on CH-OH group of donors	Metabolic process
GB18618 ↑	*htt*	Microtubule binding; protein binding	Axon cargo transport
GB19676 ↑	*CG8165*	Regulation of transcription from RNA polymerase II promoter	Transcription from RNA polymerase II promoter
GB19319 ↓	*RpI12*	Zinc ion binding; transcription regulator activity	Transcription from RNA polymerase I promoter
GB17741 ↓	*CG7849*	Unknown	Unknown
GB19681 ↓	*Ctr1A*	Copper ion transmembrane transporter activity	Copper ion transport
Forager			
GB14824 ↓	*CG7675*	Oxidoreductase activity, acting on CH-OH group of donors	Metabolic process
GB18557 ↑	*α-Spec*	Cytoskeletal protein binding	Synaptic transmission; regulation of cellular component organization and biogenesis

Arrows indicates up- or down-regulated by alarm pheromone and in AHB vs. EHB bees. Known functions of orthologs of these genes are shown. Quantitative trait loci were derived in a separate study based on an assay that measured propensity to sting in response to a disturbance to the colony (in the field), and involved different bees derived from crosses between AHB and EHB (6). —, unknown.

Table S4. Genes that were up- and down-regulated in all three experiments, i.e., responsive to heredity, alarm pheromone, and age, are shown with their fly and human orthologs

Bee oligo	Bee gene	Fly orthologs	Human orthologs
Up-regulated			
AM00762	—	—	—
AM01806	—	—	—
AM02192	—	—	—
AM02336	—		—
AM02847	GB10220	—	—
AM02986	GB10356	—	—
AM03461	GB10836	*Hsc70Cb*	*HSPA4* (*hsp70*)
AM03846	GB11223	*His3*:*CG33803*	*HIST2H3*
AM04192	GB11572	*Inos*	*AC008397.7* (*MIP synthase*)
AM04834	GB12215	*Droj2*	*DNAJA4*
AM05318	GB30506	*Gp93*	*HSP90B1*
AM05381	GB12766	*Arr2*	—
AM06103	GB13501	—	—
AM06766	GB14166	—	—
AM08435	GB15853	*Kap-α1*	*KPNA*
AM08741	GB16163	—	—
AM09086	GB16507	*CG1600*	—
AM10198	GB17624	*CHORD*	*CHORDC1*
AM10664	GB18094	*tj*	*MAF*
AM12432	GB19885	*CG1516*	*PC*
AM12790*	GB11487	*moody*	*MTNR1a*
Down-regulated			
AM02193	—	—	—
AM02582	—	—	—
AM03140	GB10513	*CG14286*	*C8orf33*
AM03851	GB11228	*His3.3A*	*H3F3B*
AM06007	GB13404	*Ts*	*TYMS*
AM06580	GB13982	*Faa*	*FAH*
AM07997*	GB15409	*Cyp6 g1*	—
AM08023	GB15436	*CG10674*	*C19orf56*
AM08856	GB16276	*SmB*	*SNRPB*
AM10511	GB17938	*CG1756*	—
AM10649	GB18078	*CG9646*	*C22orf9*
AM10750	GB18183	—	—
AM11126	GB18566	—	—
AM11255	GB18694	—	—
AM11556	GB19000	*Tim9a*	*TIMM9*
AM12010	GB19459	*Psf1*	*GINS1*
AM12536	GB19989	*CG4875*	*AC068533.6* (*RPC9*)

Asterisks indicate genes that were regulated at a slightly less stringent threshold in one experiment (*moody* was up-regulated in AHB foragers at FDR = 0.097 and *Cyp6 g1* was down-regulated by alarm pheromone at FDR = 0.057). —, unknown.

Table S5. Oxidative phosphorylation pathway downregulated in aggressive bees

	Bee gene	Fly orthologs	Alarm Pheromone	Old Bee	AHB Soldier (I)	AHB Soldier (C)
Complex I NADH-coenzyme Q oxidoreductase	GB15948	CG10320				
	GB18920	Pdsw				
	GB10916	CG9306				
	GB10474	CG12400				
	GB13526	CG3621				
	GB10859	CG2014				
	GB15438	CG15434				
	GB17095	CG9140				
	GB16917	CG12203				
	GB10406	CG6020				
	GB15102	mitochondrial acyl carrier protein 1				
Complex II Succinate-Q oxidoreductase	GB12875	CG6666				
Complex III Q-cytochrome c oxidoreductase	GB12164	CG30354				
	GB14417	CG14482				
	GB10344	Oxen				
	GB12510	CG4769				
Complex IV Cytochrome c oxidase	GB18028	CG14235				
	GB15238	CG9065				
	GB30388	cyclope				
	GB20012	CG10664				
	GB15816	Cytochrome c oxidase subunit Va				
	GB17614	CG2249				
Complex V ATP synthase	GB16494	ATPase coupling factor 6				
	GB18417	CG4692				
	GB15629	Oligomycin sensitivity-conferring protein				
	GB10989*	Vha68-2				
	GB16751	lethal (2) 06225				
	GB14791	bellwether				
	GB15291	ATP synthase-γ chain				

This was performed by using the DAVID annotation tool (http://david.abcc.ncifcrf.gov/) that presents information on molecular pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/) (7). Asterisk indicates up-regulation. I, individual genotype; C, colony genotype. Colors indicate the three different experiments.

Table S6. Primer sequences

Primer	Sequence (5′ to 3′)
c-Jun, forward	CGTGGCGGCATCCAAA
c-Jun, reverse	CCCTTCAGCAATTTAACCTTATCTTC
Uvop, forward	CTGCCATCACAATTTGCTTCTTAT
Uvop, reverse	TGTAACACCAGGTGTTAAAAGTGCTT
$G\beta_e$, forward	TGCGCTCAGCATGGGTAAT
$G\beta_e$, reverse	TGTCCATACCACCACAAGCAA
trp, forward	GAAGTGTCACCGACTACGAGGAT
trp, reverse	CTGCCGCCGGTTTGG
TpnCl, forward	TTCTTCGCAAAGCGTTCGA
TpnCl, reverse	CAATCTGAGAATATCAGCCACCAT
rcp1, forward	TCAATTAACTCGGAATCGGA
rcp1, reverse	CCTGGATTTCCCTGCTGAT
rp49, forward	GGAACTGGAAGTTTTAATGATGCA
rp49, reverse	CAACAATGGATTTACGTTTTTTACTG

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)