SUPPLEMENTAL TEXT

Honey bee experiments

Metabolic drug selection

Fenpyroximate and Tetradifon were selected because they target different complexes of the electron transport chain that showed aggression-associated changes in gene expression (1) (complex I and complex V respectively, (2-4)), they are soluble in acetone (a commonly used solvent for honey bee topical pharmacological treatments (5)), and there was an established lethal dose for worker bees, suggesting they are biologically active in honey bees (Insecticide Resistance Action Committee, Pesticides Properties Database, Integrative Pest Management Ecotoxicity Database).

Metabolic drug treatment details

We kept drug solutions in screw-capped vials and administered doses with a syringe in order to minimize acetone evaporation. Drug application techniques were modified from ref. (3). We administered a single 1 μL topical application of the appropriate drug with a PB-600-1 repeating dispenser (Hamilton Company, Reno, NV, USA) fitted with a 50μL syringe (Gastight #1705). The area of application encompassed the anterior portion of the thorax and the membranous region connecting the head to the thorax of the bee.

Following drug application, we marked bees on the thorax with Testors paint (Rockford, IL, USA) to designate each bee as drug-treated or acetone-treated control. We then returned groups of bees, again housed in 7.0 x 8.0 x 9.0 cm boxes, to the incubator and performed the Intruder Assays 24 h following treatment.

Intruder Assay behavioral details

The Intruder Assay was modified from (6). We measured six aggressive behaviors of increasing severity: 1) antennation or trophallaxis, 2) threatening the intruder with open mandibles, 3) biting, 4) abdomen flexion, in which the bee grips the intruder and flexes the abdomen without extruding the stinger, and 5) stinging. Severity indices were: 1: antennation and trophallaxis, 2: threatening the intruder with open mandibles, 3: biting, 4: abdomen flexion, 5: stinging.

We performed Intruder Assays in a constantly ventilated room maintained at 25-28°C. Ventilation cleared the room of any alarm pheromones that may have been released over the course of the assays. Just prior to the start of the assay, we collected forager-aged bees from typical outdoor colonies to be used as intruders. Because genotype as well as rearing environment can influence hive-mate recognition and thus aggression towards a foreign intruder bee (7), we did not collect intruder bees from any colonies that were used as study subject source colonies. To begin the assay, we marked the intruder bee on the thorax with paint in order to distinguish it from the study subjects, and carefully introduced the intruder into the box. We only scored aggressive behaviors that were instigated by the study subjects within the group; occasionally the intruder bee instigated aggression, i.e., biting or threatening the group members,

but this was rare. In cases in which subjects performed more than one behavior simultaneously (e.g. biting while abdomen flexing) we recorded only the most severe behavior.

Generating small field colonies with typical versus low aggression levels

We replicated the experimental approach from (8) in order to generate typical and low-aggression social environments. Briefly, we employed a chronic disturbance paradigm that included mechanical disturbances and electric shock. This treatment results in decreased aggression. We constructed 4 pairs of "single-cohort" colonies with ~4300 one-day-old bees/colony (9). One colony in each pair was subjected to the chronic disturbance paradigm and the other was left undisturbed as a control. We used the first pair of colonies as a test set to determine whether colony-level aggression differences could be assessed using the laboratory Intruder Assay. We found that bees from the chronically disturbed colony were about 33% less aggressive compared to bees from the carefully paired undisturbed control colony (Fig S6; N=18 and 19 groups respectively; Wilcoxon one-tailed test; $X^2_1 = 6.44$, $P < 0.011$).

The other three pairs of colonies were used to assess the interaction between social environment and drug treatment on aggression. We treated and assayed adult bees collected from these three pairs, but prior to collection, for each colony pair, we used a typical whole-colony field aggression assay identical to ref. (8) to validate that chronic disturbance resulted in decreased colony aggression levels (Fig S7; Wilcoxon exact test, $S_1 = 6$, $P = 0.05$, (8)).We counted the number of aggressive bees that emerged during the whole colony aggression assay as well as the number of times these bees stung a cloth patch treated with alarm pheromone (8). Both of these metrics are positively correlated with aggression.

Experiment 3: bee collections from field colonies

Once we had performed the whole-colony aggression assay on both colonies in the pair, we collected bees from each colony, one colony at a time. For the pair of test colonies, we sealed colony entrances and collected bees around the entrance using forceps. Bees were collected into 100 mm x 20 mm petri dishes (Becton, Dickinson Company, Franklin Lakes, NJ, USA). We put 8 bees in each dish (N = 18 groups from the disturbed colony and N = 19 groups from the control colony) and supplied 1.5 mL of 40% sucrose (mass/volume). The dishes were placed in an incubator and assayed 24 h later. We used petri dishes instead of boxes and 8 bees/group instead of 10 as above for logistical reasons: the small dish size and slightly smaller group made it easier to collect and transport groups of bees in the field.

For the other three pairs of colonies, we needed to collect about twice as many bees in order to obtain adequate sample sizes for both drug treatments. To do this, we collected bees using a specialized vacuum. We opened the hive and collected bees that were standing on or drinking from honeycomb filled with honey. We immediately anesthetized the bees on ice and treated them as described above in *Metabolic drug treatment details* (see Table S5 for sample sizes).

Minor differences between Experiments 1 and 3

In Experiment 1 we treated bees at age 7 days and assayed behavior on day 8, while in

Experiment 3 we shifted the timeline by one day (i.e. we collected and treated 8-day-old bees and tested behaviors on day 9) in order to match the timing of social manipulations as closely as possible to the previous study (8). In addition, we recorded aggressive behaviors slightly differently. Laboratory-reared bees (bees from Experiment 1), having spent their entire lives in a caged environment, tend to be more responsive to an intruder compared to colony-reared bees that are somewhat disoriented after being transferred into the lab environment. Because bees in Experiment 3 were less responsive, we scored aggression on a continuous basis instead of scan sampling.

Statistical Analysis

All statistical analyses were performed using JMP Pro 9.0.2. Although mortality across all experiments was relatively low, mortality in Experiment 3 varied across colony pairs, probably as a function of external environmental variables like weather. Mortality, however, did not differ across social environments. For Experiment 3 we excluded groups from the analysis if 50% or more of either drug-treated or acetone-treated bees within the group died. For all Intruder Assays involving drug treatment, aggression scores were log-transformed for normalization prior to further analysis. To assess the effects of drug treatment, we performed a two-tailed *t*-test, blocking for experimental group to account for group-to-group variation in aggression. In the comparison of groups collected from the test colonies, log transformation failed to normalize the data, and in this case a non-parametric test was used. All other statistical tests are listed next to the appropriate analysis in the RESULTS section.

Fruit fly experiments

Gene names

CG2014 and *CG9140* were renamed "*ND20-like*" and "*ND51*" in consultation with Flybase (Dr. Steven Marygold and Dr. Damiano Porcelli). These genes are orthologous to the vertebrate genes *Ndufs7b* and *Ndufv1a*, respectively.

Fly stocks

A total of 27 UAS-RNAi homozygous lines, including the lines UAS-*ND20-like*-RNAi (v50731; Table S3), were ordered from the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria). The following driver lines were ordered from the Bloomington Drosophila Stock Center (Bloomington, IN): *elav-Dcr2*-GAL4 driver line (genotype: P{w[+mW.hs]=GawB}*elav*[C155] w[1118]/P{w[+mC]=UAS-*Dcr*-2.D}2) (BDSC no. 25750) for pan-neural expression, the *repo*GAL4 driver line (genotype: w[1118]/P{w[+m*]=GAL4}repo/*TM3,Sb*[1]) (BDSC no. 7415) for pan-glia expression, and w1118 (BDSC no. 5905). A *w*/*elav*-GAL4 (genotype: w[*]/ P{w[+mC]=GAL4-*elav*.L}3) pan-neural driver line backcrossed for six generations into a white Canton-S (w-CS) background as well as a Canton-S (CS) and white Canton-S line (w-CS), for behavioral controls and cantonization procedures respectively, were kindly donated by Scott A. Kreher (Dominican University, River Forest, IL).

Fly-crossing scheme

Initially, we crossed our *w1118*/*elav-Dcr2*-GAL4 drivers with *w1118/*UAS-RNAi effector lines to quickly screen for developmental and behavioral differences relative to a *w1118* fly strain (listed in Table S3). Several *w1118*/*elav-Dcr2*-GAL4/w1118/UAS-RNAi flies failed to eclose or survive as adults, and only seven of these strains were tested in our aggression assay. These developmental abnormalities may have been caused by the enhanced RNAi-mediated knockdown of the metabolic genes tested because our *w1118*/*elav-Dcr2*-GAL4 driver line also contained a UAS-*Dicer-2* insertion to increase RNAi efficiency (10). We then switched to using a donated *w*/*elav*-GAL4 driver line, without a UAS-*Dicer-2* insertion, and a *w/repo*-GAL4 driver line that were both crossed into a white Canton-S (w-CS) genetic background for six generations. We crossed these lines with two of our UAS-RNAi effector lines (UAS-*CG2014* "*ND20-like"* and UAS-*CG9140* "*ND51*") that we also backcrossed into the same white Canton-S (w-CS) background for six generations. The developmental abnormalities observed when crossing these UAS-RNAi effector lines with the *elav-Dcr2*-GAL4 driver line disappeared when these lines were crossed with the donated *w/elav*GAL4 driver line (see Table S3). We simultaneously screened males from these crosses as well as both parental control lines and a Canton-S (CS) control line in our aggression assay. All crosses totaled approximately 20 flies as parents, and the sex ratio was close to 1:1. All the virgin females were grouped in tens and isolated for 5 d before being crossed. Crossing schemes are summarized in Table S6.

Behavioral verification of software

To verify that the set-up and software for the behavioral assay was working properly, we tested a high-aggression line as a positive control. We crossed our *Cha*GAL4/+ driver strain with our UAS-*tra/*+ effector strain to generate *Cha*GAL4*/*UAS-*Tra* male flies which have previously been shown to exhibit high male-male aggression activity due to feminized cholinergic neurons (11) (Fig S8).

Aggression assay and behavioral analyses

The fly arena was designed after (12), with several minor modifications: LED lights were used at the bottom of the arena as a light source instead of ring light bulbs for the purpose of keeping individual flies on the food floor.

Videos were qualified and trimmed in the Picture Motion Browser supplied with the Sony camcorder (Sony Corp.) and then converted from mp4 to Windows Media Video format [640 × 480 resolutions (video graphics array), high quality at 3,000 kbps]. The CADABRA software package (Caltech, Pasadena, CA) was used to track and quantify the behavioral activity of the fly pairs (12). First, the *qtrack* module was used to generate the feature files for all videos; the *analysis* module then loaded all the feature files, performed statistical analyses, and generated the figures.

Validation of gene knockdown

Pooled whole flies (3 males per test sample) of the knockdown and control lines (UAS-RNAi*/*+,

*elav*GAL4/+, and *repo*GAL4/+), were collected for quantitative polymerase chain reaction (qPCR) to validate the knockdown of target genes as described in (13). We were unable to assess mRNA levels for the UAS-RNAi/+ control for *ND20-like* due to a lack of flies. This is because we carried out the behavioral assay and qPCR assays first comparing the knockdown line with the Canton-S wild-type, and later added comparisons with the parental lines. At that time we had only enough parental flies to perform one of two parental control qPCR validations for *ND20-like*. The average final yield of RNA samples was approximately 1.5 mg, of which 200 ng was used for cDNA synthesis. mRNA levels were quantified relative to a standard reference gene, *rp49* (14). Gene expression results are shown in Fig S2-3. Primers for qPCR are listed in Table S7. We attempted to achieve a higher level of knockdown with a neuron-specific RNAi enhanced driver line, *elav-dicer2*GAL4 for 27 different OX-related genes. However, because most flies from these lines failed to develop or exhibited abnormal behavior (Table S3), the results reported here pertain only to the donated *elav*GAL4 line mentioned above. Gene expression of *ND20-like* for both *elav*GAL4/UAS-*ND20-like*-RNAi and *repo*GAL4/UAS-*ND20-like*-RNAi was reduced by about 50% compared to the relevant parental control lines (Fig. S2). Gene expression of *ND51* for both *elav*GAL4/UAS-*ND51*-RNAi and *repo*GAL4/UAS-*ND51*-RNAi was also reduced by about 50% relative to relevant parental control lines (Fig. S3).

Neuron knockdown of genes in the oxidative phosphorylation pathway by the elav-Dcr2-GAL4 *driver severely affects development*

To maximize the degree of knockdown in neuronal cells, we utilized the *elav-Dcr2*-GAL4 line (10) as the driver for genetic RNAi of target genes in the oxidative phosphorylation pathway. Preliminary screening was carried out by crossing the *elav-Dcr2*-GAL4 driver line with 27 of the UAS-RNAi lines (Table S3). Overall, progeny showed poor development. Thirteen of the 27 lines (48%) were unable to eclose to the adult stage. Five other lines eclosed, but did not survive for the minimum of 4 d for downstream behavioral screening. For example, *elav-Dcr2*-GAL4/UAS-*ND20*-RNAi showed weakness on walking, having no balance and leaning toward one side of the body. Flies in the other eight lines survived a complete life cycle, but the aggression assay showed no significant differences compared to the w^{1118} control. As a result we screened the lines using *elav*-GAL4 as opposed to *elav-Dcr2*-GAL4 whose increased knockdown resulted in developmental and/or behavioral alterations in the F1 progeny.

Statistical analyses

Non-parametric Kruskal-Wallis ANOVA tests followed by pairwise Mann-Whitney U Tests were used to compare aggression levels across RNAi flies and parental lines.

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Table S1. Drug doses and honey bee aggression scores.

Drug	Dosage (ug/bee)	Relative Dosage (effective dose = 1)	Aggression (relative to control = 1)	t	P
Fenpyroximate	0.033	0.1	1.07	0.42	0.68
	0.25	.75	1.16	1.02	0.31
	0.33	**1**	**1.28**	**2.03**	**0.050**
	0.65	2	0.85	-1.4	0.17
Tetradifon	5.2	0.25	1.06	0.17	0.87
	21.1	**1**	**1.3**	**2.1**	**0.049**
	42.2	2	0.80	-1.3	0.20

N=40 for all Fenpyroximate except for the effective dose, N=39. N=20 for all doses of Tetradifon. Bold terms are significant at $P<0.05$.

Table S2. Drug doses and honey bee survivorship.

Drug	Dosage (ug/bee)	Relative Dosage (effective dose = 1)	Survivorship (relative to control = 1)	t	P
Fenpyroximate	0.033	.1	0.96	-0.28	0.78
	0.25	.75	1.00	0.35	0.73
	0.33	**1**	**0.95**	**-2.4**	**0.019**
	0.65	**2**	**0.89**	**-3.7**	**0.004**
Tetradifon	**5.2**	**.25**	**0.96**	**-2.52**	**0.04**
	21.1	1	0.99	-0.39	0.7
	42.2	2	0.95	-1.59	0.12

Bold terms are significant at $P<0.05$.

Table S3. Results of *elav-Dcr2*-Gal4/UAS-RNAi screening tests

Oxidative phosphorylation complex	VDSC stock no.	*Drosophila* gene name	Development status of *elav-Dcr2*Gal4;UAS-RNAi	Detected as aggressive vs. w^{1118}
I	3923	*CG10664*	Failed to eclose	—
I	8837	*CG10320*	Died in early adulthood	—
I	35437	*Pdsw*	Died in early adulthood	—
I	23088	*CG9306*	Alive	No
I	37463	*CG12400*	Died in early adulthood	—
I	109816	*CG3621*	Alive	No
I	50731	*ND20-like (CG2014)*	Alive but weak	—
I	29236	*CG15434*	Alive	No
I	43183	*ND51 (CG9140)*	Died in early adulthood	—
I	101489	*CG12203*	Died in early adulthood	—
I	13130	*CG6020*	Failed to eclose	—
I	39232	*Mitochondrial acyl carrier protein 1*	Failed to eclose	—
II	6031	*CG6666*	Failed to eclose	—
III	107515	*CG30354*	Alive	No
III	33849	*CG14482*	Alive	No
III	35828	*Oxen*	Failed to eclose	—
III	9180	*CG4769*	Alive	No
IV	26848	*CG14235*	Failed to eclose	—
IV	44490	*Cytochrome c oxidase subunit Va*	Failed to eclose	—
IV	40977	*CG2249*	Alive	No
IV	13403	*Cyclope*	Alive	—
V	35385	*ATPase coupling factor 6/ATPsyn-Cf6, CG4412*	Failed to eclose	—
V	13324	*CG4692*	Failed to eclose	—
V	12792	*Oligomycin sensitivity-conferring protein*	Failed to eclose	—
V	107311	*Lethal(2)06225*	Failed to eclose	—
V	16538	*ATP synthase-gamma chain/ATPsyn-gamma*	Failed to eclose	—
V	34664	*Bellwether*	Failed to eclose	—

Dark gray genes failed at the pupal stage. Light gray genes failed before reaching 4 d old.

Table S4. Fixed effect tests for a linear mixed model for aggression score (group is a random effect)

FENPYROXIMATE

Source	DF	F	P
Pair	2	1.7594	0.1777
Drug trt	1	2.1192	0.1487
Social env	1	0.0955	0.7579
Drug trt * Social env	**1**	**4.2177**	**0.0427**
Pair * Drug trt	2	0.0242	0.9761
Pair * Soc env	2	0.8547	0.4286
Pair * Drug trt * Social env	2	0.5716	0.5665

TETRADIFON

Source	DF	F	P
Pair	**2**	**3.6688**	**0.0294***
Drug trt	1	0.1434	0.7058
Social env	1	0.8151	0.3690
Drug trt * Socal env	**1**	**5.3908**	**0.0225***
Pair * Drug trt	2	0.8318	0.4386
Pair *Soc trt	2	0.0291	0.9713
Pair * Drug trt * Social env	2	0.2883	0.7502

Fenpyroximate: model represents data from N=51 groups (social environment = disturbed) and N=51 groups (social environment = control). Tetradifon: model represents data from N=48 groups (social environment = disturbed) and N=49 groups (social environment = control). Bees for both drug treatments were collected from the same three pairs of colonies. “env” = environment, “trt” = treatment. See Table S5 for sample sizes for this analysis. Bold terms were significant in the model.

Table S5. Sample sizes for Experiment 3. Groups with 50% or greater mortality for either drug-treated or acetone-treated bees were omitted to arrive at the final sample size.

Colony Pair	Social Treatment	Drug Treatment	Final sample size (Number of groups)*
1	Disturbed	Fenpyroximate	19
		Tetradifon	15
1	Undisturbed	Fenpyroximate	18
		Tetradifon	13
2	Disturbed	Fenpyroximate	19
		Tetradifon	15
2	Undisturbed	Fenpyroximate	19
		Tetradifon	19
3	Disturbed	Fenpyroximate	13
		Tetradifon	18
3	Undisturbed	Fenpyroximate	14
		Tetradifon	17

*Each group consists of 4 bees treated with the drug and 4 treated with acetone-control.

Table S6. Schemes of *Drosophila* crosses

Genotype	Maternal parent	Paternal parent
*elav*Gal4/UAS *ND20-like* RNAi	*elav*Gal4/TM3,Sb	UAS *ND20-like* RNAi/TM3, Sb
*repo*Gal4/UAS *ND20-like* RNAi	*repo*Gal4/TM3,Sb	UAS *ND20-like* RNAi/TM3, Sb
*elav*Gal4/UAS *ND51* RNAi	*elav*Gal4/TM3,Sb	UAS *ND51* RNAi/TM3, Sb
*repo*Gal4/UAS *ND51* RNAi	*repo*Gal4/TM3,Sb	UAS *ND51* RNAi/TM3, Sb
UAS *ND20-like* RNAi/+	UAS *ND20-like* RNAi/TM3, Sb	+/+ (wCS)
UAS *ND51* RNAi/+	UAS *ND51* RNAi/TM3, Sb	+/+ (wCS)
*elav*Gal4/+	*elav*Gal4/TM3,Sb	+/+ (wCS)
*repo*Gal4/+	*repo*Gal4/TM3,Sb	+/+ (wCS)

Table S7. Primers used for RNAi qPCR validation in *Drosophila*

Primer name	Sequence
RP49-F	CCCACGGGATTCAAGAAGTTC
RP49-R	GCATGAGCAGGACCTCCAG
ND20-F	GCATTGTTCCGGTGGACATC
ND20-R	TCCGTACATTAAGGCCTCGG
ND51-F	AAGAATGCGTGCGGAACAG
ND51-R	CCGCAAATGTAAGCTCCAGC

FIGURES

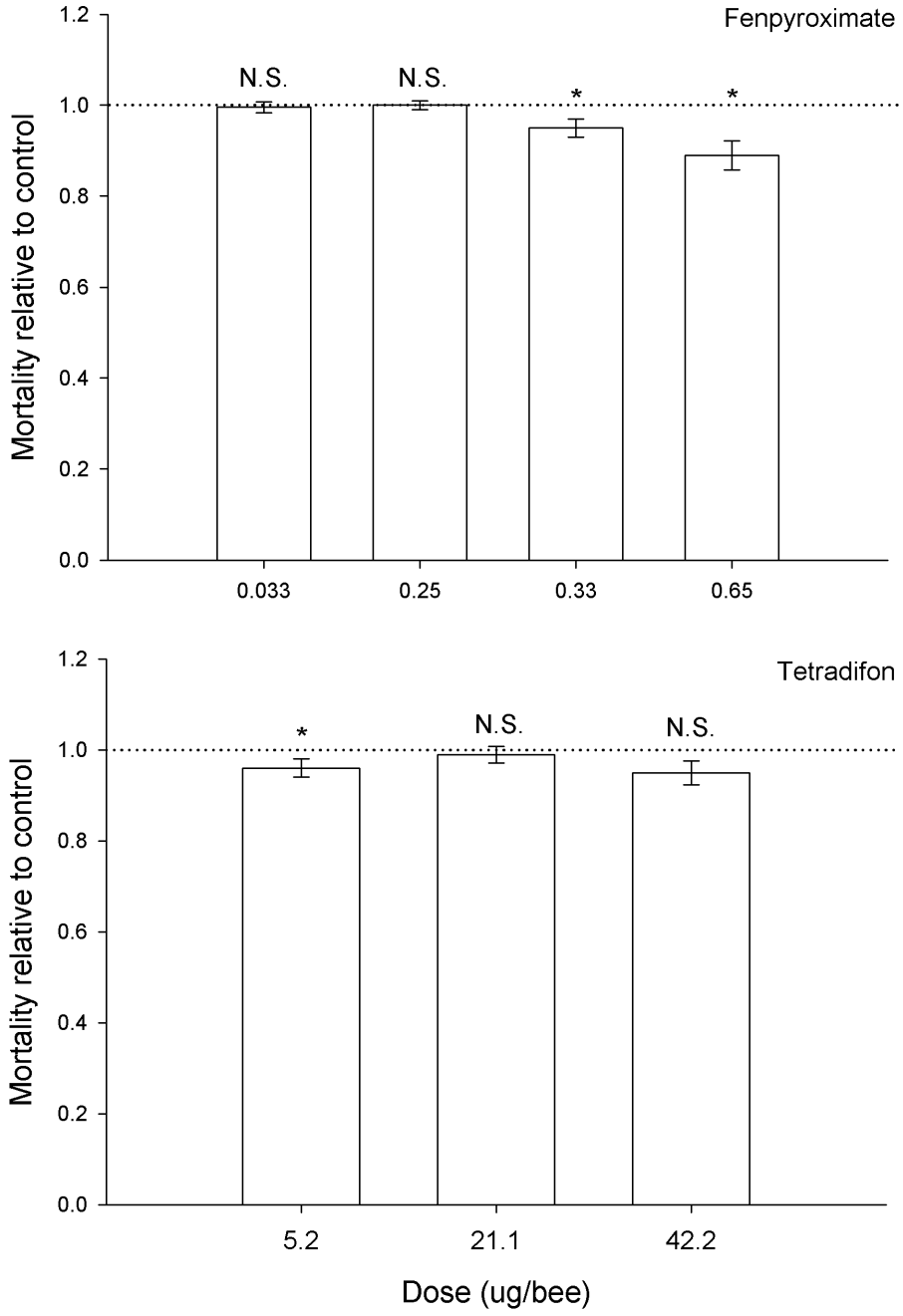

Fig S1. Survivorship relative to control for Fenpyroximate (top) and Tetradifon (bottom). Control survivorship is set to one (indicated by horizontal dotted line). Asterisks indicate a significant difference in survivorship comparing treatment to control ($P<0.05$). Data are shown in Table S2.

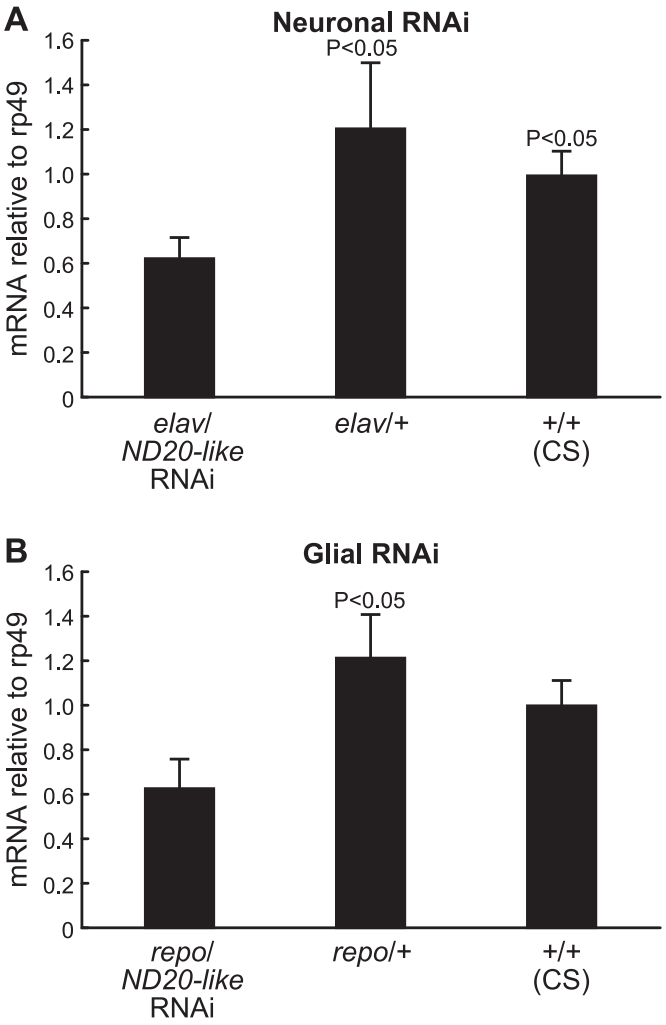

Fig S2. Validation of *ND-20-like* knockdown by qPCR. N = 30 and 15 respectively for *elav*/*ND20-like* RNAi, and *elav*/+. N = 21 and 18 respectively for *repo*/*ND20-like* RNAi and *repo*/+. +/+ (CS) is the expression level for wild-type canton-S flies (N=9). Significance scores are for *t*-Tests of expression relative to knockdown flies.

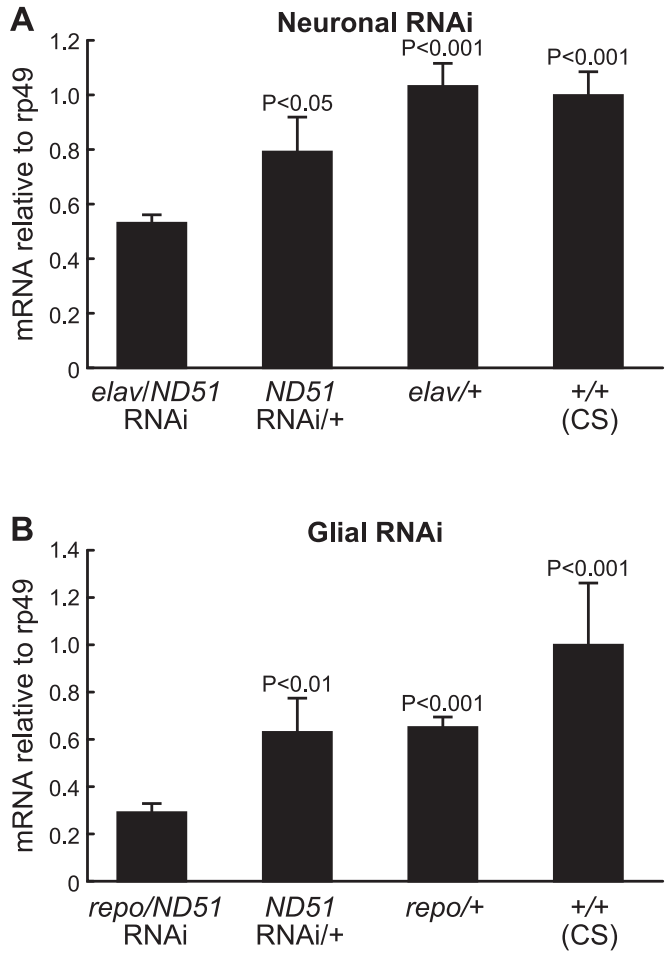

Fig S3. Validation of *ND51* knockdown by qPCR. N = 21, 9, and 15 respectively for *elav*/*ND51* RNAi, *ND51* RNAi/+, and *elav*/+. N = 12, 12, and 12 repectively for *repo*/*ND51* RNAi, *ND51* RNAi/+, and *repo*/+. +/+ (CS) is the expression level for wild-type canton-S flies (N=9). Significance scores are for *t*-Tests of expression relative to knockdown flies.

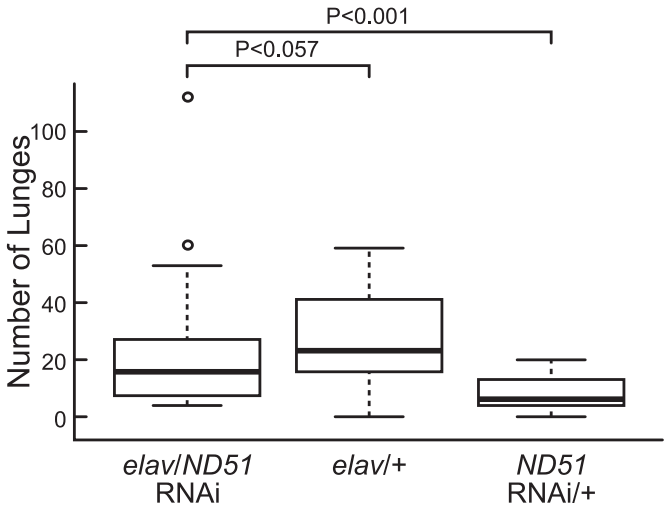

Fig S4. Effect of neuronal RNAi knockdown of metabolic genes on aggression in *Drosophila*. *ND51* neuronal knockdown flies (*elav*/*ND51* RNAi, N = 27 pairs) showed significantly more lunging compared to one of two heterozygous parent controls (*ND51* RNAi/+, N = 20 pairs, *elav*/+, N = 23 pairs, Kruskal-Wallis Test: $H_2 = 18.5$, $P < 0.0001$, post-hoc Mann-Whitney U Tests: *elav*/*ND51* RNAi versus *ND51* RNAi/+, $Z = 3.07$, $P < 0.001$; *elav*/*ND51* RNAi versus *elav*/+ $Z = -1.58$, $P < 0.057$).

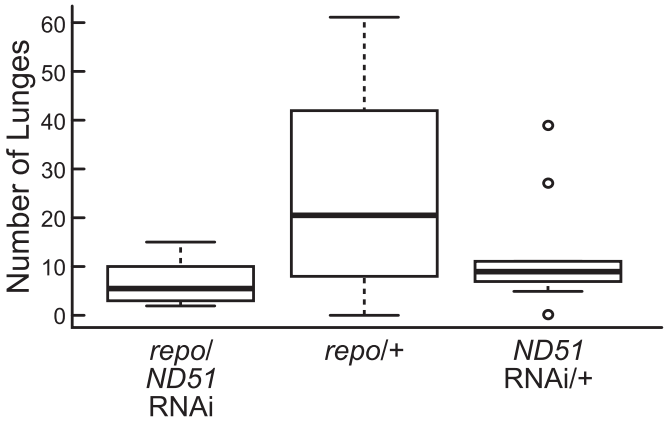

Fig S5. Effect of glia-specific RNAi knockdown of metabolic genes on aggression in *Drosophila*. *ND51* glial knockdown flies (*repo*/*ND51* RNAi, N = 10 pairs) showed no differences in lunging compared to heterozygous parent controls (*ND51* RNAi/+, N = 9 pairs, *repo*/+, N = 10 pairs, Kruskal-Wallis Test, $H_2 = 5.4$, $P = 0.07$).

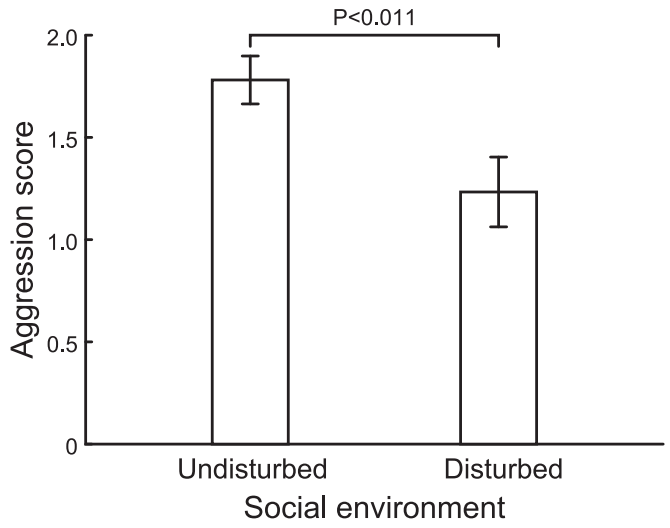

Fig S6. Sensitivity of a laboratory Intruder Assay to colony manipulations known to depress aggression in the field. Bees from a chronically disturbed low-aggression test colony (N = 18 groups collected) show decreased aggression during the Intruder Assay relative to an undisturbed control colony (N = 19 groups collected).

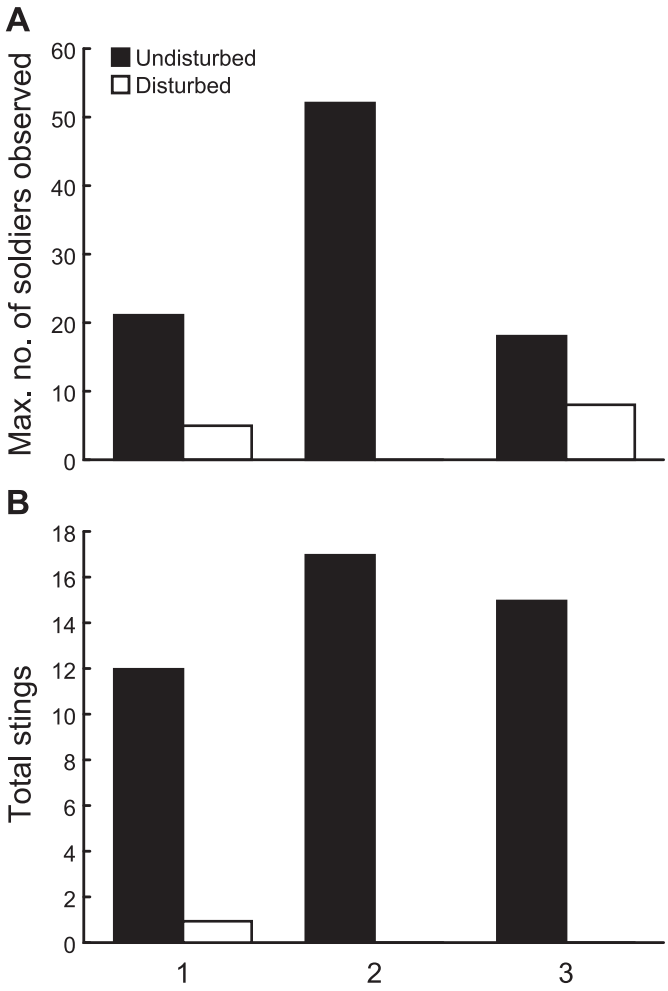

Fig S7. Chronic disturbance caused decreased honey bee aggression in a field aggression assay (N = 3 pairs of disturbed and undisturbed colonies, P = 0.05 for both maximum number of soldiers and total stings). Bees were collected from these colonies for drug treatment (Fig 4).

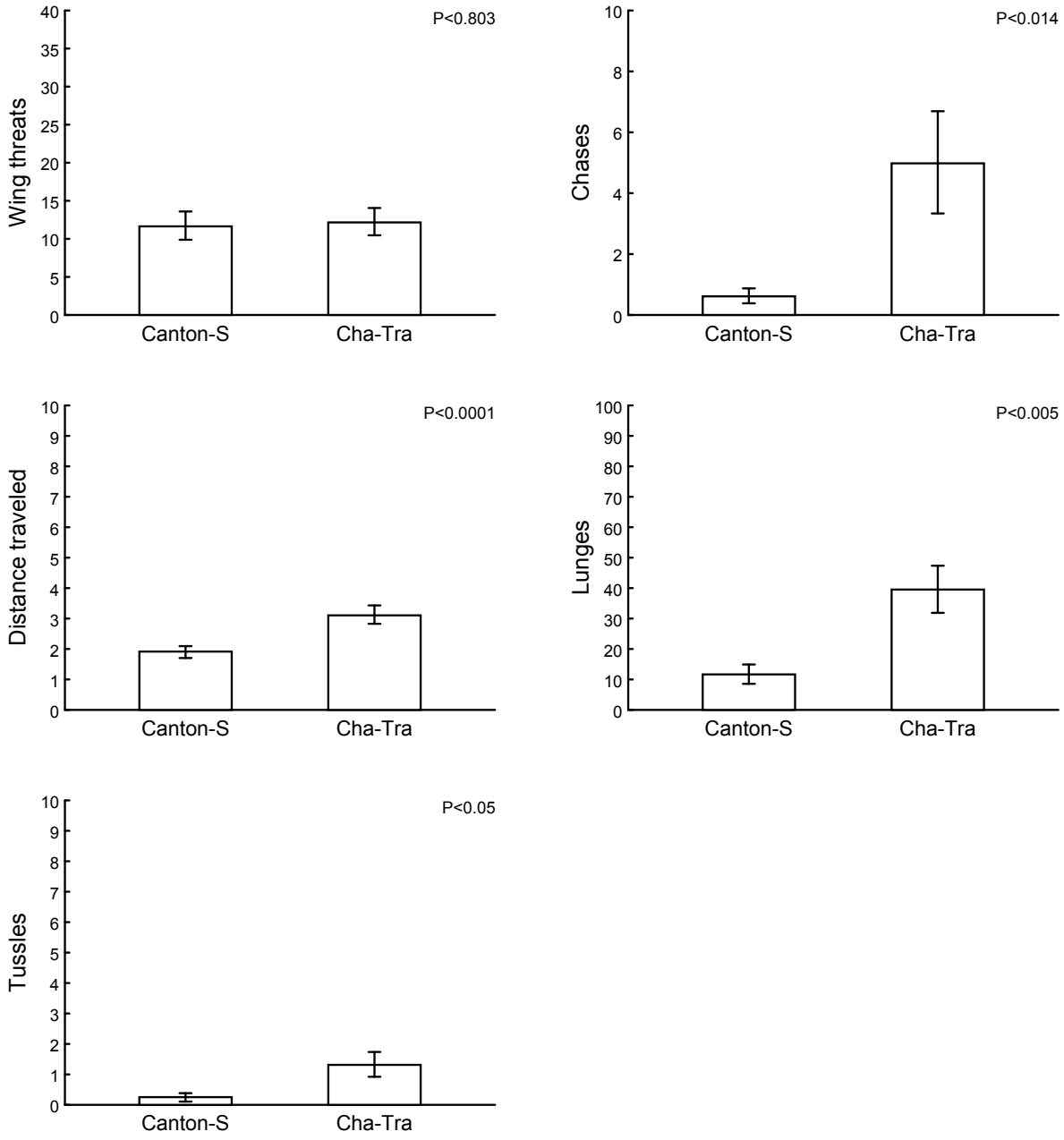

Fig S8. Compared to Canton-S (+/+) (N=12), the *Cha-Tra* positive control (N = 13) line showed significantly greater numbers of lunges, tussles, chases, as well as greater travel distances.