

# Molecular analysis of flies selected for aggressive behavior

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Aggressive behavior is pervasive throughout the animal kingdom, and yet very little is known about its molecular underpinnings. To address this problem, we have developed a population-based selection procedure to increase aggression in *Drosophila melanogaster*. We measured changes in aggressive behavior in the selected subpopulations with a new two-male arena assay. In only ten generations of selection, the aggressive lines became markedly more aggressive than the neutral lines. After 21 generations, the fighting index increased more than 30-fold. Using microarray analysis, we identified genes with differing expression levels in the aggressive and neutral lines as candidates for this strong behavioral selection response. We tested a small set of these genes through mutant analysis and found that one significantly increased fighting frequency. These results suggest that selection for increases in aggression can be used to molecularly dissect this behavior.

Aggression is a complex social behavior influenced both by genetic and environmental factors and is still poorly understood. In mammals, different types of aggression have been distinguished<sup>1</sup>, some of which are genetically separable<sup>2</sup> and may involve separate underlying circuitry. In insects, aggression often occurs in the context of competition for limited resources in the environment and frequently leads to some type of territorial defense<sup>3</sup>. Defense of a limited resource, typically food or mating partners, is likely to help individuals survive and pass on their genes through the generations, and this may explain why aggressive behavior is so widespread throughout the animal kingdom<sup>4</sup>. Not surprisingly, aggressive behavior has also been identified in *Drosophila melanogaster*<sup>5–22</sup>. Jacobs gave the first detailed description of the behavior<sup>6</sup>. Dow and von Schilcher highlighted the ecological significance of fly aggression by showing a correlation between territorial defense and mating success<sup>7</sup>. Hoffmann conducted a thorough quantitative analysis of territoriality in *D. melanogaster* and its sibling species *D. simulans*<sup>10</sup>. He further focused on the former in a series of studies investigating heritability, conditions and ecology of its territoriality<sup>11–17</sup>. Recently, a modification to the setup used in these studies has been published<sup>21</sup> and a description of female aggression has also been reported<sup>19,22</sup>.

In mice, a number of genes have been identified with effects on aggressive behavior<sup>23</sup>. Several of these genes were serendipitous findings, and many of them have been interpreted as having effects on serotonin<sup>23</sup>. A comprehensive or unbiased molecular analysis of aggression has never been attempted in any laboratory species, including *D. melanogaster*, despite its significant genetic resources. One reason for this may relate to the complexity of the behavior, making it difficult to develop a simple assay to conduct high-throughput genetic screens. Second, anecdotal evidence suggests that fly aggression is a labile phenotype. Most laboratory *D. melanogaster* strains have lower levels of aggression than wild-caught strains, which seem to lose it quite rapidly upon domestication. This would suggest

then that the loss of aggression in laboratory strains is due to relaxation of selection pressure, and it should therefore be a recoverable phenotype under renewed selection pressure.

Therefore, we developed a population-based selection procedure to enhance aggression in *D. melanogaster* in an unbiased manner. Selection for aggression represents a forward genetic approach and is limited only by the existing variation in the starting population. Through selection, we generated two aggressive and two neutral fly populations derived from a single starting population and quantified the behavioral selection response with a simple two-male arena assay. After only ten generations of selection, both aggressive selected lines were significantly more aggressive than both neutral lines, and these differences further increased under continued selection pressure. To identify candidate genes that may explain the behavioral differences between these lines, we performed microarray expression analysis on the heads of these different lines after 21 generations of selection. To confirm direct involvement of some of these candidate genes, we analyzed mutants corresponding to a subset of genes and showed that one of them is capable of independently contributing to aggressive behavior.

## RESULTS

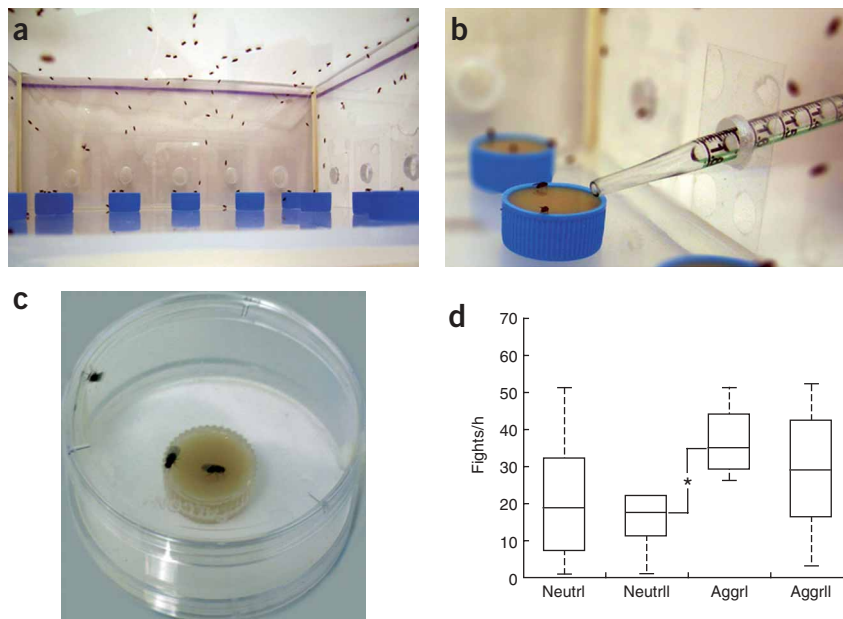
### The elements of male aggressive behavior

Male flies display a variety of aggressive behaviors, most of which have been previously described<sup>6,7,10,21</sup>. Below we describe a population-based selection system for increased aggression and two separate methods for quantifying the changes that occurred throughout the selection. To better illustrate the behaviors quantified in these different systems and to show how flies were picked for selection, we filmed animals under different conditions (for descriptions, see **Supplementary Videos 1–3**).

We also noticed a behavior that has not been previously described in *D. melanogaster* in which territorial males lower their abdomens

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**Figure 1** Population cage and territorial assay. **(a)** Frontal view of Plexiglas population cage that contains 120 males and 60 females, with 11 blue territories aligned to three of the four walls, and 13 holes (two for the corner territories) corresponding to the 11 territories. **(b)** An aspirator goes through the hole in the wall to remove flies from the blue territory (the hole is covered with a transparency with slits cut towards the middle, so that aspirator can go through without releasing flies) (see also **Supplementary Video 5** and **Supplementary Fig. 1**). **(c)** Territorial quantification assay with Eppendorf cup and two males and one mated female to count total number of fights. **(d)** Box plot of the total number of fights per hour at Gen8 ( $n = 10$  pairs per line tested). Upper and lower edges of boxes correspond to the 25% and 75% quantiles. The median (50% quantile) is shown as a horizontal line in the box, and the dashed lines show the 5% and 95% quantiles. Asterisk shows medians that are statistically significantly different ( $P < 0.05$ ), by Kruskal-Wallis ANOVA for unpaired groups.

and brush their genital region on the food surface while walking as if to mark the territory (**Supplementary Video 4**). A similar behavior, referred to as abdomen dipping, has been described in other dipteran species, in which it has been associated with territorial behavior<sup>24–26</sup>.

### Selection on escalating males

To select males for increased aggression, we designed a population cage (**Fig. 1a**) with multiple territories, from which we could easily remove the most aggressive males as they fought (**Fig. 1b**; see Methods; **Supplementary Video 5**). As a control for the aggressive selected population cage, we used an identical setup from which we removed and discarded 15 to 30 aggressive males so that both cages would be exposed to the same selection stimuli (see Methods). At the end of each experiment, we anesthetized the control cage and picked random males from the remaining population as the founders for the next generation. We started every generation by mating the males from the selected or control group with random virgin females from that same generation. We performed the entire selection experiment in duplicate and thus created four subpopulations that we kept separate throughout. Thus, two of these were aggressive selected lines and two were neutral selected, (denoted AggrI, AggrII, NeutrI and NeutrII, respectively).

### Aggr lines are more aggressive than Neutr lines

To evaluate whether the selection procedure produced a change in aggression between the different populations, we tested the four selected populations after eight generations of selection (Gen8). For this first evaluation, we used a simplified territorial assay consisting of a scaled-down version of the assay described previously<sup>7,10</sup> (**Fig. 1c**). We loaded two males and one mated female in each of ten small Petri-dish cages with a small food territory and filmed them for 1 h for each population. The total number of fights per hour per cage was higher for both Aggr lines, although this difference with the Neutr lines was significant only between AggrI and NeutrII ( $P < 0.05$ , **Fig. 1d**).

To quantify aggression by a faster, high-throughput procedure, we developed a simpler assay in which we could analyze 20 pairs of males at once in a rectangular chamber containing 20 separate arenas devoid of territorial resources (food and females) (see Methods and **Supplementary Fig. 1**). We started using this ‘arena assay’ on flies from

Gen11 and tested the selected flies again ten generations later. Four parameters were scored to obtain a detailed aggression profile for all the lines: (i) frequency of fighting (percentage of pairs that show any fighting during the observation time); (ii) latency to fighting (time until any fighting occurs averaged over the fighting pairs, not including the nonfighting pairs); (iii) fighting index (total amount of time spent fighting expressed as a percentage of total observation time for the fighting pairs); and (iv) intensity of fighting (number of high-intensity elements such as holding or tossing (**Supplementary Video 3**), again averaged over the fighting pairs). After 11 generations of selection, both Aggr lines were significantly more aggressive than both Neutr lines for fighting frequency ( $P < 0.001$ , **Fig. 2a**, white bars). The three other parameters were also significantly different when we included all pairs (including the nonfighting pairs) in the calculations (**Table 1**).

### Selection response further increases with continued selection

We continued the selection for another ten generations; at this point, both Aggr lines also became significantly different from both Neutr lines for the other aggression parameters even when nonfighting pairs were excluded (index,  $P < 0.001$ ; latency,  $P < 0.001$ ; intensity,  $P < 0.01$ , **Fig. 2a–d**, gray bars), demonstrating that the flies from the Aggr lines are not only more likely to fight but also that the flies that engage in fighting fight faster, longer and more intensely. We collapsed the data for both Neutr lines and both Aggr lines because of the small number of fighting pairs in the Neutr lines and because the trend in both Neutr and both Aggr lines were similar. The Neutr lines showed a tendency of decreased aggression for all parameters at Gen21 compared with Gen11, suggesting that there is also a downward trend of selection based on discarding a proportion of aggressive males from these control cages. However, because this trend is not significant, we consider the reference lines as neutral lines rather than lines with decreased levels of aggression. The strong increase in aggression in the Aggr lines is most obvious by comparing the average fighting index between the Aggr and Neutr lines when nonfighting pairs are included, which demonstrates a difference of more than 30-fold between the two most extreme lines, AggrI and NeutrII at Gen21 (**Table 1**).

**Figure 2** Fighting parameters in arena assay at Gen11 (white bars) and 21 (gray bars).

(a) 'Fighting frequency' represents the mean percentage of arenas that show fighting in four subpopulations at Gen11 (white) and 21 (gray). Error bars represent s.e.m. ( $n = 80$  pairs per line per generation tested). Significantly different groups are indicated by letters above the bars.

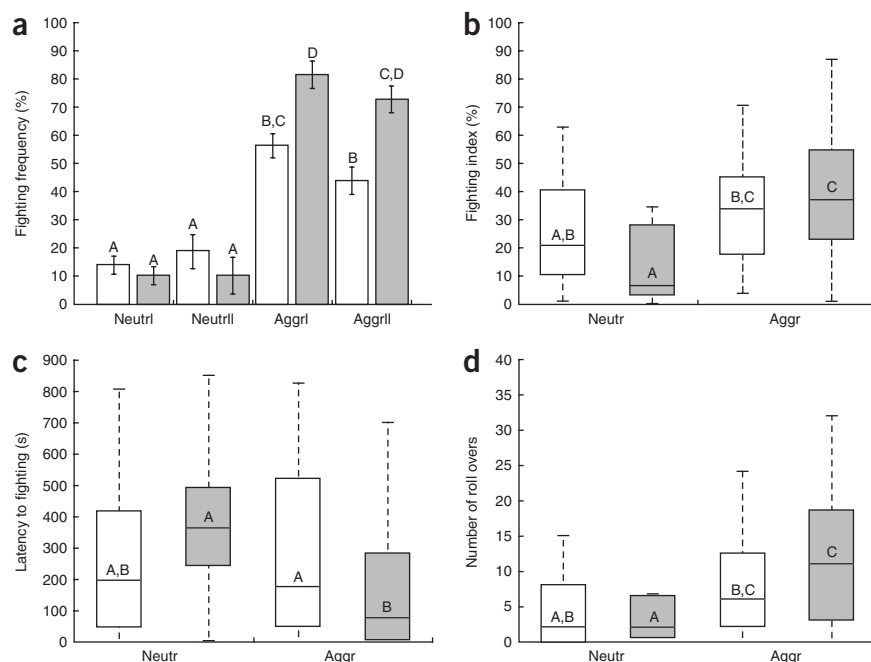
(b) 'Fighting index' represents the median percentage of fighting over the total observation period of the fighting pairs only. Neutr and Aggr groups are combined for statistical power ( $n = 23$ ,  $n = 15$ ,  $n = 63$ ,  $n = 123$ , number of pairs corresponding to each box).

Significantly different groups are indicated with letters above each median.

(c) 'Latency to fighting' represents the median latency of fighting pairs only. Neutr and Aggr groups are again combined ( $n = 23$ ,  $n = 15$ ,  $n = 63$ ,  $n = 123$ ). Significantly different groups are indicated by letters above the medians.

(d) 'Intensity of fighting' represents the median number of intense fighting elements (Supplementary Video 3) for fighting pairs only. Neutr and Aggr groups are again combined ( $n = 20$ ,  $n = 8$ ,  $n = 52$ ,  $n = 45$ ). Statistically significant groups are indicated with letters above the medians.

All groups were compared using Kruskal-Wallis ANOVA followed by *post hoc* tests to identify the significantly different groups. Horizontal lines in the boxes represent 50% quantile (median), and the upper and lower edges of the boxes denote the 25% and 75% quantiles. Dashed lines show the 5% and 95% quantiles. White plots correspond to Gen11, and gray plots to Gen21. The letters above each plot denote significantly different groups. For example, 'A' is different from 'B' but not from 'A,B', etc.



As an additional method for assessing the increase in aggression between the Aggr and Neutr lines, we directly quantified the levels of aggression in the population cage by counting the number of escalations in a given time period. Both Aggr lines had a significantly higher number of escalated encounters than the Neutr lines, in which escalations were rare ( $P < 0.001$ , Fig. 3a). These experiments also showed that there did not seem to be a circadian component to aggression, as males escalated at a similar rate throughout the day (data not shown). Finally, we looked at aggression between, rather than within, the Neutr and Aggr lines (in all Aggr-Neutr combinations) both in the population cage and in a one-on-one situation in the arena assay. In mixed population cages (with 50% Aggr and 50% Neutr flies), the Aggr flies were involved in all of the escalated encounters, whereas Neutr males almost never engaged in escalations

( $P < 0.001$ , Fig. 3b). In mixed arena assays (one Aggr male paired with one Neutr male), Aggr males dominated Neutr males far more often than the reverse ( $P < 0.001$ , Fig. 3c).

### Aggr lines are not more active than Neutr lines

To evaluate the specificity of the selected phenotype (that is, whether any other parameters might have changed between the Neutr and Aggr lines over the course of the selection) we tested the four subpopulations in a variety of other behavioral tests at Gen22 and Gen23. We first evaluated the activity of the different lines to investigate whether the Neutr lines were not simply populations of sluggish flies. We used the Trikinetics system to measure the activity profile of flies as has been previously described<sup>27,28</sup>. Although the NeutrII line tended to be more active, none of the lines differed significantly from one another in total activity counts or relative activity counts (that is, normalized for any differences that flies might show in patterns of rest and activity) ( $P = 0.1445$ , Fig. 4a). None of the lines showed any differences in circadian rhythm or phase (data not shown).

Because of the potential relevance of mating behavior to aggression<sup>7,10</sup>, we next evaluated whether the flies showed any differences in courtship or mating. Using a standard courtship paradigm, we found no differences in any of the courtship parameters (data not shown) or in total courtship index ( $P = 0.68$ , Fig. 4b). We next determined mating parameters and evaluated single-pair matings in a small mating chamber with virgin females from the background Canton-S strain. We found no differences in mating duration ( $P = 0.34$ , Fig. 4c) or latency to mating ( $P = 0.34$ , Fig. 4d), although the AggrI line had 12.5% fewer matings in the 30-min observation period ( $P = 0.068$ , data not shown). We also investigated whether flies from the Aggr lines would outperform flies from the Neutr lines in a mating competition assay with one virgin female from the background strain.

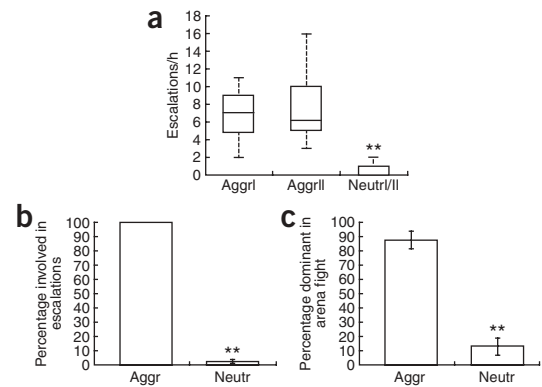
**Table 1** Mean fighting latency, index and intensity at Gen11 and Gen21 with all pairs included

	Latency (s) $n = 80$		Index (%) $n = 80$		Intensity <sup>a</sup> $n = 40$	
	Gen11	Gen21	Gen11	Gen21	Gen11	Gen21
NeutrI	807 (27)	850 (14)	2.8 (0.9)	1.5 (0.7)	0.1 (0.1)	0.6 (0.3)
NeutrII	788 (30)	840 (19)	4.8 (1.5)	1.1 (2.6)	1.5 (0.5)	0.6 (0.3)
AggrI	537 (42)	275 (33)	16.7 (2.2)	33.4 (0.5)	5.4 (1.0)	10.7 (2.6)
AggrII	634 (39)	390 (38)	13.3 (2.2)	25.9 (2.8)	3.0 (0.7)	9.7 (2.2)

Indices are as a percentage of total observation time, and intensities reflect the number of high-intensity elements such as holding and tossing (shown in Supplementary Video 3). Numbers are the means of 80 pairs (except for intensity, for which  $n = 40$ ), and numbers in parentheses reflect s.e.m. All Aggr-Neutr comparisons are statistically significant except the comparison between AggrII and NeutrII for intensity (Kruskal-Wallis ANOVA followed by multiple comparison test).

<sup>a</sup>Intensity is measured as the number of high-intensity elements.

**Figure 3** Aggression quantification in population cage at Gen22 and Aggr-Neutr mixed populations and pairs. **(a)** The median number of escalations per hour over 6 h for AggrI, AggrII, NeutrI/II combined (number of experiments per group = 3; number of escalations per group = 175, 164 and 16, respectively). The median is represented by the horizontal lines in the boxes; the upper and lower edges of the boxes represent the 25% and 75% quantiles. Dashed lines represent 5% and 95% quantiles. Asterisks indicate statistically significant differences ( $P < 0.001$ ). **(b)** Proportion of aggressive versus neutral flies involved in escalation in mixed population cages with 60 Neutr and 60 Aggr males. Bar graphs show the mean of four combinations of three replicate experiments, each 3–4 h in duration (representing all combinations between aggressive and neutral lines), and error bars represent s.e.m. Asterisks denote statistical significance ( $P < 0.001$ ). **(c)** Proportion of dominant Aggr or Neutr males from Aggr-Neutr mixed pairs tested in arena assay. Bar graphs represent mean of four combinations of 70 pairs tested (again representing all Aggr-Neutr combinations), and error bars represent s.e.m. Asterisks denote statistical significance ( $P < 0.001$ ). All groups were compared statistically using the Kruskal-Wallis ANOVA for unpaired groups **(a)** and the Wilcoxon test for paired groups **(b,c)**.



taken into account for further downstream molecular analysis (see below).

### Differential gene expression in Aggr and Neutr fly heads

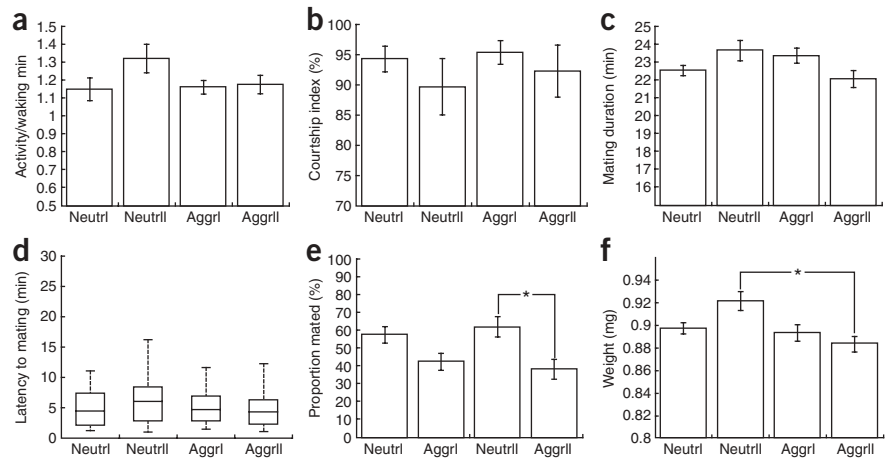
Because we found strong differences in aggression between the four selected populations, and because aggression levels are likely to be strongly influenced by the brain, we decided to evaluate the differences in gene expression in the heads of these flies to see if we could find genes expressed in their brains that might be correlated with the changes in behavior. Because all aggression parameters were significantly changed at Gen21, we performed microarray expression analysis on heads from Gen21 flies (three replicates per line, Affymetrix *Drosophila* Genome Array version 1). Using the GeneSpring platform (version 7.2), first we statistically compared the triplicate array results of any two pairs of lines to evaluate how many genes differed significantly between groups to get an idea of the divergence that had occurred between these lines throughout the selection. We found that the number of genes with significantly changed expression levels between any two lines ranged from 474–775 at a significance level of 0.05. The Aggr-Neutr comparisons showed no more expression differences than the AggrI-AggrII or NeutrI-NeutrII comparisons. To avoid pursuing candidate genes that might correlate with traits that were accidentally coselected but unrelated to aggression (discussed above; Fig. 4), we statistically compared the four lines using aggression

Notably, both Aggr lines mated less than the expected 50% when competing with males from the Neutr lines (we tested AggrI versus NeutrI and AggrII versus NeutrII; only AggrII versus NeutrII was significant ( $P < 0.01$ ); Fig. 4e). This is unexpected, as it has been previously reported that territorial males are more successful at copulating with females<sup>7,10</sup>. As our mating competition experiments were done in a mating wheel without a territory, these results suggest that mating success depends on the territorial context and is not inextricably coupled to aggression. Indeed, mating success of the Aggr and Neutr males in mixed population cages depends on whether the matings occur in the territory or elsewhere in the cage (H.A.D., unpublished observations).

Finally, we determined whether the lines showed any differences in size by weighing groups of approximately 20 flies. The NeutrII line was slightly heavier than the other lines, although significantly so only when compared with AggrII ( $P < 0.01$ , Fig. 4f).

The above-described experiments show that traits other than the ones under direct selection can vary over time, a feature that is common in artificial selection experiments<sup>29</sup>. Such random changes are undoubtedly reflected in the animal's genome and should be

**Figure 4** Control behaviors tested in selected lines at Gen22 and 23. **(a)** Bar graphs show mean activity counts ( $\pm$  s.e.m.) normalized per waking minute for 20 males per line tested for 3 d on a 12 h light/12 h dark cycle. **(b)** Bar graphs show mean courtship index ( $\pm$  s.e.m.) for 20 males per line, all tested against Canton-S females. **(c)** Bar graphs show mean mating duration ( $\pm$  s.e.m.) of 40 males per line paired with virgin females of Canton-S. **(d)** Box plots show median mating latency of 40 males per line paired with Canton-S virgin females for 30 min. Horizontal lines represent median (50% quantile), boxes represent 25% and 75% quantiles, and dashed lines denote 5% and 95% quantiles. For comparison of the medians, we performed a Kruskal-Wallis ANOVA for unpaired groups. **(e)** Mating competition between Aggr and Neutr males with Canton-S virgin females. Bar graphs show the proportion ( $\pm$  s.e.m.) of Neutr and Aggr males that mated in 40 pairs of AggrI-NeutrI and 40 pairs of AggrII-NeutrII combinations. Statistical analysis was done by *t*-test of paired samples ( $P < 0.01$ ). **(f)** Bar graphs show the mean male weight per line ( $\pm$  s.e.m.) of eight groups of 20 flies. Asterisk shows groups that are statistically significantly different ( $P < 0.01$ ). Statistical analysis comparing means was done by ANOVA. *Post hoc* tests were done to identify significantly different groups. Note that the y axes in **a**, **b**, **c** and **f** are truncated to show better detail.



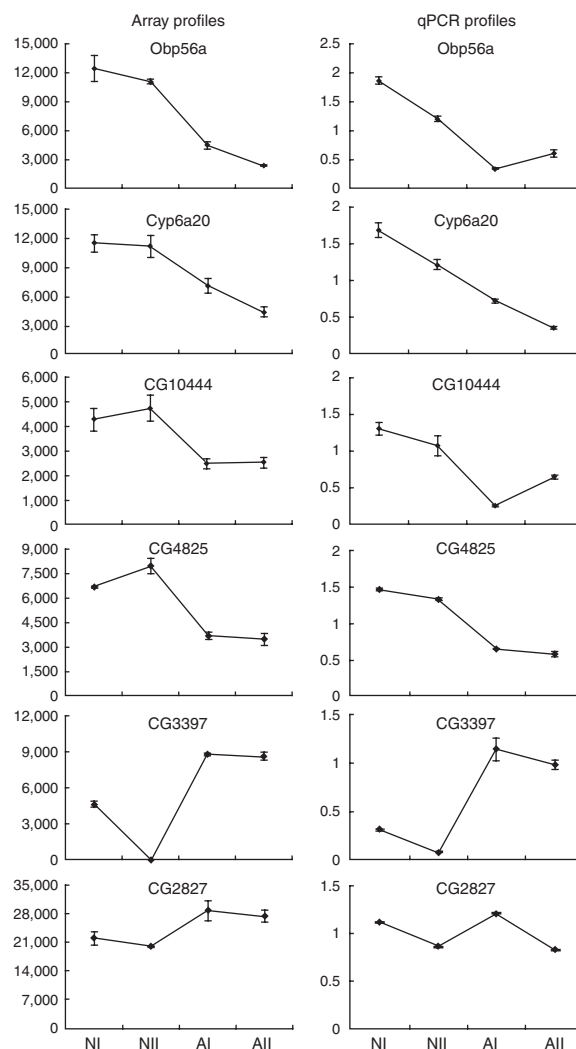


**Table 2** Genes whose expression differs  $\geq 25\%$  between Aggr and Neutr lines

Gene	Expression in Aggr (as a multiple of that in Neutr)	Gene	Expression in Neutr (as a multiple of that in Aggr)
<i>CG16978</i>	2.55	<i>Obp56a</i>	2.50
<b><i>CG3397</i></b>	2.10	<i>CG11458</i>	1.98
<i>Drs</i>	2.08	<b><i>CG4825</i></b>	1.66
<i>CG11899</i>	1.61	<i>Dh</i>	1.63
<i>CG7900</i>	1.54	<i>GNBP1</i>	1.62
<i>Est8</i>	1.53	<b><i>CG10444</i></b>	1.55
<i>CG32444</i>	1.53	<i>CG13252</i>	1.49
<i>CG5195</i>	1.48	<i>CG2555</i>	1.47
<i>CG18162</i>	1.48	<b><i>Cyp6a20</i></b>	1.46
<i>CG5955</i>	1.47	<i>kek4</i>	1.39
<b><i>CG2827</i></b>	1.44	<i>CG7529</i>	1.36
<i>Snap</i>	1.44	<i>CG10098</i>	1.35
<i>TpnC41C</i>	1.43	<i>CG8942</i>	1.34
<i>CG31475</i>	1.42	<i>Mub</i>	1.29
<i>CG11073</i>	1.41		
<i>CG5104</i>	1.38		
<i>CG9295</i>	1.38		
<i>Mlc1</i>	1.37		
<i>CG6852</i>	1.36		
<i>CG5498</i>	1.36		
<i>CG1943</i>	1.36		
<i>CG7378</i>	1.35		
<i>CG15449</i>	1.35		
<i>Treh</i>	1.35		
<i>Est1</i>	1.34		
<i>CG2767</i>	1.33		
<i>CG7331</i>	1.33		
<i>Mfas</i>	1.31		

For complete description of these genes, see ref. 32. ( $P < 0.002$ ). Boldface represents genes for which mutants were tested.

selection as the difference parameter. Consequently, we focused on genes that are changed in the same direction in both aggressive lines compared with both neutral lines. To reduce the number of false-positive candidates, we also set the significance level much lower. We found that approximately 80 genes were significantly differentially expressed between the Aggr and Neutr lines at the 0.002 significance level (**Supplementary Table 1**; raw data files are available in the Gene Expression Omnibus (GEO); see Methods). Notably, for several gene categories, expression of most of the genes was higher in the Aggr lines. These included genes involved in muscle contraction,  $\text{Ca}^{2+}$  signaling, energy metabolism and cuticle formation (**Supplementary Table 1**). Although some of them may be directly involved in aggression, it is also possible that they represent coselected responses. For example, some of these genes may allow Aggr flies to fight harder and longer without changing their tendency to fight. This is supported by the fact that we have repeatedly observed escalations in these flies to last more than 7 min, whereas typical escalations average approximately 20 s (ref. 9). We also found that none of the genes that are related to serotonin metabolism were significantly differentially expressed between both Aggr and both Neutr lines (data not shown). This was notable, as serotonin has been implicated in aggression in a wide variety of organisms. We also tested for differences in total serotonin (5HT) levels in the heads of flies from the selected lines at Gen23. Using HPLC analysis on extracts from fly



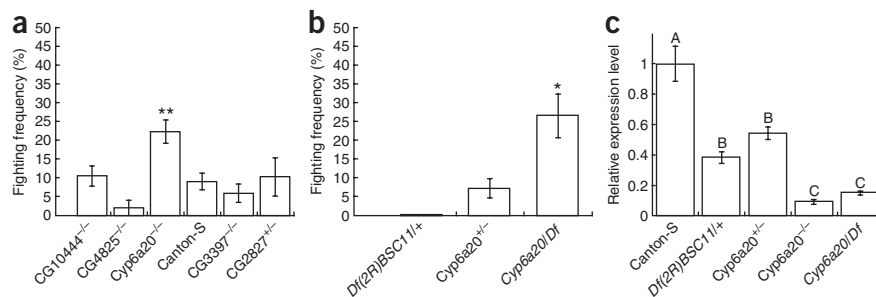
**Figure 5** Validation of array expression differences by qPCR. Graphs on the left represent expression profiles in the selected lines obtained from the array analysis. Graphs on the right represent the corresponding gene expression profiles analyzed by qPCR. y axis values represent luminescence levels on the arrays or relative expression levels (normalized to the overall average expression level) for the qPCRs. NI = NeutrI, NII = NeutrII, AI = AggrI and AII = AggrII. Error bars represent s.e.m. for a minimum of three replicate expression measurements.

heads, we did not find any changes in 5HT between any of the lines, further supporting the array results (data not shown).

Many of the significant expression changes were small. Only four genes showed an expression difference of twofold or above. Such small expression changes have been shown in both flies<sup>30</sup> and humans<sup>31</sup> to have significant behavioral effects. In **Table 2**, we list all of the genes that differ in their expression between the Aggr and Neutr lines by  $\geq 25\%$ , which represents a difference that can generally be verified molecularly using other means than arrays (D. Robinson, personal communication). Of this list, 28 genes showed higher expression in the Aggr lines, and 14 showed decreased expression. Expression profiles of these genes are shown in **Supplementary Figure 2**.

To evaluate the reliability of the array-based expression differences, we verified the expression changes of six genes in **Table 2** by quantitative RT-PCR (qPCR). Five of these six genes had expression profiles that matched the array profiles (**Fig. 5**).

**Figure 6** Fighting frequency of mutants in arena assay and mutant validation. (a) Bar graphs show mean fighting frequency ( $\pm$  s.e.m.) of five background standardized mutants tested, as well as the background strain Canton-S (minimum of 50 pairs per line tested). Statistically significant differences in median values were identified by Kruskal-Wallis ANOVA followed by multiple comparison tests ( $P < 0.001$ ). (b) Bar graphs show mean fighting frequency ( $\pm$  s.e.m.) of mutant crossed to a deficiency (*Df(2R)BSC11/SM6a*) uncovering the mutant locus as well as controls (deficiency flies outcrossed to Canton-S virgin females and heterozygote mutant males). Statistically significantly different median values were identified by Kruskal-Wallis ANOVA for unpaired groups followed by multiple comparison tests ( $P < 0.01$ ). (c) Bar graphs show relative expression level ( $\pm$  s.e.m.) of *Cyp6a20* in heads of homozygous mutants, transheterozygote mutants over deficiency, heterozygote animals, heterozygote deficiency males and males from the control background strain. Letters above each bar denote significantly different groups (ANOVA followed by *post hoc* test to identify statistically significant groups). Note that the y axes in a and b are truncated to 50%.



### Mutant analysis of candidate genes

To evaluate whether the genes in **Table 2** were directly involved in the changes in aggressive behavior that occurred during the selection, we analyzed individual mutant lines corresponding to candidate genes for which we had confirmed the expression difference by qPCR and for which mutant lines were publicly available. Three had reduced expression levels in the Aggr lines, and two had increased expression levels (boldface in **Table 2**; one of these was not confirmed by qPCR). To produce standardized lines, we picked mutants for which P-elements were available and introgressed them into the standard Canton-S background (and made them *w*<sup>+</sup>) so that they could be directly compared with each other and with the standard background strain for effects on aggressive behavior (in the arena assay). We determined fighting frequencies for the five lines that were tested and Canton-S (**Fig. 6a**). One of the lines, mutant for the gene encoding cytochrome P450 6a20 (*Cyp6a20*)<sup>32</sup>, showed a significant effect on aggressive behavior in the direction predicted by the array results ( $P < 0.001$ , **Fig. 6a**). To make sure that this gene was indeed responsible for the behavioral effect, we tested the mutant over a deficiency (*Df(2R)BSC11/SM6a*) that removes the region encompassing the gene. We found that it also showed a significant increase in fighting frequency ( $P < 0.01$ , **Fig. 6b**). To verify whether the P-element specifically disrupts the *Cyp6a20* gene, we analyzed the expression level of this gene in the homozygote, heterozygote and transheterozygote (mutant over deficiency) flies compared with the standard background strain. *Cyp6a20* expression in heads from homozygote and transheterozygote flies was downregulated to 8%–15% of the level found in their control counterparts ( $P < 0.0001$ ; **Fig. 6c**). To control for the specificity of this locus, we analyzed more than 20 other control lines and deficiencies outcrossed to the background strain (see Methods), none of which showed significant increases in fighting frequency (data not shown).

### DISCUSSION

We describe here a population-based selection procedure with which we selected flies for increased aggressive behavior. Our criterion for selection was initially based on males that engaged in repeated fights. In time, as flies evolved to be more aggressive, our selection threshold became more stringent, and we picked only males that engaged in the most aggressive but rare encounters, known as escalations. In these encounters, a territory holder is challenged by an intruder, and both males reciprocally box and tussle while standing on their hind legs in an attempt to maintain or take over sole territorial control (**Supplementary Video 1**). Both criteria were aimed at increasing the actual

level of aggression in these flies in addition to any propensity for obtaining territorial control. Over some 20 generations of selection, we observed very strong increases in aggressive behavior, as measured by several parameters: frequency of fighting, latency to fighting, fighting index and fighting intensity. For all of these measures, we observed significant changes between the Aggr and Neutr lines: average absolute fighting index (that is, when nonfighting pairs are included) increased by more than 30-fold in AggrI flies compared with NeutrII flies (**Table 1**). These aggression parameters were measured outside of the traditional context of territoriality (food plus female), which has been used in all previously described aggression work in *D. melanogaster*. We also evaluated the changes in aggression in a territorial context by measuring the frequency of escalations in the population cage, which also significantly increased between the Aggr and Neutr lines.

Hoffmann has previously described a selection experiment in *D. melanogaster* for increased territoriality by selecting males that would maintain territorial control for an extensive period of time<sup>13</sup>. Territorial lines were tested for their ability to control territories by pairing them with unselected control males. A significant increase was observed as early as five generations, and by 20 generations selected males almost completely outcompeted control males for territorial domination. Although it is not unlikely that these selected lines showed an increase in absolute aggression (that is, the number of fights and/or intensity of fights that occurred), none of the lines were measured directly for their levels of aggression. No molecular analysis was performed on these lines, and they no longer exist (A.A. Hoffmann, personal communication).

In our selection experiment, we evaluated whether the strong selection response for increased aggression was accompanied by significant changes in gene expression in the heads of the Aggr lines compared with the Neutr lines. Notably, we found that the two groups showed very few differences in expression, particularly as compared with a recently reported selection experiment for decreased mating speed in *D. melanogaster*, in which more than 20% of the genome has been implicated in the selection response<sup>33</sup>. We found that the number of significant expression differences between any two combinations of lines was much lower, ranging from 3.6%–5.9% of the genome at a significance level of 0.05, criteria that were significantly less stringent than those used in ref. 33. One explanation for this difference might be that the selection response for mating speed, particularly a decrease in mating speed, might be accomplished in a wide variety of ways, both specific and nonspecific. The set of genes identified in that particular

array analysis might therefore encompass nonspecific changes as well. This is supported by the fact that 25% of the differentially expressed genes occurred in males only, whereas males of the different groups showed no difference in mating speed<sup>33</sup>. Another factor that may contribute to the large difference in significant expression changes is the different genetic starting material used for the selection experiment. We used a mixture of an inbred laboratory strain, Canton-S, whereas the authors in ref. 33 used a mixture of 60 wild-caught, recently domesticated isofemale lines. However, our own previous microarray experiments on selected lines have shown percentages of differentially expressed genes between selected lines similar to those in the current experiment<sup>34</sup>. Moreover, other successful selection experiments have used laboratory strains as their starting point<sup>35</sup>. Regardless, the strength of our selection response strongly suggests that there was enough genetic variation in our starting population to have allowed for such a robust behavioral response.

To increase the chances of pursuing genes directly involved in aggressive behavior, we focused our further analysis on genes that showed significant differences in expression between both Aggr and both Neutr lines at a significance level of 0.002 and that had differences in expression of at least 25% (Table 2). We found that only one of a set of candidates that was further analyzed had a detectable effect on the phenotype as a single mutant. It is possible that the disruptions of the genes that we analyzed are not strong enough or specific enough to cause a phenotype. Alternatively, some of the genes with changed expression could be simply polymorphic expression variations that are either incidentally distributed between the lines or that are linked to loci of interest. Other genes might correlate to additional phenotypic changes in the lines that were accidentally coselected in both groups. We evaluated this in part by looking at other behavioral and related responses in our selected lines, although we found no evidence of this. Finally, it is possible that some genes had no effects because they only show an effect in a complex network of other changes that has been assembled through selection. Such cryptic genetic variation is pervasive but generally underestimated<sup>36</sup>.

The one gene that produced a direct effect on aggression encodes a cytochrome P450 (ref. 32). These enzymes are involved in a variety of fundamental physiological functions as varied as growth, development, reproduction, detoxification and pheromone recognition<sup>37</sup>. Some members of the CYP6 family have been shown to be enriched in olfactory tissues in *D. melanogaster* and *Phyllopertha diversa*<sup>38,39</sup>, in which they might be involved in pheromone signaling. If the *Cyp6a20* mutant is defective in pheromone degradation, its increased fighting frequency might be explained by abnormal sensitivity to male pheromones eliciting an aggressive response. *Cyp6a20* has been shown to undergo circadian fluctuation<sup>40</sup>. Our results, however, suggest that the *Cyp6a20* effect is not due to a phase shift in circadian behavior but rather to an effect of this mutant on aggression.

Another notable change in the list of significant expression differences is the downregulation of *Obp56a* in the aggressive lines (Table 2), which represents one of the strongest responders in this selection experiment. Odor-binding proteins have also been implicated in pheromone signaling between flies and have been shown to affect complex behavior<sup>41</sup>. In this regard, in our population cage, we noticed a previously undescribed behavior in which males drag their genital area on the food surface while walking as if making a territorial mark (Supplementary Video 5). This abdomen dipping has been previously reported in other dipterans, in which it has been shown to be associated with territorial attraction of females<sup>25,26</sup>. Although premature, it is tempting to speculate that this marking might also

act as a repellent to other males and that a decrease in gustatory detection of this repellent might make aggressive males less sensitive to it, eliminating one inhibitory component towards an eventual territorial collision.

Many of the genes that have been implicated to date in aggressive behavior in one or another organism are somehow related to serotonin metabolism (reviewed in ref. 23). We find it curious that none of the genes involved in biogenic amine metabolism in *D. melanogaster* have differing expression profiles among the selected lines generated in our experiment. Although we cannot currently exclude effects other than expression changes in these pathways, the behavioral response in these lines does not seem to be dependent on the biogenic amine pathways. We find it interesting in this regard that a previous study on *D. melanogaster* could not find any effects of serotonin manipulation on aggression<sup>20</sup>. Further investigations will be necessary to evaluate the role of serotonin in aggression in *D. melanogaster* in general and in these selected lines in particular.

## METHODS

**Stocks and breeding conditions.** The flies used as the starting population for selection were a mixture of two Canton-S lines that had been kept separate in the laboratory for several years. They were reciprocally crossed using equal numbers (approximately 90 individuals) of males and females of each line for two generations, and selection was started on the third generation from two replicate populations of 180 flies. Flies were reared in pairs of ten males and ten females in plastic bottles on yeast, dark corn syrup and agar food at room temperature ( $23.2 \pm 0.5^\circ\text{C}$ ) on a 12 h light/12 h dark cycle. Each generation, males were selected and mated with random virgin females from the same generation. Offspring were derived from a minimum of 30 pairs of flies per line every generation to maintain a minimal amount of genetic variation necessary for selection. No overlapping generations were allowed.

Mutant lines were obtained from the Bloomington Stock Center unless otherwise noted. The following mutants were background standardized and tested:  $y^1w^{67c23}$ ;  $P\{y[+mDint2] w[BR.E.BR]=SUPor-P\}CG10444^{KG04633}$ ,  $y^1$ ;  $P\{y[+mDint2] w[BR.E.BR]=SUPor-P\}CG4825^{KG06018}$ ,  $ry^{506}/TM3$ ,  $Sb^1Ser^1$ ,  $y^1w^{67c23}$ ;  $P\{w[+mC] y[+mDint2]=EPgy2\}EY04742$ ,  $y^1w^{67c23}$ ;  $P\{y[+mDint2] w[BR.E.BR]=SUPor-P\}KG04665$ ,  $w^{1118}$ ,  $P\{w[+mC]=EP\}Ta^{EP489}$ . Background standardization was performed by swapping the non-mutation bearing chromosomes using cantonized balancer lines followed by eight backcrosses of the P-bearing chromosome to a white Canton-S line so that <0.5% of the original chromosome remained. In a final step, all the lines were made  $w^+$  so that possible effects due to eye color were avoided. For lines that had ethylmethane sulfonate (EMS)-induced point mutations, only the non-mutant chromosomes were swapped, leaving the mutant chromosome not standardized. The following control strains were standardized using the same protocol:  $P\{lacW\}mth^1$  and  $P\{lacW\}Csp^1$  (provided by S. Benzer, California Institute of Technology),  $P\{lacW\}Itp-r83A^{5B4}$  (provided by Y. Yan, University of California, San Francisco),  $P\{lacW\}Adf^{1nal}$  (provided by T. Tully, Cold Spring Harbor Laboratory),  $per^{01}$ ,  $w^{74g}$ ;  $P\{w[+mC]=lacW\}Trf^1/CyO$ ,  $y^1w^{67c23}$ ;  $P\{w[+mC] y[+mDint2]=EPgy2\}CG9543^{EY03980}$ ,  $w^{1118}$ ;  $P\{w[+mC]=EP\}CG9543^{EP2027}$ ,  $y^1w^{67c23}$ ;  $P\{w[+mC] y[+mDint2]=EPgy2\}CG5439^{EY03606}$ ,  $Amy-p^A Amy-d^n$ ,  $Ca^{n1}/TM3$ ,  $Sb^1Ser^1$ . The following deficiencies were also obtained from the Bloomington Stock Center and tested as F1 heterozygotes obtained from a cross to virgin females from the background Canton-S strain:  $Df(2R)BSC11/SM6a$ ,  $w^{1118}$ ,  $Df(2R)Exel7162P+PBac\{XP5.WH5\}Exel7162/CyO$ ,  $Df(3R)T-32$ ,  $(kni^{r11})$   $cu^1sr^1e^1/MRS$ ,  $Df(3R)ry^{615}/TM3$ ,  $Sb^1Ser^1$ ,  $Df(3R)Scr$ ,  $p^e/TM3$ ,  $Sb^1$ ,  $Df(3R)by10$ ,  $red^1e^1/TM3$ ,  $Sb^1Ser^1$ ,  $Df(3L)ri-79c/TM3$ ,  $Sb^1$ ,  $Df(3R)M-Kx1/TM3$ ,  $Sb^1$ ,  $Df(3L)rdgC-co2$ ,  $th^1st^1in1$   $kni^{r11}p^1/TM6C$ ,  $cu^1Sb^1Tb^1ca^1$ ,  $Df(3L)XS533/TM6$ ,  $Sb^1Tb^1ca^1$ .

**Selection procedure.** For selection, virgin flies were collected on the day of eclosion and grouped by sex in vials of up to 40 males or females. The flies were allowed to age 4–7 d (generally 5 d) before selection. Selection was performed in a Plexiglas box of  $26.7 \times 20.3 \times 10.2$  cm (L  $\times$  W  $\times$  H), covered with nylon mesh (Fig. 1a). To maintain humidity, a 1-cm layer of a melted 1% agarose

solution was poured into the box on the evening before selection. The next day, 11 food territories (2-cm caps of a 15-ml conical tube filled with fly food) were spaced evenly along three of the walls of the rectangular box in a zigzag pattern approximately 2.5 cm apart. Across from each territory, a hole was drilled 1 cm in diameter in the Plexiglas wall and was covered with a piece of transparency with approximately nine pie-shaped slits cut toward the middle (**Supplementary Fig. 1**). On the morning of the day of selection (typically 8 to 9 a.m.), 120 males and 60 virgin females were gently loaded into the population cage through one of the holes in the wall using an aspirator. After an initial mating phase, flies eventually came down on the food territories and males typically started defending them. In the initial rounds of selection, the time required for flies to come down on the territories was approximately 2 h, and this interval gradually decreased for the Aggr lines to approximately 40 min by Gen21. Throughout the day, aggressive males were removed from the territories by gentle aspiration through the holes in the wall and stored in a vial for later mating. Initially, for the first seven rounds of selection, we selected males that won repeated encounters on a territory within a 10-min observation period; this selection process was repeated every hour. Occasionally, an escalated encounter would occur where two males would stand on their hind legs and box and tussle in an attempt to maintain or take over the territory, as has been previously described<sup>10</sup>. In this case, both males were aspirated while tussling, which was particularly easy as they did not notice the invading aspirator (**Supplementary Video 5**). After the first seven generations, we removed only these escalating males for selection, as determined by continuous monitoring of the cage throughout the day.

For each round of selection, we set up a reference population cage next to the Aggr cage, and we removed and discarded 15 to 30 aggressive males so that both populations were subjected to the same stimuli throughout the selection. At the end of each experiment, we anesthetized all the flies in this control cage with CO<sub>2</sub>, picked 30 random males from the remainder and mated them with an equal number of random virgin females from that same generation to serve as the founders for the next generation. The removal of aggressive males from this cage might be expected to lead to a decrease in aggression in this population. However, as we observed only a trend of decreased aggression that was not significant, we labeled them as Neutral lines. We also refer to this population as a control for the Aggr lines, although strictly speaking, they did undergo downward selection pressure and as such are not a traditional control group.

The entire experiment was performed in duplicate so that a total of four subpopulations were derived, two of which were selected for increased aggression and two for decreased or neutral aggression. These populations are labeled AggrI, AggrII, NeutrI and NeutrII.

**Aggression assays.** To quantify the behavior of the selected lines, we developed two assay systems. The first one is a simplified scaled-down version of the territorial assay described by Dow and von Schilcher<sup>7</sup> and later used and modified by Hoffmann<sup>10</sup>. Briefly, two males and one mated female were loaded into a plastic Petri dish cylinder of 2.5 × 2 cm (depth × height) made of two 2.5-cm Petri dish lids separated by a 2-cm wall made of a piece of transparency, containing an Eppendorf cap filled with fly food as the territory in the middle of the cage (**Fig. 1c**). The flies used in this assay were collected on the day of eclosion and were allowed to age for 4 to 6 d before being used in the assay. The mated females were mated for 1–2 d before setup. Ten territorial cages for each of the selected lines were filmed for 1 h, and all the fights in that 1 h were counted and plotted as box plots. This assay is tedious and time-consuming.

Thus, we developed a second, simpler assay to make quantitative analysis more straightforward and detailed, referred to as the arena assay because it consists of an arena with just two males without a female or food territory. We designed an arena chamber made of a 1.3-cm-thick Plexiglas rectangular plate (11.4 × 8.9 cm) containing 20 evenly spaced 1.6-cm-diameter cylindrical arenas arranged in four rows of five (**Supplementary Fig. 1**). The assay is performed as follows. The insides of the cells are coated with Fluon (Northern Products) by briefly pipetting the solution in and out of each cell 1 d before each experiment. Fluon coating prevents the flies from walking on the walls of the cell. The next morning the chamber is placed on a bed of 2% agarose and covered with a plastic lid. Two males are introduced in each arena through a loading hole (aligned with the top of each arena) in the cover plate that is plugged with a small cotton plug. When all the males are loaded, the cover plate

is gently moved up so that the loading holes now align just above each arena and the cotton plugs are removed. The chamber is then gently placed under a camera and the flies are allowed to adjust for 5 min before being videotaped for 15 min. All males are collected on the day of eclosion, allowed to age for 5–7 d and then isolated 1 d before analysis in the arena assay, as isolation has been shown to increase aggression<sup>15</sup>. All assays were performed in the morning (between 8:00 and 11:00 a.m.), and all selected lines were analyzed in succession with a rotation schedule over subsequent repeat experiments so that all lines were assayed first, second, third and fourth, although there was no detectable difference in the scores depending on the loading order. We tested 80 pairs of males for every line at the Gen11 and Gen21 time points of the selection. Four parameters were used for quantification in the arena assay. Only unambiguous fighting elements were scored: these included wing threat, charging or lunging, holding and tussling, but not sideways fencing, as described in ref. 21, because this behavior is not necessarily aggressive in nature.

For quantification of escalations in mixed populations, we set up population cages as described above, but half the flies were now derived from one line and the other half was derived from a second line (for example, a combination of AggrI and NeutrI). The males of the different lines were distinguished by a small double wing cut. The wing cuts were balanced so that Neutr and Aggr lines were equally represented with and without wing cuts. Three replicate cages were set up per combination for all reciprocal combinations. For one-on-one mixed pairs, a small double wing cut was applied to one of the two flies. We analyzed 70 pairs per combination, and wing cuts were again balanced, so that for half the pairs the Aggr male had a wing cut and for the other half the Neutr male had a wing cut. Data were pooled because the trends were similar in all combinations.

**Other behavioral assays.** Activity was analyzed as in ref. 28. We analyzed 20 males per line for 3 d in a 12 h light/12 h dark environment at 25 °C.

We performed courtship assays as in ref. 42, analyzing 20 males per line as they courted wild-type Canton-S females.

Flies were weighed in groups of approximately 20 males, and each group was weighed three times. Eight groups originating from different cultures were weighed for each line, and the average weight per fly was calculated. We then calculated the total average ± s.e.m.

Mating assays were done in a mating wheel as described in ref. 43. Briefly, males were loaded in the top half of a mating wheel with ten circular mating chambers (0.9 cm in diameter), and females were loaded in the bottom half. When all the flies were loaded, the two halves were rotated together so that in each cell, one male and one female were joined. The mating wheel was then filmed for 30 min, and tapes were analyzed for mating latency and duration. We tested 40 males per line in four replicate experiments. Mating competition assays were done in the same way, except that two males were added to each mating cell instead of one. Males of different lines were distinguished by a small wing-cut of the left or right wing so that both males had an identical disability. We tested 40 males for each line in four replicate experiments, and left or right wing cuts were made reciprocally, so that 20 males of each line competed with a small cut on the left wing and 20 with a defect on the right wing against a male with an opposite wing cut.

**Microarray analysis.** For microarrays, we isolated heads from groups of 30 to 40 age matched flies (that were randomly collected from the stock bottle) frozen in liquid nitrogen, and we extracted RNA by homogenization as previously described<sup>28</sup>. Three replicate microarray experiments were performed for each line using Affymetrix technology (*Drosophila* Genome Array version 1) according to the manufacturer's protocols (Affymetrix).

Quantitative RT-PCR (qPCR) was performed as previously described<sup>28</sup> using RNA extracted from heads of independent groups of flies (a minimum of 20) used for the microarray experiments. A minimum of two replicate reverse transcription reactions were performed, followed by a minimum of three replicate PCR reactions for each gene that was tested.

**Statistical analysis.** Most of the aggression data are not normally distributed, and for these data, medians were statistically compared using the nonparametric Kruskal-Wallis analysis of variance (ANOVA) for unpaired groups. We used the Tukey-Kramer honestly significant difference (HSD) test as our default



*post hoc* multiple comparison tests to identify those groups that differed to a statistically significant extent. For paired groups, the nonparametric Wilcoxon test was applied. Normally distributed data were analyzed using ANOVA, followed by multiple comparison tests (Tukey-Kramer HSD as default) to identify the significantly different groups. Nonparametrically distributed data are generally shown as box plots in which the box represents the 25% and 75% quantiles, with the median (50% quantile) shown as a horizontal line in the box. The dashed lines represent the 5% and 95% quantiles showing the entire spread of the data. Population proportions (percentages) are plotted as bar graphs showing the mean (bar) with s.e.m. (error bars), because proportions are more clearly visualized by the mean than the median (that is, the exact number of pairs that fight can be discerned). All parametric data are also presented as bar graphs representing the mean (bar) and s.e.m. (error bars).

Array data were analyzed using the GeneSpring platform (version 7.2). Default transformation of Affymetrix data was performed. First, the raw array signals were log transformed, followed by 'per chip' normalization in which all signals are divided by the median value on each chip. Normalized array data were then grouped according to selection response, and ANOVA was performed to find statistically significant differences in expression levels between Aggr and Neutr lines at a stringent *P* value of 0.002. Only Affymetrix P- or M-flagged signals were included in the analysis, leading to a projected false positive rate at this level of significance of approximately 13 genes.

**Accession codes.** The raw array data files are available online at <http://www.ncbi.nlm.nih.gov/projects/geo/> under accession numbers GSM120838 and GSM120858–GSM120868.

*Note: Supplementary information is available on the Nature Genetics website.*

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#### AUTHOR CONTRIBUTIONS

This study was designed by H.A.D. and R.J.G. H.A.D. performed the experiments, analyzed the data and wrote the manuscript.

#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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