

# A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*

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**Environmental and genetic factors can modulate aggressiveness, but the biological mechanisms underlying their influence are largely unknown. Social experience with conspecifics suppresses aggressiveness in both vertebrate and invertebrate species, including *Drosophila*. We searched for genes whose expression levels correlate with the influence of social experience on aggressiveness in *Drosophila* by performing microarray analysis of head tissue from socially isolated (aggressive) vs. socially experienced (non-aggressive) male flies. Among  $\approx 200$  differentially expressed genes, only one was also present in a gene set previously identified by profiling *Drosophila* strains subjected to genetic selection for differences in aggressiveness [Dierick HA, Greenspan RJ (2006) *Nat Genet* 38:1023–1031]. This gene, *Cyp6a20*, encodes a cytochrome P450. Social experience increased *Cyp6a20* expression and decreased aggressiveness in a reversible manner. In *Cyp6a20* mutants, aggressiveness was increased in group-housed but not socially isolated flies. These data identify a common genetic target for environmental and heritable influences on aggressiveness. *Cyp6a20* is expressed in a subset of nonneuronal support cells associated with pheromone-sensing olfactory sensilla, suggesting that social experience may influence aggressiveness by regulating pheromone sensitivity.**

aggression | cytochrome P450 | pheromone

Aggression is critical for the survival and reproduction of many animal species (1–3). Although aggression is an innate behavior subject to genetic influences, levels of aggressiveness are subject to environmental modifications as well. An important unanswered question is whether these influences act by independent or shared biological mechanisms. Although genes underlying heritable differences in aggressiveness are beginning to be identified (4, 5), very little is known about the molecular mechanisms underlying environmental influences on aggression.

Environmental influences on aggressiveness have been well documented in a variety of animal models. Social status established by previous agonistic experience influenced subsequent aggression-related behavior in crayfish (6) and crickets (7). Resident female Mediterranean fruit flies (*Ceratitidis capitata*) located at a resource had a higher probability of defeating an intruder, suggesting that experience on a resource may increase aggressiveness (8). Male fruit flies (*Drosophila melanogaster* and *Drosophila simulans*) raised at high density failed to successfully defend their territories against males raised at low density, an effect potentially related to differences in body size (9).

Social experience with conspecifics is one environmental influence on aggressiveness that is common to many species, including humans (3). Socially isolated male mice are more aggressive than group-housed males (10). Similar phenomena have been reported in rats (11), cichlid fish (*Haplochromis burtoni*) (12), and other vertebrate species. Effects of social experience on aggressiveness have also been described in invertebrates. Hoffman (13) reported that male *D. melanogaster* held in isolation exhibited more aggressive behaviors and required

less time to establish their territories than males held in groups, suggesting that social experience suppresses aggressiveness in the fruit flies. Analogous observations have also been reported for female *D. melanogaster* (14).

Taken together, these data suggest that the effect of social experience on aggressiveness is shared among many species. However, the nature of the molecular mechanisms mediating this effect and whether they are evolutionarily conserved are poorly understood. Here, we have used fruit flies, *D. melanogaster*, a genetically tractable organism in which aggression has been well characterized (15–17) to investigate the molecular basis of the influence of social experience on aggressiveness.

## Results

### Group Housing Suppresses Aggressiveness in a Reversible Manner.

Flies raised in isolation after eclosion are more aggressive than those raised in groups (13). To quantify more easily this behavioral difference, we modified a fight chamber (18) to permit multiplex analysis of aggressiveness [supporting information (SI) Fig. S1]. A pair of male flies of similar age and social experience (raised in isolation shortly after eclosion or in groups of 10 male flies for 3 days before the test) was transferred into a fighting arena containing a small food patch. Consistent with earlier reports (17), we observed that lunging behavior, in which one fly rears up on its hind legs and charges the other fly, was the predominant form of aggression (18). Therefore, we counted lunges as a measure of aggressiveness during a 20-min observation period. Three different parameters were measured: (i) the fighting frequency, defined as the percentage of fly pairs that exhibited at least one lunge (Fig. 1 Lower A and B); (ii) the lunging intensity, defined as the average number of lunges, calculated for all pairs that exhibited at least one lunge (Fig. 1 Lower C); and (iii) the average latency to the first lunge (Fig. 1 Lower D). Flies single-housed for 3 days exhibited a mean fighting frequency of  $\approx 50\%$  ( $53.3 \pm 14.3\%$ ) during the 20-min observation period, whereas group-housed flies did not exhibit any lunges (Fig. 1 Lower A,  $P < 0.01$ ). A similar difference was observed between flies group- or single-housed for 6 days (but transferred to a new vial at day 3) (Fig. 1 Lower B; G  $\rightarrow$  G vs. S  $\rightarrow$  S,  $P < 0.05$ ). These data confirm that, in flies, as in other animals including mice, social experience suppresses aggressiveness.

The effect of social experience on aggressiveness was reversible. When flies were single-housed for 3 days, followed by 3 days of

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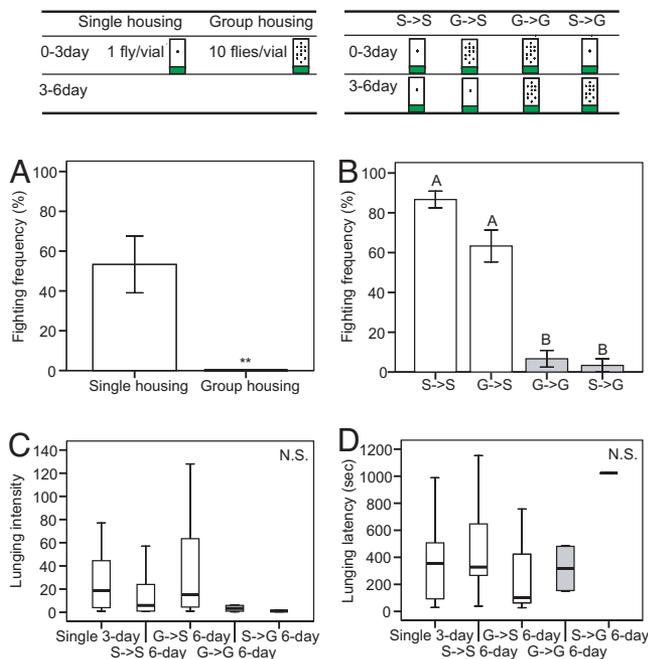
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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE6994).

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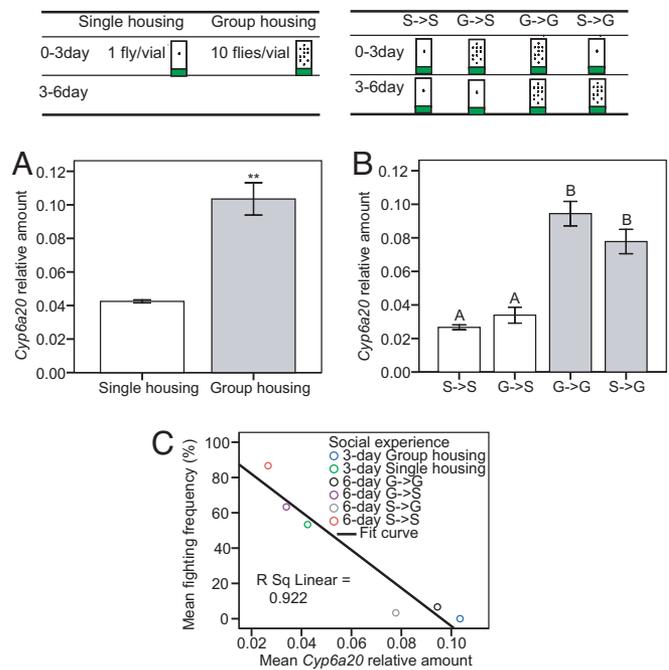


**Fig. 1.** Social experience influences *Drosophila* aggressiveness. (Upper) Experimental manipulations. (Lower) (A) Mean fighting frequencies in 3-day-old, single-housed and group-housed flies (mean  $\pm$  SEM;  $n = 6$  experiments each containing five fly pairs; \*\*,  $P < 0.01$ ). (B) Mean fighting frequencies in 6-day-old, S  $\rightarrow$  S, G  $\rightarrow$  S, G  $\rightarrow$  G, and S  $\rightarrow$  G flies (mean  $\pm$  SEM.  $n = 6$  experiments each containing five pairs. Significant differences ( $P < 0.05$ ) are indicated by letters above bars). (C) Median lunging intensities of 3-day-old, single-housed flies, and 6-day-old, S  $\rightarrow$  S, G  $\rightarrow$  S, G  $\rightarrow$  G, and S  $\rightarrow$  G flies [ $n = 16, 25, 19, 2, 1$  pairs, respectively; N.S., not significantly different ( $P > 0.05$ )]. (D) Median lunging latencies of 3-day-old, single-housed flies and 6-day-old, S  $\rightarrow$  S, G  $\rightarrow$  S, G  $\rightarrow$  G, and S  $\rightarrow$  G flies [ $n = 16, 25, 19, 2, 1$  pairs, respectively; N.S. ( $P > 0.05$ )]. Comparisons between groups were made by using the Mann–Whitney  $U$  test (A), ANOVA followed by post hoc test (B) and Kruskal–Wallis ANOVA (C and D).

group housing before the test, the fighting frequency was as low as that of flies group-housed for 6 days (Fig. 1 Lower B, G  $\rightarrow$  G vs. S  $\rightarrow$  G,  $P > 0.05$ ). Conversely, when flies were group-housed for 3 days, followed by 3 days of single housing, their aggressiveness was approximately as high as that of flies single-housed for 6 days (Fig. 1 Lower B, S  $\rightarrow$  S vs. G  $\rightarrow$  S,  $P > 0.05$ ). The median lunging intensity and latency among pairs exhibiting at least one lunge were not significantly different across all social conditions (Fig. 1 Lower C and D,  $P > 0.21, P > 0.12$ ).

**Cyp6a20 Shows Differential Expression Levels in Single- vs. Group-Housed Flies.** To investigate the molecular basis of social influences on aggressiveness, we performed comparative gene expression profiling on heads from 6-day-old group- vs. single-housed male flies. Using criteria of fold change  $> 1.25$  and  $P < 0.002$ , we identified 141 probe sets that were up-regulated and 48 probe sets that were down-regulated in single- vs. group-housed males (Dataset S1). The differentially expressed genes fell into diverse ontological and biological categories, including neurotransmitter metabolism, immunity, and olfaction.

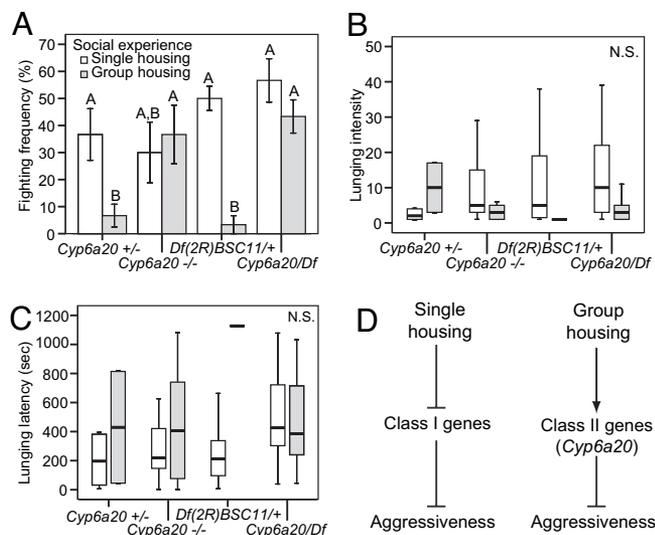
While this study was underway, a report (4) appeared describing a microarray comparison, using head mRNA, between *Drosophila* strains selected for increased aggressiveness (AggrI and AggrII) and strains selected for decreased aggressiveness (NeutrI and NeutrII). Multiple differentially expressed genes were identified, which reflect allelic variations underlying heritable differences in aggressiveness. To determine whether there is any commonality in the molecular mechanisms through which aggressiveness is modified by heritable and environmental fac-



**Fig. 2.** *Cyp6a20* expression is correlated with social experience. (Upper) Experimental manipulations. (Lower) (A) Relative levels of *Cyp6a20* mRNA (normalized to *Ddc* mRNA levels) in 3-day-old, single-housed and group-housed flies (mean  $\pm$  SEM;  $n = 4$ ; \*\*,  $P < 0.01$ ). (B) Relative levels of *Cyp6a20* mRNA in 6-day-old, S  $\rightarrow$  S, G  $\rightarrow$  S, G  $\rightarrow$  G, and G  $\rightarrow$  S flies (mean  $\pm$  SEM;  $n = 4$ ; significant differences are indicated by letters above each bar). (C) Negative correlation between relative levels of *Cyp6a20* mRNA and fighting frequency. The linear regression plot ( $R^2 = 0.922$ ) is compiled by using the data in A and B and Fig. 1 Lower A and B. Comparisons between groups were made by using student's  $t$  test (A), or ANOVA followed by a post hoc test (B).

tors, we compared the differentially expressed genes identified in our social experience experiments, with those identified by selective breeding. Applying the same criteria (fold change  $> 1.25$ ;  $P < 0.002$ ), we identified *Cyp6a20*, a cytochrome P450 gene, as the only gene similarly regulated in both datasets. *Cyp6a20* was expressed at relatively lower levels both in AggrI&II vs. NeutrI&II (4) and socially isolated vs. group-housed flies. An independent microarray experiment identified many genes differentially expressed in flies selected for increased aggressiveness, but *Cyp6a20* was not among them (5). However, that study differed in several important details from the methods used in our own experiments and those described in ref. 4, including the conditions under which flies were tested and the criteria used to define aggressive behavior.

These data led us to further investigate the role of *Cyp6a20* in the effect of social experience on aggressiveness. We first confirmed the correlation between *Cyp6a20* expression levels and social experience, using quantitative RT-PCR. *Cyp6a20* expression was almost 3-fold lower in flies single-housed for 3 days than in group-housed flies of same age (Fig. 2 Lower A,  $P < 0.01$ ). Furthermore, in flies switched from single housing to group housing after 3 days or vice versa, the levels of *Cyp6a20* expression changed in parallel with, but in the opposite direction to, the changes in aggressiveness caused by these social manipulations (Fig. 2 Lower B). There is, on one hand, a positive correlation between levels of *Cyp6a20* expression and social experience (Fig. 2 Lower A and B) and, on the other hand, a negative correlation between social experience and aggressiveness (Fig. 1), suggesting the *Cyp6a20* expression levels might be negatively correlated with aggressiveness. Indeed, a plot of *Cyp6a20* mRNA levels vs. aggressiveness was well fit by a linear

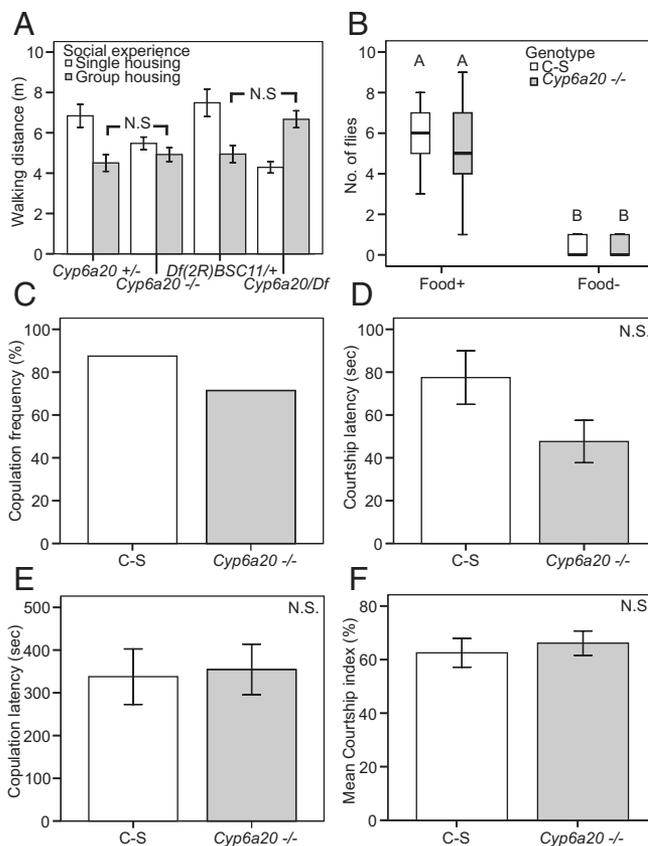


**Fig. 3.** *Cyp6a20* mutants exhibit increased aggressiveness only under group-housing conditions. (A) Mean fighting frequencies of 6-day-old, single-housed and group-housed flies, of the indicated genotypes [mean  $\pm$  SEM;  $n = 6$  experiments each containing five pairs; significant differences ( $P < 0.05$ ) are indicated by letters above the bars]. Comparison between groups was made by using ANOVA followed by a post hoc test. (B) Median lunging intensities of 6-day-old, single- or group-housed flies of the indicated genotypes ( $n = 10, 2, 9, 11, 15, 1, 17, 13$  pairs, respectively; N.S.,  $P > 0.05$ ). (C) Median lunging latencies of 6-day-old, single- or group-housed flies of different genotypes ( $n = 10, 2, 9, 11, 15, 1, 17, 13$  pairs, respectively; N.S.,  $P > 0.05$ ). Comparisons between groups were made by using Kruskal-Wallis ANOVA. (D) Two classes of negative genetic regulators of aggressiveness and their interaction with social experience. Class I genes, when mutated, increase aggressiveness under single-housing conditions, where their expression levels are normally relatively lower, but this phenotype is not observed in group-housing. Class II genes, when mutated, increase aggressiveness under group- but not single-housing conditions, overriding the effect of social experience to suppress aggressiveness. *Cyp6a20* is a class II gene.

regression function, with a correlation coefficient equal to  $-0.96$  (Fig. 2 Lower C).

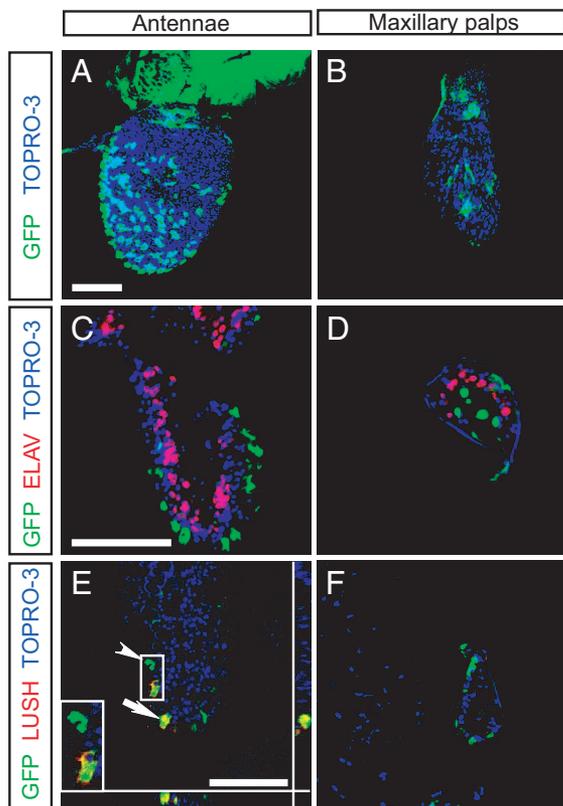
***Cyp6a20* Mediates the Suppressive Effect of Group Housing on Aggressiveness.** If *Cyp6a20* plays a role in mediating the effect of social experience, then flies deficient in *Cyp6a20* should exhibit higher fighting frequencies under group-housing but not single-housing conditions. Indeed, group-housing flies bearing a homozygous P-element insertion in the *Cyp6a20* locus showed a significantly higher fighting frequency than group-housed *Cyp6a20*<sup>+/-</sup> flies (Fig. 3A, gray bars; *Cyp6a20*<sup>-/-</sup> vs. *Cyp6a20*<sup>+/-</sup>,  $P < 0.05$ ), whereas there was no significant difference between these genotypes under single-housing conditions (Fig. 3A, white bars, *Cyp6a20*<sup>-/-</sup> vs. *Cyp6a20*<sup>+/-</sup>,  $P > 0.05$ ). Furthermore, the fighting frequency of group-housed *Cyp6a20*<sup>-/-</sup> mutant flies was as high as that of single-housed *Cyp6a20*<sup>-/-</sup> mutant flies, whereas heterozygous *Cyp6a20*<sup>+/-</sup> flies (like wild-type Canton-S flies) showed a significantly reduced fighting frequency under group-housing conditions (Fig. 3A). There was no statistically significant difference in locomotor activity between *Cyp6a20*<sup>+/-</sup> and *Cyp6a20*<sup>-/-</sup> flies under group-housing conditions as measured by the total distance traveled during a 20-min filming period (Fig. 4A). Furthermore, group-housed *Cyp6a20*<sup>-/-</sup> flies exhibited normal odor-guided behavior (Fig. 4B) and normal courtship behavior toward wild-type virgin females (Fig. 4C–F), arguing that the mutation in *Cyp6a20* does not cause general deficits in olfaction or social behavior.

To confirm that the selective increase in aggressiveness under group-housing conditions was indeed caused by the P-element



**Fig. 4.** Group-housed *Cyp6a20* mutants exhibit normal locomotor, olfactory and courtship behavior. (A) Mean walking distances of 6-day-old, single-housed and group-housed flies of the indicated genotypes ( $n = 20$ ; N.S.,  $P > 0.05$ ). Comparisons between groups were made by using ANOVA followed by post hoc test. (B) Median number of flies of the indicated genotypes ( $n = 15$ ) trapped in food-containing (food+) vs. empty (food-) traps. Significant differences are indicated by letters above the boxes). Comparisons between groups were made by using Kruskal-Wallis ANOVA followed by a post hoc test. (C–F) *Cyp6a20* mutants have normal courtship behavior. (C) Percentage of fly pairs of the indicated genotypes that copulated in 30 min. (D) Mean courtship latency of flies of the indicated genotypes ( $n = 21$  and 20, respectively; N.S.,  $P > 0.05$ ). (E) Mean copulation latency of flies of the indicated genotypes ( $n = 21$  and 20, respectively; N.S.,  $P > 0.05$ ). (F) Mean courtship index of flies of the indicated genotypes ( $n = 21$  and 20, respectively; N.S.,  $P > 0.05$ ). For D–F, comparisons between groups were made by using Student's *t* test.

insertion in the *Cyp6a20* locus, we tested the *Cyp6a20* insertion over a deficiency spanning the *Cyp6a20* gene, *Df(2R)BSC11* (4). Like *Cyp6a20*<sup>-/-</sup> flies, *Cyp6a20*<sup>Df/-</sup> mutant flies showed a significantly higher fighting frequency than *Cyp6a20*<sup>Df/+</sup> hemizygous flies under group-housing conditions (Fig. 3A, gray bars; *Df(2R)BSC11/+* vs. *Cyp6a20/Df*,  $P < 0.05$ ), whereas there was no significant difference between these genotypes under single-housing conditions (Fig. 3A, white bars; *Df(2R)BSC11/+* vs. *Cyp6a20/Df*,  $P > 0.05$ ). In addition, the fighting frequency of *Cyp6a20*<sup>Df/-</sup> flies under group-housing conditions was as high as that under single-housing conditions (Fig. 3A). There was no statistically significant difference in locomotor activity between *Cyp6a20*<sup>Df/+</sup> and *Cyp6a20*<sup>Df/-</sup> flies under group-housing conditions (Fig. 4A). The lunging intensity and latency remained unchanged in all of the four genotypes (Fig. 3B and C,  $P > 0.11$ ;  $P > 0.08$ ). Previous experiments have shown that the levels of *Cyp6a20* mRNA in *Cyp6a20*<sup>-/-</sup> and *Cyp6a20*<sup>Df/-</sup> flies are only 8–15% of those in *Cyp6a20*<sup>+/-</sup> and *Cyp6a20*<sup>Df/+</sup> flies (4). Taken together, these data suggest that the phenotype of the P-element



**Fig. 5.** *Cyp6a20* expression in olfactory sensory organs. (A and B) GFP expression in the antennae (A) and palps (B). Whole-mount antennae and palps were stained with rabbit anti-GFP (green). (C and D) GFP<sup>+</sup> cells are nonneuronal. Frozen sections were stained with rabbit anti-GFP (green) and rat anti-ELAV (red). (E and F) a subset of GFP<sup>+</sup> cells in the antennae (E), but not in the palps (F), coexpressed LUSH. Frozen sections were stained with chick anti-GFP (green) and rabbit anti-LUSH (red). Confirmation of colabeling of one cell (white arrow) by z-series analysis is shown below and to the right of E. Inset in E is a higher magnification view of the boxed region (arrowhead), illustrating GFP<sup>+</sup> LUSH<sup>-</sup> and GFP<sup>+</sup> LUSH<sup>+</sup> cells. In all images, TOPRO-3 (blue) was used for nuclear staining. (Scale bars, 50  $\mu$ m.)

insertion indeed reflects a reduction in *Cyp6a20* expression or function, although rescue experiments will be required to formally confirm this. Thus, in flies with reduced levels of *Cyp6a20* expression, group housing is much less effective in suppressing aggressiveness (for more detailed analysis, see Fig. S2 and SI Text). These genetic data, when taken together with the observation that *Cyp6a20* mRNA levels are up-regulated by group housing (Fig. 2 Lower A and B), suggest that *Cyp6a20* mediates the effect of social experience to suppress aggressiveness (Fig. 3D).

#### ***Cyp6a20* Is Specifically Expressed in a Subset of Olfactory Sensory Organs.**

As an initial step toward investigating the mechanism of action of *Cyp6a20*, we investigated where the gene is expressed, using an enhancer trap line, *P[GawB]NP2593*, in which *Gal4* is integrated into the *Cyp6a20* locus. In *P[GawB]NP2593/UAS-mCD8GFP* adult flies, the reporter was expressed in the antennae and maxillary palps, the two main *Drosophila* olfactory sensory organs (Fig. 5 A and B). Scattered GFP signal was also seen in the brain (data not shown). Thus, like other P450 genes described in *Drosophila* (19), *Cyp6a20* appears antennal-enriched. We wished to verify that the *Gal4*-targeted GFP expression faithfully recapitulated the expression pattern of endogenous *Cyp6a20* mRNA. However, the small number and inaccessibility of GFP<sup>+</sup> cells in the antennae precluded a com-

parison of *Cyp6a20* mRNA levels between GFP<sup>+</sup> and GFP<sup>-</sup> cells, and *Cyp6a20* expression was undetectable by *in situ* hybridization (data not shown). We therefore examined the expression of *Cyp6a20* mRNA in larvae, where strong GFP expression was observed specifically in the salivary gland of *P[GawB]NP2593/UAS-mCD8GFP* specimens (Fig. S3A). RT-PCR experiments performed on larval tissues confirmed that *Cyp6a20* transcripts were enriched in salivary gland (Fig. S3B). Thus, the *P[GawB]NP2593* insertion in *Cyp6a20* correctly reports expression in larval tissues, making it reasonably likely that the same holds true for the adult, although further experiments will be required to confirm this.

Intriguingly, the GFP<sup>+</sup> cells in the adult antennae were preferentially if not exclusively associated with trichoid sensilla, which are thought to be involved in pheromone detection (20). Such trichoid sensillar-specific expression has been described for two P450 genes in the moth *Mamestra brassicae* (21) but has not been reported in *Drosophila*. More surprisingly, although it is often assumed that antennal-specific P450 enzymes are expressed by olfactory receptor neurons, GFP<sup>+</sup> cells in both the antennae and palps did not coexpress ELAV, a neuronal marker (22) (Fig. 5 C and D). However, a subset of GFP<sup>+</sup> cells coexpressed LUSH (Fig. 5 E and F), an odorant binding protein that marks a subpopulation of nonneuronal support cells (23). Thus, *Cyp6a20* is a P450 gene specifically expressed in a subset of olfactory sensory support cells.

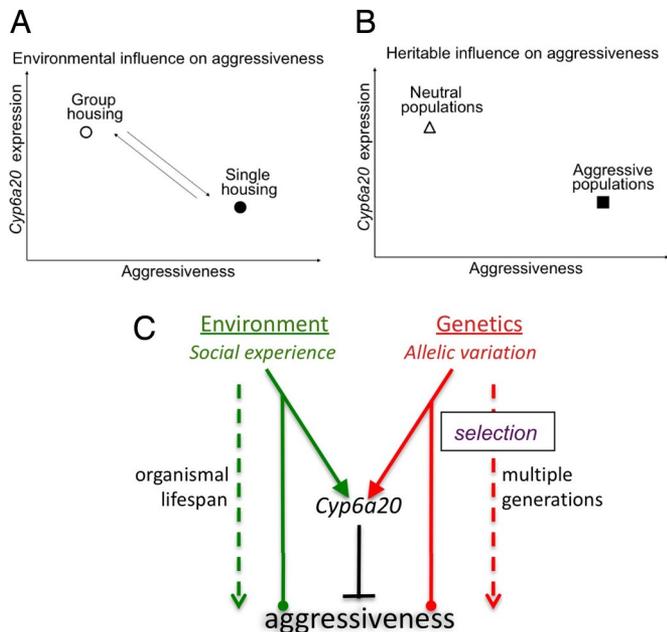
#### **Discussion**

Both genes and environment can influence aggressiveness; however, whether there is a commonality to the underlying biological mechanisms has not been clear. Using *Drosophila* as a model system, we show that an evolutionarily conserved environmental influence on aggressiveness, social experience, is associated with changes in gene expression. Detailed analysis of one of the regulated transcripts, *Cyp6a20*, indicates that it is up-regulated by social experience in a manner that correlates with the effects of social experience to suppress aggressiveness. Genetic experiments confirm that *Cyp6a20* is a negative regulator of aggressiveness (4) but reveal that its influence is only observed under conditions of group housing, where its expression is relatively higher. These data suggest that *Cyp6a20* is required to mediate the effect of group housing to suppress aggressiveness. *Cyp6a20* was the only gene in our dataset in common with a set identified in an independent expression profiling analysis of *Drosophila* populations selected for differential levels of aggressiveness (4). Taken together, these data suggest that *Cyp6a20* represents a common genetic target of heritable and environmental influences on aggressive behavior in fruit flies (Fig. 6C). Whether *Cyp6a20* is the only such target remains to be determined.

#### **Social Experience Influences Aggressiveness by Regulating Gene Expression.**

Prior social experience with conspecifics influences numerous aspects of animal behavior. Social isolation causes behavioral abnormalities in rodents, including anxiety and hyperaggressiveness (10, 24, 25). *D. melanogaster* reared as groups exhibit circadian rhythm coherence (26), and longer periods of daytime sleep (27). Social experience has also been shown to regulate courtship behavior in fruit flies (28), as well as aggression (13, 14). Further investigations are required to determine the extent to which the effects of social experience on these different behaviors are mediated by divergent or common pathways.

In cichlid fish, social interactions can regulate the brain expression of genes encoding neuropeptides (29) and steroid hormone receptors (30), but it has been difficult to extend such observations from correlation to causality. Here, we have demonstrated that extended male-male social interactions regulate gene expression in *Drosophila*, a system affording facile genetic manipulations. Recent studies have shown that rapid-onset



**Fig. 6.** *Cyp6a20* is a common genetic target of environmental and heritable influences on aggressive behavior. (A) Social experience influences aggressiveness in a reversible manner (bidirectional arrows), mediated by differential expression of *Cyp6a20*. (B) Genetic selection over multiple generations establishes neutral and aggressive populations with differential levels of aggressiveness, which correlate with differential *Cyp6a20* expression (4). (C) *Cyp6a20* regulation constitutes a common molecular target of environmental and genetic influences on aggressiveness. Circular arrowheads indicate that both positive and negative influences are possible. Environmental influences act on a time scale of the lifespan of the organism (Left), whereas genetic influences act over multiple generations as a consequence of selection (natural or artificial) (Right).

changes in gene expression accompany male–female courtship in *Drosophila* (31). (We have not yet examined the influence of male–female interactions in group housing on aggressiveness.) Further investigation of these genes may lead to a more comprehensive understanding of the effect of social experience on animal behaviors.

Under our stringency conditions, we identified 141 probe sets exhibiting higher expression in socially isolated than in group-housed flies and 48 probe sets exhibiting higher expression under group-housing conditions. Because aggressiveness is higher in socially isolated flies (13), genes in the first category are candidate positive regulators of aggressiveness, whereas those in the second category are candidate negative regulators. Loss-of-function mutations in candidate negative regulators should increase aggressiveness, as shown for *Cyp6a20* mutants (4). The present analysis confirms this but reveals that flies homozygous for a hypomorphic allele of *Cyp6a20* only show increased aggressiveness under group-housing conditions.

Interestingly, flies bearing mutations in two other candidate negative regulators of aggressiveness that we identified in a similar analysis, using a different fly strain, exhibited increased aggressiveness under single-housing conditions, but their aggressiveness could still be suppressed by group housing (L.W. and D.J.A., unpublished observations). These data suggest that there are at least two classes of genes that negatively regulate aggressiveness and whose expression levels are relatively higher in group-housed compared with single-housed flies: (i) genes, such as *Cyp6a20*, a hypomorphic allele of which overrides the effect of group-housing to suppress aggressiveness but which does not increase aggressiveness under single-housed conditions (Fig. 3A and D, class II); and (ii) genes such as those identified in our

second screen, hypomorphic mutations in which cause increased aggressiveness under single-housing conditions but not under group-housing conditions (Fig. 3D, class I). In principle, a third category of negative regulators may promote constitutively increased aggressiveness when mutated, under both single- and group-housing conditions, but we have not yet identified exemplars of this class. Recent studies have implicated serotonin, octopamine, and neuropeptide F in the control of aggressiveness in *Drosophila*. Genes related to these neuromodulatory pathways were not among those identified in our screen (18, 32–34).

**Environmental and Heritable Influences on Aggressiveness.** Genes can influence behavior both through polymorphic variation, on which natural selection can act, and by environmentally regulated changes in expression that occur within the lifetime of an individual. For example, naturally occurring polymorphisms in the *foraging* gene, which encodes a guanosine 3', 5'-monophosphate (cGMP)-dependent protein kinase (PKG), cause modifications of feeding behavior in *D. melanogaster* (35), whereas developmental changes in the expression of its honey bee ortholog, *Amfor*, modulate feeding behavior during the life history of single individuals (36). It is not yet known whether naturally occurring polymorphisms in *Cyp6a20* itself or rather in genes that encode upstream regulators of its expression underlie the differences in *Cyp6a20* transcript levels between the Neutr and Aggr strains selected in ref. 4. Nevertheless our results, taken together with the genetic selection experiments in ref. 4, identify a common genetic target of environmental and heritable influences on aggressive behavior within a single species (Fig. 6).

Studies have shown that increased aggressiveness promotes enhanced mating success (37). This raises the question of why, if aggressive behavior provides a general selective advantage, the ability of social experience to suppress aggressiveness is not eventually lost over many generations and replaced by constitutively aggressive populations. One explanation is that there may be positive selection for the ability of social experience to suppress aggressiveness. For example, under conditions where food resources are scarce and flies tend to feed in groups, individuals engaged in ongoing aggressive activity, despite this enriched social experience, might divert their energy and attention from feeding and reproductive behavior, thereby reducing their likelihood of reproductive success.

**Control of Aggressiveness by Social Regulation of Cytochrome P450s.**

Social experience suppresses aggressiveness in many species, leaving open the question of whether the underlying molecular mechanisms are also conserved. Although *Cyp6a20* does not have a clear vertebrate ortholog and appears to function in an insect-specific olfactory pathway (see the next paragraph), it encodes a cytochrome P450, which encompasses a large family of proteins with diverse enzymatic activities (38). In vertebrates, one member of this family is aromatase, which converts testosterone to estrogen and is required for inter-male aggressiveness (39, 40). Interestingly, the expression of brain aromatase has been shown to be regulated by social experience and other environmental influences (41, 42). Although flies lack testosterone, it is interesting that, in addition to *Cyp6a20*, five additional cytochrome P450 genes exhibited significant differential expression, by our criteria, between single-housing vs. group-housing conditions (Dataset S1). Thus, the general role of aromatases in mediating environmental influences on aggressiveness may be conserved, even if the pathways in which they act are not.

The antennal-specific expression of *Cyp6a20*, taken together with its up-regulation by group housing and its functional role as revealed by genetics, suggests that social experience may cause changes in olfactory sensitivity via regulation of *Cyp6a20*. Consistent with this interpretation, studies have shown that the effect

of social experience on male-male interactions requires pheromonal perception (28). Whether changes in *Cyp6a20* expression caused by social experience indeed influence pheromonal sensitivity and, if so, in what direction, is not yet clear. Pharmacologic inhibition of antennal-enriched cytochrome P450s eliminates pheromone sensitivity in some insects (43), suggesting a requirement for these proteins for maintaining olfactory sensitivity. If *Cyp6a20* were a positive regulator of pheromone sensitivity, then its increased expression under group-housing conditions might enhance sensitivity to an aggression-suppressing pheromone, resulting in a lower level of aggressiveness. Such a model would be consistent with the suggestion that inhibitory pheromones are used to suppress male-male interactions (28). Alternatively, as suggested in ref. 4, *Cyp6a20* may function to decrease sensitivity to an aggression-promoting pheromone. Distinguishing these hypotheses will require identification of the relevant pheromones and their functional role in aggression.

## Materials and Methods

**Fly Stocks and Rearing Conditions.** All fly stocks were reared in plastic vials containing yeast, corn syrup, and agar medium at 25°C, 60% humidity, and a 12-h light:12-h dark cycle. Newly eclosed males were reared either individually (single housing) or at 10 flies (group housing) per vial [2.4 cm (diameter) × 9.4 cm (height)] for 3 or 6 days before performing the behavioral assay. Wild-type Canton-5 (CS) flies were used for all experiments unless otherwise indicated. *Cyp6a20<sup>-/-</sup>* was introgressed into the CS background from *y<sup>1</sup>w<sup>67c23</sup>; P[y<sup>+</sup>mDint2] w[BR.E.BR] = SUPor-P}KG04665* as described in ref. 4. *Cyp6a20<sup>+/-</sup>* flies were generated by crossing *Cyp6a20<sup>-/-</sup>* males with Canton-5 females. *Cyp6a20<sup>Df/+</sup>* (*Df(2R)BSC11/+*) flies were generated by crossing *Df(2R)BSC11/SM6a* males with Canton-5 females. *Cyp6a20<sup>Df/+</sup>* (*Cyp6a20/Df*) flies were generated by crossing *Df(2R)BSC11/SM6a* males and *Cyp6a20<sup>-/-</sup>* females. P[GawB]NP2593 flies were from the *Drosophila* Genetics Resource Center, Kyoto Institute of Technology, Kyoto, Japan.

**Aggression Assay.** A multiplex aggression apparatus containing five arenas was constructed as illustrated in Fig. S1. Two males of the same age and social experience but from different vials were introduced into each well by gentle aspiration without anesthesia. The behavior of five pairs was video-captured for 20 min and analyzed manually, by counting the number of lunges in each arena. Lunging behavior was defined as described in ref. 17. The temperature and humidity of the apparatus was set to ≈25°C and ≈40%, respectively. Aggression assays were performed between 5 p.m. and 12 a.m.

**Microarray Analysis.** Three biological replicates were performed for both single-housing and group-housing conditions. For each replicate, 20 heads were isolated from 6-day-old, single-housed or group-housed male flies that were collected and frozen at 5 pm–6 pm on different days. Total RNA was prepared by using TRIzol as described in ref. 44. The following steps, including RNA quality test, reverse transcription, cRNA labeling, fragmentation and hybridization (Affymetrix *Drosophila* Genome Array 2.0) were performed by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at California Institute of Technology. The raw data from all arrays are available online at <http://www.ncbi.nlm.nih.gov/project/geo> under series GSE6994.

**Quantitative RT-PCR.** Four biological replicates were performed for each group indicated in Fig. 2. For each replicate, 20 heads were isolated from flies that were collected and frozen at 5 pm–6 pm on different days. Total RNA was prepared by using Qiagen RNeasy Micro Kit. For each biological replicate, three RT-PCR (technical) replicates were performed.

**Other Behavioral Assays.** Locomotor activity of flies was measured by using a customized program written in Matlab (Dankert et al., in preparation). For each measurement, a pair of 6-day-old male flies was introduced into a fighting arena with a food patch and agar, videotaped for 20 min and analyzed. To facilitate the automatic tracking of fly trajectories, the fighting arena used here was square-shaped instead of round-shaped as Fig. S1. For food trap assay, male flies were group-housed (10 flies/vial) for 5 days and wet-starved for an additional day on 1% agar medium before the test. On the day of the test, 10 flies were carefully aspirated into a plastic cylinder with the same dimensions as the fighting arena (Fig. S1) containing two odor traps (one with food and one without food). The trap was made by inserting a P1000

pipette tip into a 5-ml glass serum bottle [1.6 cm (diameter) × 3.4 cm (height)], containing 1 ml of standard fly food, or, as a control, 1% agar. After 2 hours, the number of flies trapped in each trap was counted and analyzed. For courtship assay, male flies were raised at 10 flies/vial for 5–6 days before testing. On the day of the test, one male fly of a given genotype, and a wild-type (C-5) virgin female of a similar age were carefully aspirated into a square-shaped fighting arena and videotaped for 30 min. Fly pairs that did not perform copulation were not included in the analysis. Three parameters were analyzed: courtship latency (the latency to the first courtship behavior exhibited toward the female), copulation latency (the latency of copulation), and courtship index (the percentage of time spent on courtship, including copulation, during the first 10 min of videotaping).

**Statistical Analysis of Behavioral Data.** For two-group comparisons, Student's *t* test (Fig. 4 D–F) or the Mann–Whitney *U* test (Fig. 1 A) were performed for parametrically or nonparametrically distributed data. For comparisons of more than two groups, ANOVA (Figs. 1B, 3A, and 4A) or Kruskal–Wallis ANOVA (Figs. 1 C and D, 3 B and C, and 4B) was performed for parametrically or nonparametrically distributed data, respectively. We used Student–Newman–Keuls test (Fig. 3A) or Dunnett's C test (Figs. 1B and 4A and B) as post hoc tests after ANOVA (and Kruskal–Wallis ANOVA for Fig. 4B) to identify significantly different groups with or without the assumption of homogeneity of variance, respectively. Significance levels for ANOVA and Kruskal–Wallis ANOVA were set to 0.05. Bar graphs are used to illustrate comparisons of means, with error bars representing SEM. Boxplots are used to illustrate comparisons of medians, with the lower and upper edges of the boxes representing the 25% and 75% quantiles, respectively, and the whiskers representing the 5% and 95% quantiles. The only exception was Fig. 1A, in which a bar graph (mean ± SEM) is used for illustrative purposes, whereas the statistical comparison was made between medians. This was done for the consistency with the other “fighting frequency” graphs (Figs. 1B and 3A).

**Statistical Analysis of Microarray and Quantitative RT-PCR Data.** Array data were analyzed by using the Rosetta Resolver platform through the default processing pipeline, including normalization, grouping, and intergroup comparison. A fold-change of 1.25 and a *P* value of 0.002 were used to identify differently expressed genes (Dataset S1). For quantitative RT-PCR, Student's *t* test was performed for the comparison between two normally distributed datasets (Fig. 2A). ANOVA followed by Student–Newman–Keuls post hoc test was used for the comparison between four normally distributed datasets with homogeneity of variance assumed (Fig. 2B).

**Immunohistochemistry.** Whole-mount antibody staining was adapted from ref. 45. Three- to 6-day-old male P[GawB]NP2593/UAS-*mCD8GFP* flies were anesthetized and dissected in PBS. Antennae (both second and third segments) and palps were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature (RT), washed two times for 10 min in PBS, incubated in PBS containing 5% Triton X-100 (5% PBT) for 5 min, washed three times for 10 min in 0.3% PBT, blocked for 1 h in 0.3% PBT containing 5% heat inactivated normal goat serum (0.3% PBT/S), and incubated with primary antibody in 0.3% PBT/S overnight at 4°C. On the following day, samples were incubated in 0.3% PBT/S at RT for 1 h after washing three times for 10 min in 0.3% PBT. Samples were then incubated with secondary antibody and TOPRO-3 in 0.3% PBT/S for 2 h at RT in dark, washed three times for 5 min in 0.3% PBT, mounted in Vectashield (Vector Laboratories), and imaged on confocal microscope (Leica).

Antibody staining of frozen sections was adapted from ref. 46. Three- to 6-day-old male P[GawB]NP2593/UAS-*mCD8GFP* flies were anesthetized, aligned by using a Martin Heisenberg-style fly collar, mounted in frozen Tissue Tek OCT, sectioned at 14 μm, fixed in 4% paraformaldehyde in PBS at RT for 7 min, washed two times for 10 min in PBS, penetrated in 0.1% PBT for 30 min, blocked in 0.1% PBT/S for 30 min, and incubated with primary antibodies in 0.1% PBT/S overnight at 4°C. On the following day, slides were washed three times for 10 min in 0.1% PBT, blocked in 0.1% PBT/S for 30 min, incubated with secondary antibodies and TOPRO-3 in 0.1% PBT/S for 2 h in dark, washed three times for 5 min in 0.1% PBT, mounted in Vectashield, and imaged on confocal microscope (Leica).

Antibodies were used diluted as follows: rabbit anti-GFP (Invitrogen; 1:800), chicken anti-GFP (Chemicon; 1:300), rabbit anti-LUSH [D. P. Smith (University of Texas Southwestern Medical Center, Dallas, TX); 1:20], rat anti-ELAV (DSHC 7E8A10; 1:10), Alexa488/Cy3-conjugated secondary antibodies (Molecular Probes; 1:500), and TOPRO-3 (1:2,000).

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