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An assay of behavioral plasticity in *Drosophila* larvae

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Abstract

Stress, or threats to homeostasis, is a universal part of life. Organisms face changing and challenging situations everyday, and the ability to respond to such stress is essential for survival. When subjected to acute stress, the body responds molecularly and behaviorally in order to recover a steady state. We developed a simple and robust assay of behavioral plasticity for *Drosophila* larvae in which well-defined behavioral responses and recovery can be observed and quantified. After experiencing different control and bright light treatments, populations of photophobic fly larvae were placed a defined distance from a food source to which they crawled. Half-times ($t_{1/2}$), or times at which half the total number of larvae reached the food, were used to compare different treatments and larval populations. Repeated control treatments with a main experimental strain gave tight, reproducible $t_{1/2}$ ranges. Control treatments with the wild type strains *Oregon R* and *Canton S*, the "rover" and "sitter" alleles of the *forager* locus, and *eyeless* mutants gave comparable results to those of the experimental strain. Exposure to bright light for a defined time period resulted in a reproducible slowing of locomotion. However, given a defined recovery period, the larvae recover full, normal locomotion. In addition, bright light treatments with *Canton S* gave comparable results to those of the experimental strain. *Eyeless* mutants, which are partially blind, do not show a response to bright light treatment. Thus, our assay measures the behavioral responses to bright light in *Drosophila* larvae and therefore might be useful as a general assay for studying behavioral plasticity and, potentially, adaptation to a stressful stimulus.

Keywords: Drosophila; Stress; Behavior; Assay

1. Introduction

1.1. Stress background

Hans Selye (1956) first coined the term "stress" in his book *The Stress of Life* and described it as the "non-specific response of the body to any demand." By definition, stress is a state in which homeostasis is threatened in either a perceived or physical manner (Pacak and Palkovits, 2001). When the cause of stress is uncontrollable, unpredictable, and of short duration (acute stress), the body reacts in an adaptive, compensatory manner in order to regain or maintain its homeostatic state. This natural stress response can be molecular and/or behavioral, and the organism may recover from the acute event without any lasting effects. This process involving the adaptive physiological response to acute stress is referred to as allostasis (Sterling and Eyer, 1988).

The ability to adapt to changing, challenging situations and environments is integral to an organism's survival (McEwen, 1999). The body's molecular and behavioral responses to stressful circumstances are advantageous because they allow for brisk CNS changes followed by rapid restoration of homeostasis. However, these responses are a "double-edged sword" (McEwen, 1998) – while they promote survival, they can also have long-term, detrimental effects on neuronal function. When the uncontrollable stressor is repeated or of longer duration, the stress becomes chronic and can lead to allostatic load and molecular changes in the brain. The stress becomes remembered and learned, and the stress response can be provoked by non-threatening events, such as in post-traumatic stress disorder. In *Aplysia*, a transient

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shock is translated to a chronic 'anxiety' state, both behaviorally and molecularly. In the three forms of learning examined in the *Aplysia*—habituation, sensitization, and classical conditioning—two stages of memory storage were observed: a transient memory that lasts minutes and an enduring memory that could last days or even weeks (Pinsker et al., 1970, 1973; Carew et al., 1972; Frost et al., 1985). Short-term memory stems from changes in synaptic strength between interconnected neurons (Castellucci et al., 1970; Kupfermann et al., 1970), while the conversion of a short-term memory to a long-term one requires protein synthesis and the formation of new neural connections (Castellucci et al., 1989). In studying the molecular biology behind this phenomenon, it was found that the neurotransmitter serotonin plays a key role in learning and the formation of both short and long term memories. Serotonin increases presynaptic cAMP, which activates PKA and leads to synaptic strengthening (Byrne and Kandel, 1996). Repeated puffs of serotonin activate PKA and lead to a tightly controlled cascade of gene activation that gives rise to the growth of new synaptic connections (Schacher et al., 1988; Dash et al., 1990; Glanzman et al., 1990; Bailey et al., 1992; Bacskai et al., 1993; Bailey and Kandel, 1993; Kaang et al., 1993; Martin et al., 1997a), and long-term changes in synaptic function and structure are confined to synapses stimulated by serotonin (Martin et al., 1997b; Casadio et al., 1999).

1.2. *Drosophila* larval behavior

The *Drosophila melanogaster* larva undergoes two stages before pupation and metamorphosis: foraging and wandering (Sokolowski et al., 1984). The foraging stage spans most of the larva's life, from the beginning of first instar to late third instar, in which it is feeding and burrowed deep into the food substrate. During this time, *Drosophila* larvae are photophobic and will actively move away from bright light (Lilly and Carlson, 1990; Gordesky-Gold et al., 1995; Sawin-McCormack et al., 1995). Approaching late third instar, larvae enter the wandering stage where they leave the food to find an appropriate pupation site. At the onset of wandering, their repulsion to light decreases until the larvae behave indifferently towards bright light stimuli (Sawin-McCormack et al., 1995).

1.3. *Drosophila* larval chemosensory and visual system

The *Drosophila* larva possesses a simple olfactory system (Python and Stocker, 2002). The major components of the larval chemosensory system consist of the dorsal organ, the terminal organ, the ventral organ, and a series of pharyngeal sensilla (Stocker, 1994; Cobb, 1999). The dorsal and terminal organs together form the antennomaxillary complex and are involved in olfaction and taste, respectively (Singh and Singh, 1984; Heimbeck et al., 1999; Oppliger et al., 2000). The dorsal organ, which consists of the larval antenna and main olfactory organ, contains 21 odorant receptor neurons, while the terminal organ contains roughly 80 gustatory neurons (Tissot et al., 1997; Heimbeck et al., 1999; Python and Stocker, 2002).

Bolwig's organ, the larval eye, is the light-sensing organ of the *Drosophila* larva and comprises the larval visual system (Bolwig, 1946). It is composed of two bilateral clusters of 12 photoreceptor cells in the larval mouth hooks (Steller et al., 1987; Hofbauer and Campos-Ortega, 1990). The larval optic nerve is formed by the photoreceptors' axons and innervates the optic lobe primordium portion of the brain lobes (Green et al., 1993; Campos et al., 1995).

1.4. An assay for behavioral plasticity

Many different assays have been developed and used to study behavior in *Drosophila* larvae. For example, choice assays have been used to study photobehavior (Lilly and Carlson, 1990), olfactory response (Shaver et al., 1998), gustatory response (Heimbeck et al., 1999), visual learning (Gerber et al., 2004), olfactory learning (Scherer et al., 2003), and thermobehavior (Liu et al., 2003). Path length assays have been used to examine foraging behavior (Pereira and Sokolowski, 1993; Pereira et al., 1995; Sokolowski et al., 1997) and photobehavior (Busto et al., 1999). Locomotion, crawling, and turning behavior have been studied using touch-sensitive assays (Caldwell et al., 2003; Tracey et al., 2003) and plate assays (Heiman et al., 1996; Yang et al., 2000; Suster et al., 2003).

Hypergravity exposure (Le Bourg and Minois, 1999) and starvation/desiccation (Hoffmann and Harshman, 1999) have been used to examine stress responses in adult flies. However, a method has not been developed to examine stress responses in *Drosophila* larvae. Here we have developed a locomotion assay and scoring method that is not only useful in studying behavior, but can also be used in conjunction with bright light to examine behavioral responses in *Drosophila* larvae.

2. Materials and methods

2.1. Fly stocks and harvest of synchronized larvae

Fly strains were maintained at room temperature ($25 \pm 2\,^{\circ}C$) in plastic vials or glass bottles containing a standard cornmeal/molasses *Drosophila* medium. Eggs from adult flies 1–10 days old were collected on fresh egg plates (molasses-agar media in 35 mm × 10 mm dishes) with a small amount of yeast paste in the center. The plates were replaced after 24-hour incubation periods and kept at room temperature, while the hatched larvae were allowed to grow. Early third instar larvae (72–78 h) from these plates were tested in the experiments.

Homozygous strains of *UAS-mCD8-GFP; ddc-GAL4* flies were used for the treatments and assays. In addition, the following strains were also examined as controls: wild type strains *Canton S* and *Oregon R*, the "rover" (for^R/for^R)

and "sitter" (for^S/for^S) alleles of the *forager* locus, *eyeless* (*Drosophila* pax-6 homolog, *ey[2]/ey[2]*).

2.2. Collection and washing of Drosophila larvae

Because larvae spend most of their lives burrowed in food, they are covered in food substrate when immediately removed from the medium. This poses a problem in crawling assays, as larvae covered in yeast will leave yeast trails as they crawl, causing other larvae to follow their paths or be attracted to them. To avoid this problem, the larvae were washed in distilled water after collection.

Using a small moistened paintbrush, approximately 200–400 larvae were collected from the molasses agar plates and placed in a small amount of distilled water. After gently stirring the water with the brush to aid in washing the larvae, the water was removed and drained using a 1000 μL Pipetman. Clean distilled water was added again, and the washing procedure was repeated two to three times until the larvae were clean of yeast.

2.3. Behavioral assay and data collection

The effects of the bright light treatments on larvae crawling were assessed and quantified using a locomotion assay. The apparatus was a 100 mm × 15 mm dish composed of 2.3% agar with a circular hole (25 mm diameter) dug out in the center. A small amount of cold yeast-water paste (50:50, yeast from Lesaffre Yeast Corporation) was spread along the edges of the hole prior to running the assay. In addition, the larvae were gathered and put onto a spatula for transfer onto the plate with a brush. At the start of the assay, the larvae were placed and spread out 5 mm from the edge of the plate. The assay was run for 60 min. To allow multiple simultaneous runs of the assay and faster counting of the larvae, the assays were recorded in Quicktime movie format (.mov) using Apple iSight webcams and SecuritySpy software on a Macintosh computer.

The larvae were scored by counting the number to reach the edge of the yeast within each minute of the assay. Larvae that crawled out of the yeast were scored only once. Those larvae that did not make it to the yeast but were still mobile within the 60 min were marked with an infinity time. Those that were not crawling (from possible injury during collection/washing or treatment) were disregarded from the assay.

No density-dependent effects were observed in any of the assays. Increasing or decreasing the number of larvae tested in the assays did not affect crawling speed or arrival times to the yeast.

2.4. Data analysis and statistics

For each assay, an arrival-time or distribution plot (number of larvae scored over time) was drawn. In addition, a "half-time", or $t_{1/2}$, was manually determined by interpolation from the raw data and used as a comparison tool. The half-time corresponds to the time at which half the total number of larvae in the assay reached the yeast.

Average arrival times and logarithmic slopes were also determined in these experiments as potential comparison measures. Although all gave similar statistical results, the $t_{1/2}$ was used as the main comparison measure.

All data were normally distributed and were analyzed using one-way Analyses of Variance (ANOVA). To determine which data sets had significantly different means, the Tukey–Kramer Multiple Comparisons Test was performed as a post-test.

3. Results

3.1. Establishing the method and protocol

3.1.1. UAS-mCD8-GFP; ddc-GAL4 line

Larvae from homozygous strains of *UAS-mCD8-GFP; ddc-GAL4* flies were used as the main experimental strain in the treatments and assays. This particular strain was chosen because it is isogenic and well-characterized. As serotonergic and dopaminergic neurons are labeled with green fluorescent protein in this strain, it will be used for future anatomical investigations.

3.1.2. Stress source and duration

Because larvae are repulsed by light, we hypothesized that bright light from a Fostec high intensity light source applied directly onto the larvae would be an effective cause of stress for the animals. The larvae were kept in approximately 500 μL of distilled water during the light exposure, both to buffer environmental temperatures changes and to prevent migration away from the light source.

In a set of pilot experiments, the duration of light to use was determined. Periods of 0 min (no light, wild type), 10, 20, and 30 min of light were applied onto the larvae immediately following washing, after which the larvae were observed in the locomotion assay and compared. Ten minutes of light gave the maximum behavioral response. However, increasing the duration of light gave a response that reverted back to a wild type, no-light response. The larvae's loss of response to increased amounts of light is probably due to desensitization to the light after 10 min of exposure. After the initial 10 min, the larvae may start recovering from the light and thus begin to show more wild-type responses.

3.1.3. Delay and assay duration

Because we were looking to develop an assay that characterizes both stress and behavioral plasticity in fruit fly larvae, we hypothesized that the larvae would be able to recover after light exposure. In another set of pilot experiments, we tested the larvae's recovery from the bright light and determined an amount of delay time that resulted in a fully recovered

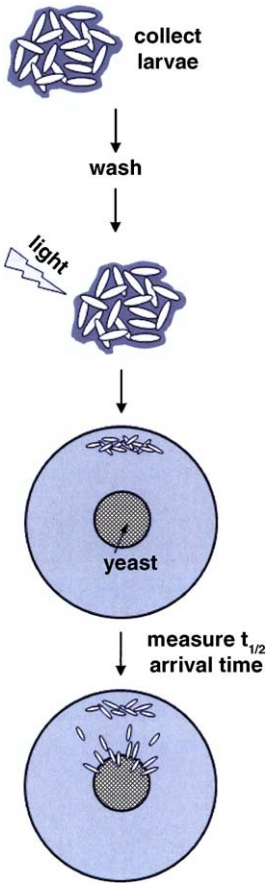

Fig. 1. Overview of the experimental protocol. A large population of early third instar larvae (72–78 h) is collected from a molasses egg plate using a small moistened paintbrush. The larvae are then washed and rinsed in a small amount of distilled water. Immediately after washing, the larvae undergo a 50-min treatment period that includes a 10-min exposure to bright light administered at the beginning, middle, or end of the period as well as 40 min of rest. The larvae are tested in a 60-min locomotion assay after treatment, where they are placed 5 mm from the edge of an agar plate with a yeast paste hole dug out in the center. As the larvae crawl towards the yeast, the number of larvae to reach the edge of the yeast within each minute of the assay is recorded. A $t_{1/2}$ arrival time, or the time at which half the total number of larvae in the assay reached the yeast, is then determined.

response. After exposure to 10 min of light, the larvae were given rest periods in which they were allowed to roam freely in a covered and empty 35 mm × 10 mm Petri dish. A 40-min delay or rest period after light was sufficient to give responses that were consistent with wild type responses.

Initially, an assay time of 30 min was sufficient to account for almost all of the larvae and minimize the number marked with an infinity time. However, after determining bright light and recovery times and incorporating them into the methods, a much longer assay time was needed, especially for the bright light assays. An assay time of 60 min was found to be sufficient.

An overview of the complete experimental protocol is shown in Fig. 1.

Table 1
Repeated control assays of *UAS-mCD8-GFP; ddc-GAL4* line

	Treatment		
	Rest before water	Intermediate water	Water before rest
Half-time ($t_{1/2}$)	4.099	4.031	3.866
	4.239	3.904	3.898
	3.931	3.975	4.303
	4.635	4.333	4.660
	3.894	4.257	4.500
	4.134	4.048	3.667
	4.464	4.357	3.961
Mean ± S.E.	4.199 ± 0.1025	4.129 ± 0.0691	4.122 ± 0.1392

Populations of early third-instar larvae of the *UAS-mCD8-GFP; ddc-GAL4* line were tested in the locomotion assay after experiencing three different control treatments: rest before water, intermediate water, and water before rest. From the raw data, half-times ($t_{1/2}$), or times in which 50% of the larval population reached the yeast, were manually determined by interpolation. Statistical tests were performed using one-way Analyses of Variance (ANOVA). The $t_{1/2}$ values of the three control populations were not significantly different from one another ($P > 0.05$).

3.2. Basics of the behavioral assay – tight data and reproducibility

3.2.1. Control treatments

In order to verify that the washing and periods in distilled water had no significant effects on larval behavior in our assay, we conducted several sets of control experiments without light. Before being tested in the behavioral assay, the larvae underwent a 50-min treatment period that included 10 min in distilled water (approximately 500 μL) and a total of 40 min of rest, all of which were conducted in partial dark. These intervals were determined based on the observations and results from the pilot experiments described above. The 10 min in distilled water were administered at three different time points: 40 min (rest before water), 20 min (intermediate water), and 0 min (water before rest). During the rest periods, the larvae were allowed to roam freely in a covered and empty 35 mm × 10 mm dish. Small amounts of distilled water were used to help collect the larvae after rest periods. A diagram of the control treatments can be seen in Fig. 2A.

3.2.2. Repeated control assays of UAS-mCD8-GFP; ddc-GAL4 line

Using larvae from the homozygous *UAS-mCD8-GFP; ddc-GAL4* lines, each control treatment was tested in the behavioral assay multiple times. A raw data plot from a typical control assay is shown in Fig. 3A. The half-times, or $t_{1/2}$ values, of all the control assays are listed in Table 1. The $t_{1/2}$ values of all sets of experiments gave a tight range and reproducible data (Fig. 4). The data yielded comparable results and were not significantly different from one another (one-way ANOVA, $P > 0.05$). Therefore, the washing and treatments do not significantly affect larval behavior in this assay.

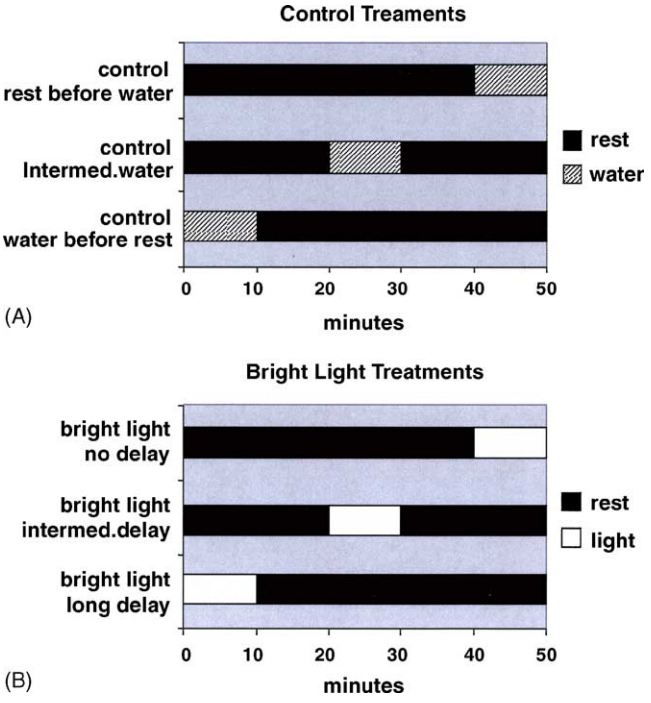

Fig. 2. Determined treatments for the assay. All treatment periods were 50 min in duration and were conducted prior to the locomotion assay. (A) Control treatments were used for non-light-treated *UAS-mCD8-GFP; ddc-GAL4* and *eyeless* larvae populations, the *forager* alleles "rover" and "sitter", and wild type strains *Oregon R* and *Canton S.* In the treatments, the larvae experienced "rest" periods (indicated by the dark gray) totaling 40 min in duration and "water" periods (blue) 10 min in duration. During the rest periods, larvae were allowed to roam freely in a covered and empty 35 mm × 10 mm dish, and in the water periods, the larvae were kept in distilled water (approximately 500 mL). Both periods were conducted in partial dark. The 10-min water period was administered at the end, beginning, and middle of the entire treatment period, corresponding to the "rest before water," "intermediate water," and "water before rest" control treatments, respectively. (B) Bright light treatments were used for *UAS-mCD8-GFP; ddc-GAL4* and *eyeless* larvae populations and consisted of rest periods and light periods. Rest periods were the same as described in (A), totaling 40 min in duration and conducted in the dark. Light periods were the same as the water periods in (A) except the larvae and distilled water were exposed to bright light for 10 min instead of being kept in the dark. Light periods were administered at the same time points as the water periods in (A), giving the bright light treatments "no delay," "intermediate delay," and "long delay". For interpretation of the references to color in this figure legend, please refer to the web version of the article.

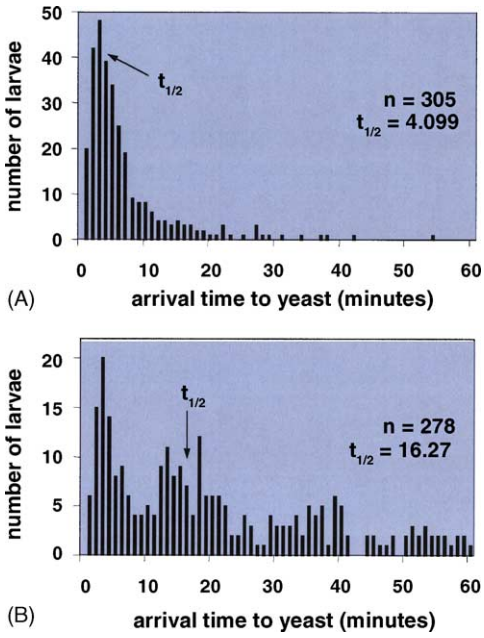

Fig. 3. Raw data from a control assay and a bright light assay. Bright light populations yielded noisier distribution plots and higher half-times ($t_{1/2}$) than control populations. (A) and (B) are distribution plots from a select control population and a select bright light population, respectively. Distribution plots were obtained by counting the number of larvae scored per minute during the 60-min assay. (A) Control populations yielded severely left-skewed distributions. This particular distribution, derived from a "rest before water" control population, gave a $t_{1/2}$ value of 3.724 min, indicating that half the total number of larvae ($n = 131$) arrived to the yeast in less than 4 min. (B) Bright light populations also produced left-skewed distributions, but there was much more noise throughout the curve. This particular distribution, derived from a "bright light, no delay" population, gave a $t_{1/2}$ value of 13.266 min (see Section 2), indicating that half the total number of larvae ($n = 278$) arrived to the yeast in more than 13 min.

3.3. Introducing bright light

3.3.1. Bright light treatments

Bright light treatment experiments followed the same procedures as the control treatments outlined in Section 3.2.1, except larvae were exposed to bright light instead of partial dark while kept in distilled water. The larvae were still kept in partial dark during rest periods. Three modes of bright light and delay were examined. To examine these modes, bright light periods for the duration of 10 min were administered at three different time points within a 50-min window: at 40 min (no delay), 20 min (intermediate delay), and 0 min (long delay). Therefore, all larvae experience matching amounts of light and rest but at different times and orders. A diagram of the bright light treatments can be seen in Fig. 2B.

3.3.2. Bright light and recovery

Populations that endured bright light exposure with no delay yielded half-times that were significantly different from those of the control populations. A raw data plot from a typical bright light, no delay assay is shown in Fig. 3B. These differences subsided with a long delay period from the light. Populations with intermediate delay gave results that were midway between no and long delay. Therefore, the intermediate and long delay periods administered after bright light exposure resulted in intermediate and full recovery from the light, respectively. Populations with no delay experienced no recovery. These results are listed in Table 2 and are shown in Fig. 4.

All controls yielded comparable results and were not significantly different from one another (one-way ANOVA, $P > 0.05$). The differences among the bright light, no recovery populations and the controls were significant (one-way ANOVA, Tukey comparison, $P < 0.001$). Bright light populations that experienced full recovery were not significantly

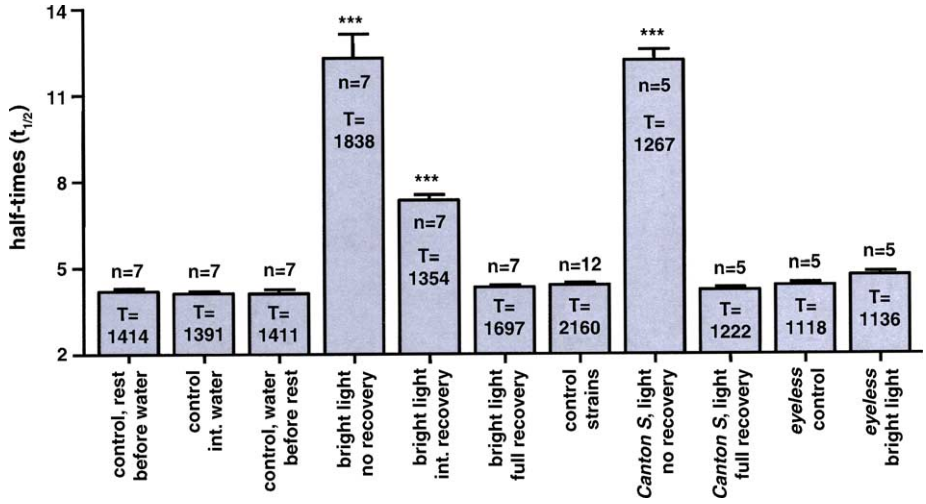

Fig. 4. Half-times ($t_{1/2}$) of control populations, bright light populations, control strains, and *eyeless* populations. Columns represent means of populations, and error bars represent standard errors of populations. The sample size of each set is indicated by *n*, and the total number of larvae used is indicated by *T*. Pure strains of the *UAS-mCD8-GFP; ddc-GAL4* line were used in the control populations and bright light populations that are represented in the first six columns. The control populations were tested under three different control treatments: rest before water, intermediate water, and water before rest. The data ranges of these controls, shown in the first three columns, were comparable and were not significantly different from one another (one-way ANOVA, $P > 0.05$). Treatments used with the bright light populations consisted of no recovery, intermediate recovery, and full recovery from light. Half-times of the no recovery and intermediate recovery light populations were significantly higher from those of the control populations (one-way ANOVA, Tukey comparison, $P < 0.001$), indicated by the triple-asterik (***) over the fourth and fifth columns. Bright light populations that experienced full recovery, shown in the sixth column, were not significantly different from the controls ($P > 0.05$). The wild type strains *Oregon R* and *Canton S* and the "rover" and "sitter" alleles of the *forager* locus were tested as control strains and are represented in the seventh column. The range of $t_{1/2}$ values of the control strains fit nicely into the ranges of the $t_{1/2}$ values of the control populations of the *UAS-mCD8-GFP; ddc-GAL4* line and was not significantly different from them ($P > 0.05$). To demonstrate behavioral plasticity in a wild type strain, *Canton S* was also tested in the bright light, no recovery and bright light, full recovery treatments. The half-times of the no recovery light populations of *Canton S*, represented in the eighth column, were significantly different from those of the *UAS-mCD8-GFP; ddc-GAL4* control populations ($P < 0.001$, indicated by ***) but not significantly different from those of the *UAS-mCD8-GFP; ddc-GAL4* bright light, no recovery populations ($P > 0.05$). The half-times from the full recovery light populations of *Canton S*, represented in the ninth column, were not significantly different from those of the controls and full recovery light populations of *UAS-mCD8-GFP; ddc-GAL4* ($P > 0.05$ for both). This indicates that the no recovery and full recovery bright light populations from both *Canton S* and *UAS-mCD8-GFP; ddc-GAL4* yield comparable results. *Eyeless* strains were tested in the control and bright light treatment assays to verify light as the source of the behavioral response. These are represented in the last two columns. Both control and bright light *eyeless* populations gave $t_{1/2}$ values that were not significantly different from the control *UAS-mCD8-GFP; ddc-GAL4* populations ($P > 0.05$).

Table 2
Bright light and recovery, *UAS-mCD8-GFP; ddc-GAL4*

Treatment	Half-time ($t_{1/2}$)[a]	*n* [total larvae]
Control, rest before water	4.199 ± 0.1025	7 [1414]
Control, intermediate water	4.129 ± 0.0691	7 [1391]
Control, water before rest	4.122 ± 0.1392	7 [1411]
Bright light with no recovery	$12.29 \pm 0.8328^{***}$	7 [1838]
Bright light with intermediate recovery	$7.345 \pm 0.1840^{***}$	7 [1354]
Bright light with full recovery	4.313 ± 0.0837 (n.s.)	7 [1697]

Populations of early third-instar larvae of the *UAS-mCD8-GFP; ddc-GAL4* line were tested in the locomotion assay after experiencing the six control and bright light treatments. From the raw data, half-times ($t_{1/2}$), or times in which 50% of the larval population reached the yeast, were manually determined by interpolation.
Statistical tests were performed using one-way Analyses of Variance (ANOVA). The no recovery and intermediate recovery light populations were significantly different from the control populations (Tukey comparison, $P < 0.001$), indicated by ***. The full recovery light populations were not significantly different from the controls ($P > 0.05$), indicated by n.s.
n represents the sample size, or number of assays run. The total number of larvae tested is indicated in brackets [ ].
[a] Mean ± S.E. of the $t_{1/2}$ values.

different from the controls ($P > 0.05$). The bright light, intermediate recovery populations were significantly different from the controls ($P < 0.001$) as well as from the bright light, no recovery populations ($P < 0.001$).

3.4. Wild type strains and the forager locus

3.4.1. Control assays of Oregon R, Canton S, and the forager locus

The control treatments "rest before water" and "water before rest" were roughly tested with the wild type strains *Canton S* and *Oregon R* and the *forager* alleles for^R and for^S. The results from all the strains, listed in Table 3, yielded a narrow data range (values between 4.043 and 4.573) which fit nicely into the *UAS-mCD8-GFP; ddc-GAL4* controls range (Fig. 4).

3.4.2. Bright light, no recovery and bright light, full recovery assays with Canton S

The bright light treatments with no recovery and full recovery were each tested five times with the wild type strain *Canton S.* These results are listed in Table 4. The half-times from the bright light with no recovery *Canton S* populations

Table 3
Control assays of wild type strains and the *forager* locus

Strain	Half-time ($t_{1/2}$) (no. of larvae)	
	Rest before water	Water before rest
Oregon R	4.460 [137]	4.396 [197]
	4.265 [207]	4.276 [238]
Canton S	4.500 [135]	4.247 [274]
	4.475 [162]	4.043 [163]
Rover	4.573 [170]	4.570 [210]
Sitter	4.412 [150]	4.434 [117]

Populations of early third-instar larvae from wild type strains *Oregon R* and *Canton S* and *forager* alleles "rover" and "sitter" were tested in the locomotion assay after experiencing the "rest before water" and "water before rest" control treatments. From the raw data, half-times ($t_{1/2}$), or times in which 50% of the larval population reached the yeast, were manually determined by interpolation. The number of larvae tested in each of the assays is indicated in brackets [ ].
The $t_{1/2}$ values from the wild type and *forager* populations fit into the data range of the *UAS-mCD8-GFP; ddc-GAL4* control populations and as a whole did not significantly differ from those of the control populations.

were significantly different from those of the *UAS-mCD8-GFP; ddc-GAL4* control populations (one-way ANOVA, Tukey comparison, $P < 0.001$) but not significantly different from those of the *UAS-mCD8-GFP; ddc-GAL4* bright light, no recovery populations ($P > 0.05$). This indicates that the no recovery bright light populations from both *Canton S* and *UAS-mCD8-GFP; ddc-GAL4* yield comparable results.

In addition, the half-times from the full recovery light populations of *Canton S* were not significantly different from those from the controls and full recovery light populations of *UAS-mCD8-GFP; ddc-GAL4* ($P > 0.05$ for both). This indicates that the full recovery bright light populations from both

Table 4
Bright light assays of the wild type strain *Canton S*

	Treatment	
	Bright light with no recovery	Bright light with full recovery
Half-time ($t_{1/2}$) (no. of larvae)	12.43 [289]	4.500 [221]
	12.63 [235]	4.253 [260]
	13.09 [277]	4.292 [245]
	11.90 [233]	4.121 [264]
	10.93 [233]	3.957 [232]
Mean ± S.E. (total larvae)	12.20 ± 0.3691 [1267]	4.225 ± 0.0905 [1222]

Populations of early third-instar larvae from the wild type strain *Canton S* were tested in the locomotion assay after experiencing bright light treatments with no recovery and full recovery. From the raw data, half-times ($t_{1/2}$), or times in which 50% of the larval population reached the yeast, were manually determined by interpolation. The number of larvae tested is indicated in brackets [ ].
Statistical tests were performed using one-way Analyses of Variance (ANOVA). The no recovery light populations were significantly different from the *UAS-mCD8-GFP; ddc-GAL4* control populations (Tukey comparison, $P < 0.001$) but not significantly different from the *UAS-mCD8-GFP; ddc-GAL4* bright light, no recovery populations ($P > 0.05$). The full recovery light populations were not significantly different from the controls and full recovery light populations of *UAS-mCD8-GFP; ddc-GAL4* ($P > 0.05$ for both).

Table 5
Eyeless mutants

Treatment	Half-time ($t_{1/2}$)[a]	*n* [Total larvae]
Control, rest before water	4.382 ± 0.0991 (n.s)	5 [1118]
Control, intermediate water	3.898	1 [285]
Control, water before rest	4.085	1 [222]
Bright light with no recovery	4.733 ± 0.1259 (n.s.)	5 [1136]
Bright light with intermediate recovery	4.023	1 [185]
Bright light with full recovery	4.500	1 [211]

Early third-instar larvae from *eyeless* mutant populations were tested in the locomotion assay after experiencing the six control and bright light treatments. From the raw data, half-times ($t_{1/2}$), or times in which 50% of the larval population reached the yeast, were manually determined by interpolation.
Statistical tests to compare the $t_{1/2}$ values of the "rest before water" control and the "bright light with no recovery" populations were performed using one-way Analyses of Variance (ANOVA). The populations did not differ significantly from one another ($P > 0.05$), indicated by n.s. In addition, they did not differ significantly from the control populations of the *UAS-mCD8-GFP; ddc-GAL4* line ($P > 0.05$).
n represents the sample size, or number of assays run. The total number of larvae tested is indicated in brackets [ ].
[a] Mean ± S.E. of the $t_{1/2}$ values.

Canton S and *UAS-mCD8-GFP; ddc-GAL4* yield comparable results.

3.5. Eyeless

3.5.1. Using eyeless to test the behavioral response in Drosophila larvae

Drosophila larval populations show reduced migration toward food after bright light exposure. To confirm the bright light as the source of the behavioral responses seen in the *UAS-mCD8-GFP; ddc-GAL4* line, *eyeless* mutant strains were also tested. Due to an impaired visual system, *eyeless* mutants are partially blind. If the reduced migration resulted from bright light exposure, *eyeless* mutants should not exhibit as large of an adaptive response to the bright light.

3.5.2. Phenotype test of eyeless mutants

In a rough preliminary phenotype test to demonstrate their partial blindness, approximately 100–150 *eyeless* larvae were placed (after washing) on a 100 mm × 15 mm agar plate on which a beam of light 15 mm in diameter was shone. This was also done for *UAS-mCD8-GFP; ddc-GAL4* larvae, which were used as the control. The number that crossed the beam of light, as measured by the number of trails left in the agar, was much lower for the control than for the *eyeless* mutants. This confirms a reduced visual input in the *eyeless* larvae. The phenotype tests were conducted in partial dark.

3.5.3. Eyeless in the six treatment assays

In the light-treated groups, the *eyeless* mutants showed greatly reduced behavioral responses compared to those of the *UAS-mCD8-GFP; ddc-GAL4* line. The results are listed in Table 5 and shown in the last two columns of Fig. 4. The "rest before water" control and the "bright light with no recovery"

treatments were each repeated five times, while the other four treatments were tested once. The control treatments gave half-times (4.382 ± 0.0991) that were comparable to those from the control treatments of the wild type strains, the "rover" and "sitter" alleles of the *forager* locus, and the *UAS-mCD8-GFP; ddc-GAL4* line. The bright light, no recovery *eyeless* populations produced half-times (4.733 ± 0.1259) that were somewhat increased, but not significantly different, from the control treatments and significantly less than the bright light *UAS-mCD8-GFP; ddc-GAL4* populations. The bright light *eyeless* population with intermediate recovery gave a $t_{1/2}$ value (4.023) comparable to that of the *eyeless* intermediate control (3.898). The bright light *eyeless* population with full recovery gave a $t_{1/2}$ value (4.500) comparable to that of the "water before rest" *eyeless* control population (4.085).

The "rest before water" control and the "bright light with no recovery" populations with the *eyeless* mutants were not significantly different from one another (one-way ANOVA, $P > 0.05$) or from the control and full recovery populations of the *UAS-mCD8-GFP; ddc-GAL4* line ($P > 0.05$) but were significantly different from the bright light, no recovery and bright light, intermediate recovery populations of the *UAS-mCD8-GFP; ddc-GAL4* line ($P < 0.001$).

3.5.4. Light is the source of behavioral response

All treatments of the *eyeless* mutant produced results that emulated those of the control treatments for the *UAS-mCD8-GFP; ddc-GAL4* line, the wild type strains, and the "rover" and "sitter" alleles of the *forager* locus. Exposure to light did not give a significant behavioral response. Therefore, the behavioral response of non-visually impaired larvae is due to a light responsivity and not due to high temperature and other potential effects of the treatment.

4. Discussion

4.1. Summary of experiment

In our experiments, we developed a locomotion assay for *Drosophila* larvae that can be used to assess behavioral effects and various alterations in the nervous and sensory systems during *Drosophila* development. Using an agar plate with a yeast paste hole dug out in the center, large quantities of larvae were tested in a behavioral assay after undergoing different control and bright light treatments. The treatments were compared using the half-times, or times at which half the total number of larvae reached the yeast, of the populations.

4.2. Simple, stable, and robust

Our treatments and locomotion assay test the behavioral response and recovery from bright light in *Drosophila* larvae. The assay is simple and proved to be robust. In our experiments, a well-defined behavioral response was observed – a significant increase in the $t_{1/2}$ value of the bright light populations from the non-light populations. Full recovery from the light was also observed. Bright light as the source of the behavioral response was also confirmed and demonstrated using *eyeless* mutants. One caveat with the *eyeless* experiments is that the *eyeless* gene may have other minor functions in addition to larval vision, although they are not revealed in our experiments with the *eyeless* mutant larvae.

Control treatments with the experimental strains, the wild type strains *Oregon R* and *Canton S*, the "rover" and "sitter" alleles of the *forager* locus, and the *eyeless* mutants all gave comparable results. The data from the strains collectively fit into a tight range and were very reproducible, demonstrating the assay's steadiness and giving a stable baseline without alterations in behavior. In *Canton S*, bright light treatments with no recovery gave comparable behavioral responses to those observed with the experimental strain *UAS-mCD8-GFP; ddc-GAL4*. Bright light with full recovery in *Canton S* also gave fully recovered responses that were comparable to those observed with the experimental strain. Thus, the assay was not background specific. There was no genetic contribution to the results, which did not differ among different strains and genotypes undergoing the same assay treatments.

4.3. Meaning of larval behavioral plasticity

Drosophila larvae in the foraging stage prefer dark areas and are repulsed by light. When a control population of larvae is left to crawl towards yeast, the majority of the larvae (approximately 80%) reach the yeast within the first 10 min, while the rest trickle in during the remaining time. This leaves a severely left-skewed distribution in a plot of larvae scored versus time (Fig. 3A). When a population of larvae is left to crawl towards yeast after exposure to bright light, there is an increase in the number of larvae that reach the yeast later in the assay time period; thus, there is more noise in the tail of the distribution (Fig. 3B). This leads to the significant increase in the $t_{1/2}$ value, which corresponds to the behavioral response exhibited in our experiments. It is not at all clear what causes the behavioral change in response to light. The fact that larvae rapidly adapt to this noxious condition may indicate that the response is a form of allostasis, or adaptation to stress.

4.4. A behavioral model for stress?

Our assay may be a behavioral model for stress. It quantifies the adaptive behavioral responses to bright light in *Drosophila* larvae and is a measure of behavioral plasticity. Given the robustness of our assay and its simplicity, it could be used in a genetic screen for mutants in behavioral plasticity with respect to their abilities to adapt to bright light. While a well-defined behavioral response is observed in the assay, we do not know what it is or what changes

are taking place in the larvae to cause the response. Is it a loss of appetite or motor function due to stress? In addition, because our experiments involve populations of larvae, it is not clear whether all larvae respond in the same manner. Are the larvae stressed at the molecular level despite not showing a behavioral response to the light? Conversely, are the few larvae from control populations that reach the yeast later in the assay undergoing stress, or are there always a select few that will always be slow? Further investigations using this assay may answer these questions and give additional insights into behavioral plasticity and adaptation to stress.

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References

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, et al. Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. Science 1993;260:222–6.

Bailey CH, Kandel ER. Structural changes accompanying memory storage. Annu Rev Physiol 1993;55:397–426.

Bailey CH, Montarolo P, Chen M, Kandel ER, Schacher S. Inhibitors of protein and RNA synthesis block structural changes that accompany long-term heterosynaptic plasticity in Aplysia. Neuron 1992;9:749–58.

Bolwig N. Senses and sense organs of the anterior end of the house fly larvae. København: C.A. Reitzel; 1946.

Busto M, Iyengar B, Campos AR. Genetic dissection of behavior: modulation of locomotion by light in the *Drosophila melanogaster* larva requires genetically distinct visual system functions. J Neurosci 1999;19:3337–44.

Byrne JH, Kandel ER. Presynaptic facilitation revisited: state and time dependence. J Neurosci 1996;16:425–35.

Caldwell JC, Miller MM, Wing S, Soll DR, Eberl DF. Dynamic analysis of larval locomotion in *Drosophila* chordotonal organ mutants. Proc Natl Acad Sci USA 2003;100:16053–8.

Campos AR, Lee KJ, Steller H. Establishment of neuronal connectivity during development of the *Drosophila* larval visual system. J Neurobiol 1995;28:313–29.

Carew TJ, Pinsker H, Kandel ER. Long-term habituation of a defensive withdrawal reflex in Aplysia. Science 1972;175:451–4.

Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, et al. A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell 1999;99:221–37.

Castellucci V, Pinsker H, Kupfermann I, Kandel ER. Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in Aplysia. Science 1970;167:1745–8.

Castellucci VF, Blumenfeld H, Goelet P, Kandel ER. Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of Aplysia. J Neurobiol 1989;20:1–9.

Cobb M. What and how do maggots smell? Biol Rev 1999;74:425–59.

Dash PK, Hochner B, Kandel ER. Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. Nature 1990;345:718–21.

Frost WN, Castellucci VF, Hawkins RD, Kandel ER. Mono-synaptic connections made by the sensory neurons of the Gill-Withdrawal and Siphon-Withdrawal reflex in Aplysia participate in the storage of long-term-memory for sensitization. Proc Natl Acad Sci USA 1985;82:8266–9.

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, et al. Visual learning in individually assayed *Drosophila* larvae. J Exp Biol 2004;207:179–88.

Glanzman DL, Kandel ER, Schacher S. Target-dependent structural changes accompanying long-term synaptic facilitation in Aplysia neurons. Science 1990;249:799–802.

Gordesky-Gold B, Warrick JM, Bixler A, Beasley JE, Tompkins L. Hypomorphic mutations in the larval photokinesis A (lphA) gene have stage-specific effects on visual system function in *Drosophila melanogaster*. Genetics 1995;139:1623–9.

Green P, Hartenstein AY, Hartenstein V. The embryonic development of the *Drosophila* visual system. Cell Tissue Res 1993;273:583–98.

Heiman RG, Atkinson RC, Andruss BF, Bolduc C, Kovalick GE, Beckingham K. Spontaneous avoidance behavior in *Drosophila* null for calmodulin expression. Proc Natl Acad Sci USA 1996;93:2420–5.

Heimbeck G, Bugnon V, Gendre N, Haberlin C, Stacker RF. Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. J Neurosci 1999;19:6599–609.

Hofbauer A, Campos-Ortega JA. Proliferation pattern and early differentiation of the optic lobes in Drosophila-Melanogaster. Rouxs Arch Dev Biol 1990;198:264–74.

Hoffmann AA, Harshman LG. Desiccation and starvation resistance in *Drosophila*: patterns of variation at the species, population and intrapopulation levels. Heredity 1999;83(Pt 6):637–43.

Kaang BK, Kandel ER, Grant SG. Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in Aplysia sensory neurons. Neuron 1993;10:427–35.

Kupfermann I, Castellucci V, Pinsker H, Kandel E. Neuronal correlates of habituation and dishabituati on of the gill-withdrawal reflex in Aplysia. Science 1970;167:1743–5.

Le Bourg E, Minois N. A mild stress, hypergravity exposure, postpones behavioral aging in *Drosophila melanogaster*. Exp Gerontol 1999;34:157–72.

Lilly M, Carlson J. Smellblind: a gene required for *Drosophila* olfaction. Genetics 1990;124:293–302.

Liu L, Yermolaieva O, Johnson WA, Abboud FM, Welsh MJ. Identification and function of thermosensory neurons in *Drosophila* larvae. Nat Neurosci 2003;6:267–73.

Martin KC, Michael D, Rose JC, Barad M, Casadio A, Zhu H, et al. MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. Neuron 1997a;18:899–912.

Martin KC, Casadio A, Zhu H, Yaping E, Rose JC, Chen M, et al. Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell 1997b;91:927–38.

McEwen BS. Protective and damaging effects of stress mediators. N Engl J Med 1998;338:171–9.

McEwen BS. Stress and hippocampal plasticity. Annu Rev Neurosci 1999;22:105–22.

Oppliger FY, Guerin PM, Vlimant M. Neurophysiological and behavioural evidence for an olfactory function for the dorsal organ and a gustatory one for the terminal organ in *Drosophila melanogaster* larvae. J Insect Physiol 2000;46:135–44.

Pacak K, Palkovits M. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev 2001;22:502–48.

Pereira HS, Sokolowski MB. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. Proc Natl Acad Sci USA 1993;90:5044–6.

Pereira HS, MacDonald DE, Hilliker AJ, Sokolowski MB. Chaser (Csr), a new gene affecting larval foraging behavior in *Drosophila melanogaster*. Genetics 1995;141:263–70.

Pinsker H, Kupfermann I, Castellucci V, Kandel E. Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. Science 1970;167:1740–2.

Pinsker HM, Hening WA, Carew TJ, Kandel ER. Long-term sensitization of a defensive withdrawal reflex in Aplysia. Science 1973;182:1039–42.

Python F, Stocker RF. Adult-like complexity of the larval antennal lobe of D-melanogaster despite markedly low numbers of odorant receptor neurons. J Comp Neurol 2002;445:374–87.

Sawin-McCormack EP, Sokolowski MB, Campos AR. Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. J Neurogenet 1995;10:119–35.

Schacher S, Castellucci VF, Kandel ER. cAMP evokes long-term facilitation in Aplysia sensory neurons that requires new protein synthesis. Science 1988;240:1667–9.

Scherer S, Stocker RF, Gerber B. Olfactory learning in individually assayed *Drosophila* larvae. Learn Mem 2003;10:217–25.

Selye H. The stress of life. New York: McGraw-Hill; 1956.

Shaver SA, Varnam CJ, Hilliker AJ, Sokolowski MB. The foraging gene affects adult but not larval olfactory-related behavior in *Drosophila melanogaster*. Behav Brain Res 1998;95:23–9.

Singh RN, Singh K. Fine-structure of the sensory organs of Drosophila-Melanogaster Meigen larva (Diptera, Drosophilidae). Int J Insect Morphol Embryol 1984;13:255–73.

Sokolowski MB, Kent C, Wong J. *Drosophila* larval foraging behavior—developmental stages. Anim Behav 1984;32:645–51.

Sokolowski MB, Pereira HS, Hughes K. Evolution of foraging behavior in *Drosophila* by density-dependent selection. Proc Natl Acad Sci USA 1997;94:7373–7.

Steller H, Fischbach KF, Rubin GM. Disconnected: a locus required for neuronal pathway formation in the visual system of *Drosophila*. Cell 1987;50:1139–53.

Sterling P, Eyer J. Allostasis: a new paradigm to explain arousal pathology. In: Fisher S, Reason J, editors. Handbook of life stress, cognition and health. New York: John Wiley & Sons; 1988. p. 629–49.

Stocker RF. The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res 1994;275:3–26.

Suster ML, Martin JR, Sung C, Robinow S. Targeted expression of tetanus toxin reveals sets of neurons involved in larval locomotion in *Drosophila*. J Neurobiol 2003;55:233–46.

Tissot M, Gendre N, Hawken A, Stortkuhl KF, Stocker RF. Larval chemosensory projections and invasion of adult afferents in the antennal lobe of *Drosophila*. J Neurobiol 1997;32:281–97.

Tracey Jr WD, Wilson RI, Laurent G, Benzer S. Painless, a *Drosophila* gene essential for nociception. Cell 2003;113:261–73.

Yang P, Shaver SA, Hilliker AJ, Sokolowski MB. Abnormal turning behavior in *Drosophila* larvae. Identification and molecular analysis of scribbler (sbb). Genetics 2000;155:1161–74.