



## Behavioral dissection of *Drosophila* larval phototaxis

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### ABSTRACT

A behavior generally comprises multiple processes. Analyzing these processes helps to reveal more characteristics of the behavior. In this report, light/dark choice-based *Drosophila* larval phototaxis is analyzed with a simplistic mathematical model to reveal a fast phase and a slow phase response that are involved. Larvae of the strain  $w^{1118}$ , which is photophobic in phototaxis tests, prefer darkness to light in an immediate light/dark boundary passing test and demonstrate a significant reduction in motility in the dark condition during phototaxis tests. For  $tim^{01}$  larvae, which show neutral performance in phototaxis tests, larvae unexpectedly prefer light to darkness in the immediate light/dark boundary passing test and demonstrate no significant motility alteration in the dark condition. It is proposed that *Drosophila* larval phototaxis is determined by a fast phase immediate light/dark choice and an independent slow phase light/dark-induced motility alteration that follows.

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### Introduction

Preference is basic and crucial for many animal behaviors. For example, in some olfactory and visual learning and memory study paradigms in *Drosophila*, avoidance of heat or odor punishment is fundamental for conditioning [1,2]. In some other cases, however, rewarding is involved [3]. Unraveling the mechanisms underlying behavioral preference is important for the understanding of those complex behaviors that involve preference.

Phototaxis is generally considered as a form of light-dependent preference behavior in animals. In the fruit fly *Drosophila melanogaster*, it is well known that wild-type adults show positive phototaxis while negative phototaxis is seen in larvae. Adult phototactic behavior is a polygenic trait and is affected by factors like age and rhabdomere structure in compound eyes [4,5]. Genetic mutants defective in normal adult phototaxis have been isolated, including some mutants that exhibit negative phototaxis [5,6]. On the other hand, the photophobic responses of fly larvae represent a composite of various responses [7]. When the light is switched on or off, wild-type larvae show immediate stop and head-swing responses that are implemented in phototaxis behavior.

In the present study, *Drosophila* larval phototaxis was analyzed using an equilibrium-based mathematical model. Using  $w^{1118}$  and  $tim^{01}$  larvae, the phototaxis of *Drosophila* larva was shown to involve an immediate ability to pass the light/dark boundary and motility alteration during the phototaxis test. The fast phase light/dark choice and the slow phase motility alteration appeared to be independent of each other.

### Materials and methods

**Fly stocks.** The fly strains  $w^{1118}$  and  $tim^{01}$  (kindly provided by A. Guo) were used. They were raised on standard medium [8] and normal light/dark (LD) cycles. In all experiments, 3rd-instar larvae 72–96 h after egg laying were used.

**Behavioral assays.** All behavioral tests were performed at room temperature (22–24 °C) between 10:00 am and 5:00 pm. (1) *Ten minutes phototaxis test*: the 10-min phototaxis test was performed following the protocol introduced by Mazzoni et al. except that the light intensity was 550 lux and test time was 10 min [9]. The light preference index (PI) in the 10-min phototaxis test was calculated as  $PI = (\text{number of larvae in the dark half} - \text{number of larvae in the light half}) / (\text{number of larvae in the dark half} + \text{number of larvae in the light half})$ . Specifically for the larval dynamic distribution analysis, the test started with all larvae in the light or dark half but at distances of no more than 1 cm from the dark/light boundary. (2) *Immediate LD boundary passing test*: a larva was positioned at a distance of about 1 cm from the light/dark(LD) boundary with the head towards the boundary line in a half-covered testing plate. The larva was then allowed to cross the boundary line. If the whole body of the larva passed the midline and did not return to the starting side within 2 s, the test was counted as a successful pass. If the larva turned back when its head touched the boundary line, or crossed the line and returned within 2 s, the test was counted as a failed pass. One larva was allowed to try only once. In each group, 20 larvae were subjected to the boundary passing test in directions of light to dark (L → D) and dark to light (D → L). The proportion of larvae with successful passes in each group was scored as the immediate LD boundary passing rate. They were then subjected to a 10-min phototaxis test before another round of immediate

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boundary passing tests in both directions. The sequence of L → D and D → L tests was random. (3) *Two minutes* midline accessing test: for this test, a circle with a radius of about 0.75 cm was drawn at the center of the “dark half” of the testing plate. Two lines running parallel to the LD boundary at distances of about 1/8 (N line) and 7/8 (F line) of the radius length were drawn in the “dark half” of the plate, as shown in Fig. 1A. During the test, both halves of testing plate were exposed to light (550 lux) or dark (5 lux) condition without cover. Twenty larvae were allowed to move on the testing plate for 2 min to become familiar with the new environment. All 20 larvae were then lined up along the F (or N) line and allowed to move freely for 2 min. The percentage of larvae that touched the LD boundary line was scored as the midline accessing rate from the far line (RM<sub>F</sub>). All the larvae were then collected and lined up along the N (or F) line and allowed to move freely for 2 min. The percentage of larvae that touched the LD boundary was scored as midline accessing rate from the near line (RM<sub>N</sub>). These 20 larvae were then subjected to the 10-min phototaxis test with the “dark half” of the testing plate covered, as described above. By the end of the 10-min phototaxis test, the larvae in the light half were gently transferred to the 0.75-cm circle at the center of the dark half. The larvae were then allowed to move freely for

2 min in the same light condition as before the 10-min phototaxis test. The proportion of larvae that touched the LD boundary line was scored as the midline accessing rate (RM). The sequence of F-line and N-line tests was random.

*Statistics.* All comparisons were made based on either a one-sample or two-sample *t*-test.

## Results

### Larval distribution varied before reaching equilibrium during the 10-min phototaxis test

The *w*<sup>1118</sup> strain was used as representative of flies that show normal larval photophobia responses. Fig. 1B shows the results of representative experiments beginning with all larvae either on the dark or light side. During the 10-min tests, the distribution of larvae in the light and dark halves was not constant, but kept changing before finally reaching equilibrium and becoming steady in 10 min. Since the starting side did not affect the final distribution pattern, all the subsequent 10-min phototaxis test started with all the larvae placed along the LD boundary.

### Larval distribution could be explained with a simplistic model

One natural way to explain the final larval distribution is as follows. When the distribution equilibrium was reached, the amount of larvae that left one half to the other half should be equal to the amount of larvae that entered that half from the other half during a certain time period. We use  $N_L$  to indicate the number of larvae in the light half and  $N_D$  those in the dark.  $P_{LD}$  is used to indicate the probability for a larva passing the light/dark (LD) boundary from light to dark and  $P_{DL}$  from dark to light. With this nomenclature:

$$N_L \times P_{LD} = N_D \times P_{DL} \quad (1)$$

Because only some of the larvae on each side actually try to cross the LD boundary, the equation is actually

$$N_{LE} \times P_{LD} = N_{DE} \times P_{DL} \quad (2)$$

in which  $N_{LE}$  and  $N_{DE}$  are the number of larvae that effectively tried to cross the LD boundary from the light half and the dark half, respectively. However,  $N_{LE}$  and  $N_{DE}$  could not be easily measured. What could be easily counted were the numbers of larvae on the light and dark halves,  $N_L$  and  $N_D$ . Thus, new factors were introduced and a third equation was obtained:

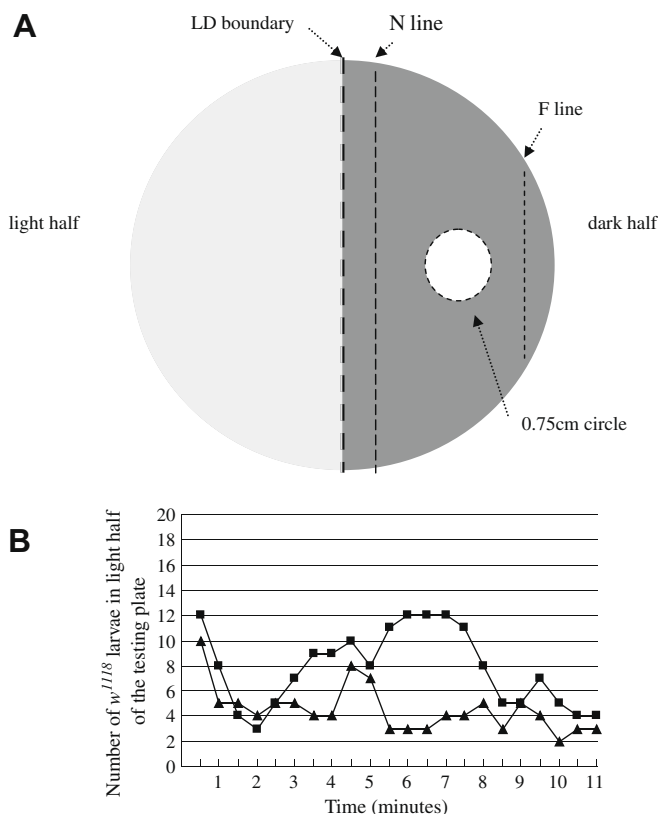
$$N_L \times P_L \times P_{LD} = N_D \times P_D \times P_{DL} \quad (3)$$

in which  $P_L = N_{LE}/N_L$  and  $P_D = N_{DE}/N_D$ .  $P_L$  and  $P_D$  measure the proportion of larvae that effectively try to pass the light/dark boundary in each half.

### *w*<sup>1118</sup> larvae were photophobic in an immediate LD boundary passing test

To estimate the  $P_{LD}$  and  $P_{DL}$ , the immediate LD boundary passing test was done before and after the 10-min phototaxis test. The average values were used to estimate  $P_{LD}$  and  $P_{DL}$  during the 10-min phototaxis test. As shown in Fig. 2A, both before and after the 10-min phototaxis test, nearly 100% of the *w*<sup>1118</sup> 3rd-instar larvae crossed the LD boundary line successfully from the light side, whereas averagely only about 75% of the larvae succeeded in crossing the line from the dark side in the immediate LD boundary passing test. Thus, at least in this immediate LD boundary passing test, *w*<sup>1118</sup> larva preferred darkness to light.

At same time, an average light preference index of 0.70 was obtained in the 10-min phototaxis test. This meant that 17 larvae



**Fig. 1.** Larval phototaxis test. (A) Schematic representation of the testing plate. The light half of the testing plate is shown in light gray and the dark half is in deep gray. N (near) line and F (far) line are the starting lines for the larvae to move in the 2-min midline accessing test before the 10-min phototaxis test, and the 0.75-cm circle is the area for the transferred larvae to start in the 2-min midline accessing test after the 10-min phototaxis test. See Materials and methods for more details on the immediate LD boundary passing test and the 2-min midline accessing test. (B) Oscillation of *w*<sup>1118</sup> 3rd-instar larvae distribution in the light and dark halves of the testing plate during the 10-min phototaxis test. Twenty larvae were placed on either the light or dark side of the LD boundary at the beginning of the test. The number of larvae in the light half was counted every 0.5 min. The testing time of 11 min was used to demonstrate the larval distribution equilibrium by 10 min. Triangle, test started with all larvae in the light half. Rectangle, test started with all larvae in the dark half.

were on the dark side and three were on the light side, on average, by the end of the test (Fig. 2B). The following approximate values were obtained:

$$N_L = 3; \quad N_D = 17; \quad P_{LD} = 1.0; \quad P_{DL} = 0.75$$

It was noticed that

$$N_L \times P_{LD} = 3$$

and

$$N_D \times P_{DL} = 12.75$$

Therefore

$$N_L \times P_{LD} \neq N_D \times P_{DL}$$

Eq. (1) clearly could not hold, suggesting that the asymmetrical LD boundary passing rate was not the only factor that decided the final distribution of larvae.

*The motility of  $w^{1118}$  larvae in dark condition decreased during the 10-min phototaxis test*

The equations

$$N_L \times P_L \times P_{LD} = N_D \times P_D \times P_{DL} \quad (3)$$

and

$$(N_D \times P_{DL}) / (N_L \times P_{LD}) = P_L / P_D \quad (4)$$

were then checked against our experimental data. Experimental data were substituted into Eq. (4) as follows:

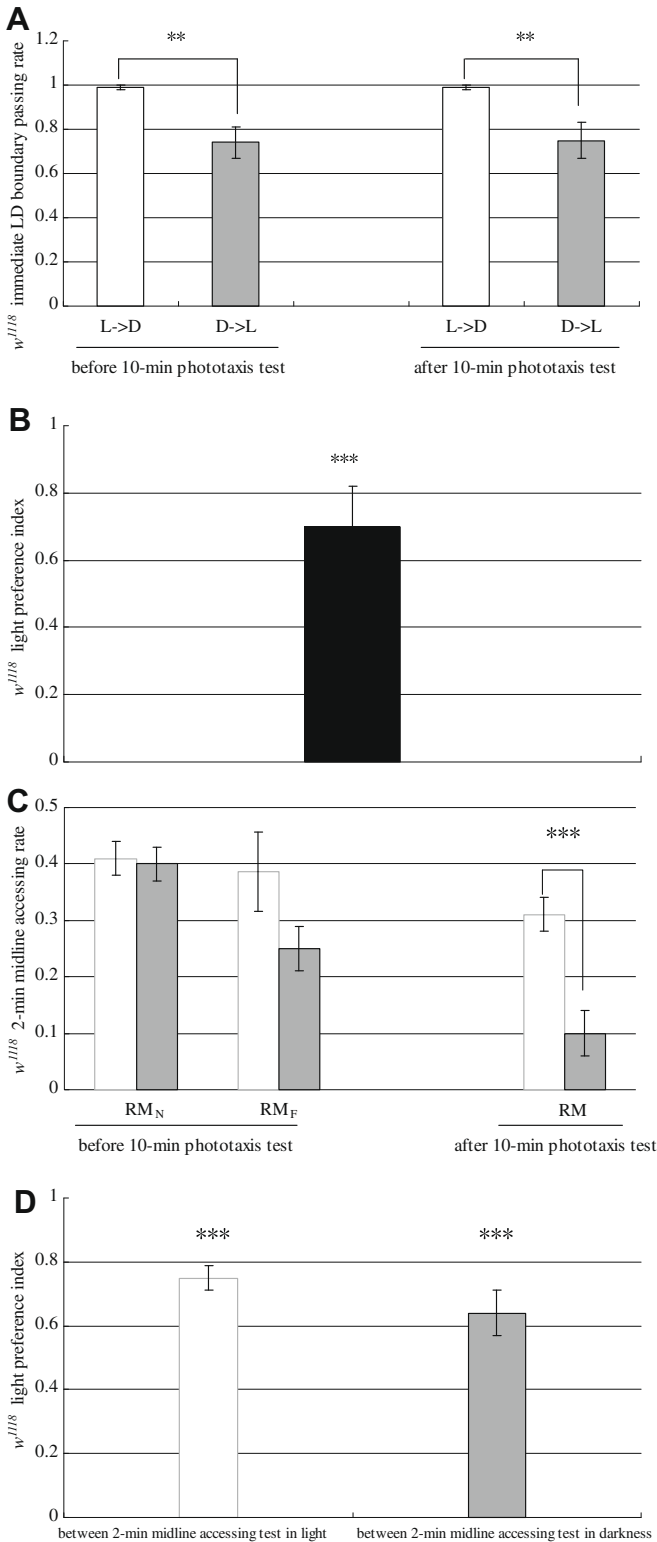
$$3 \times P_L \times 1.0 = 17 \times P_D \times 0.75$$

and

$$P_L / P_D = (17 \times 0.75) / (3 \times 1.0) = 4.25$$

This means that, at equilibrium, the probability of larvae in the light half of the testing plate trying to cross the LD boundary was more than four times that of larvae in the dark half trying to cross the LD boundary.

Since  $P_L$  and  $P_D$  were difficult to measure directly by experiment, factors that might affect  $P_L$  and  $P_D$  were analyzed. Larval motility might be an important underlying factor. Larval motility in light and dark conditions was evaluated using a 2-min midline accessing test. The 2-min tests in which the larvae started from a starting line far from ( $RM_F$ ) or near to ( $RM_N$ ) the LD boundary were performed for under- and overestimations of larval motility respectively. As shown in Fig. 2C, the  $RM_F$  and  $RM_N$  in the light/dark conditions before the 10-min phototaxis test were not significantly different. After the 10-min phototaxis test, the midline accessing rate (RM) with larvae averagedly starting from the center of one testing plate half was used to estimate motility. Here, RM,



**Fig. 2.**  $w^{1118}$  larval light responses before and after the 10-min phototaxis test. (A) Immediate LD boundary passing rates of  $w^{1118}$  3rd-instar larvae before and after the 10-min phototaxis test. The LD boundary passing rates in the L → D direction are significantly higher than those in the D → L direction both before (0.99 ± 0.01 vs. 0.74 ± 0.07,  $p < 0.01$ ) and after (0.99 ± 0.01 vs. 0.75 ± 0.08,  $p < 0.01$ ) the 10-min phototaxis test. Open bar, LD boundary passing rate in direction of light to dark (L → D), gray bar, LD boundary passing rate in direction of dark to light (D → L). (B) Photophobic performance of the same larvae in the 10-min phototaxis test carried out between the immediate LD boundary passing tests (0.70 ± 0.12 vs. 0,  $p < 0.001$ ). (C) Two minutes midline accessing rates of  $w^{1118}$  3rd-instar larvae in light and dark conditions before and after the 10-min phototaxis test.  $RM_F$  and  $RM_N$  before the 10-min phototaxis test as well as the RM after the 10-min phototaxis test in both light and dark conditions are shown. Before the 10-min phototaxis test, the 2-min midline accessing rates in light condition are not significantly different from those in dark condition (for  $RM_N$ , 0.41 ± 0.03 vs. 0.40 ± 0.04,  $p > 0.05$ ; for  $RM_F$ , 0.38 ± 0.07 vs. 0.22 ± 0.04,  $p > 0.05$ ). After the 10-min phototaxis test, the RM in the dark condition is significantly lower than that in the light condition (0.31 ± 0.03 vs. 0.1 ± 0.04,  $p < 0.001$ ); Open bars represent tests carried out in the light condition, gray bars represent tests carried out in the dark condition. (D) Photophobic performance of the same larvae in 10-min phototaxis tests carried out between the 2-min midline accessing tests in light or dark conditions (for the 10-min phototaxis test between midline accessing tests performed in the light condition, 0.75 ± 0.04 vs. 0,  $p < 0.001$ ; for the 10-min phototaxis test between midline accessing tests performed in the dark condition, 0.64 ± 0.07 vs. 0,  $p < 0.001$ ; average preference index, 0.70). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .  $n = 10$  for all groups.

but not  $RM_F$  and  $RM_N$ , was tested to minimize the arousal effect of brush disturbance on larval motility. After the 10-min phototaxis test, a more drastic difference between motility in the dark and light conditions was observed (Fig. 2C).

If the average values of RM in light and dark conditions were used to obtain a simplified quantitative estimation of  $P_L/P_D$ , approximately

$$P_L/P_D = (\text{RM in light})/(\text{RM in darkness}) = 0.31/0.10 = 3.1$$

could be obtained. From Fig. 2D, the average light preference index was 0.70, meaning that 17 larvae were on the dark side and three were on the light side by the end of the 10-min phototaxis test. If the average  $P_{LD}$  and  $P_{DL}$  values from Fig. 2A were applied, approximately

$$N_L = 3; \quad N_D = 17; \quad P_{LD} = 1.0; \quad P_{DL} = 0.75$$

could be obtained. These values were then substituted into the left part of Eq. (4) and

$$(N_D \times P_{DL})/(N_L \times P_{LD}) = (17 \times 0.75)/(3 \times 1.0) = 4.25$$

was obtained. On the right part of Eq. (4),  $P_L/P_D = 3.1$ . Eqs. (3) and (4) still do not strictly hold, but they have a good chance to hold if the error in estimation and experimental error are considered.

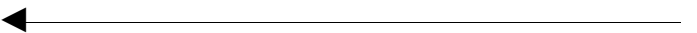
Thus, the motility of  $w^{1118}$  larvae decreased more in the dark condition than in light condition after the 10-min phototaxis test. Such a decrement contributed significantly to the final larval distribution on the testing plate.

*tim<sup>01</sup>* larvae were photophilic in the immediate LD boundary passing test and lacked motility alteration in the phototaxis test

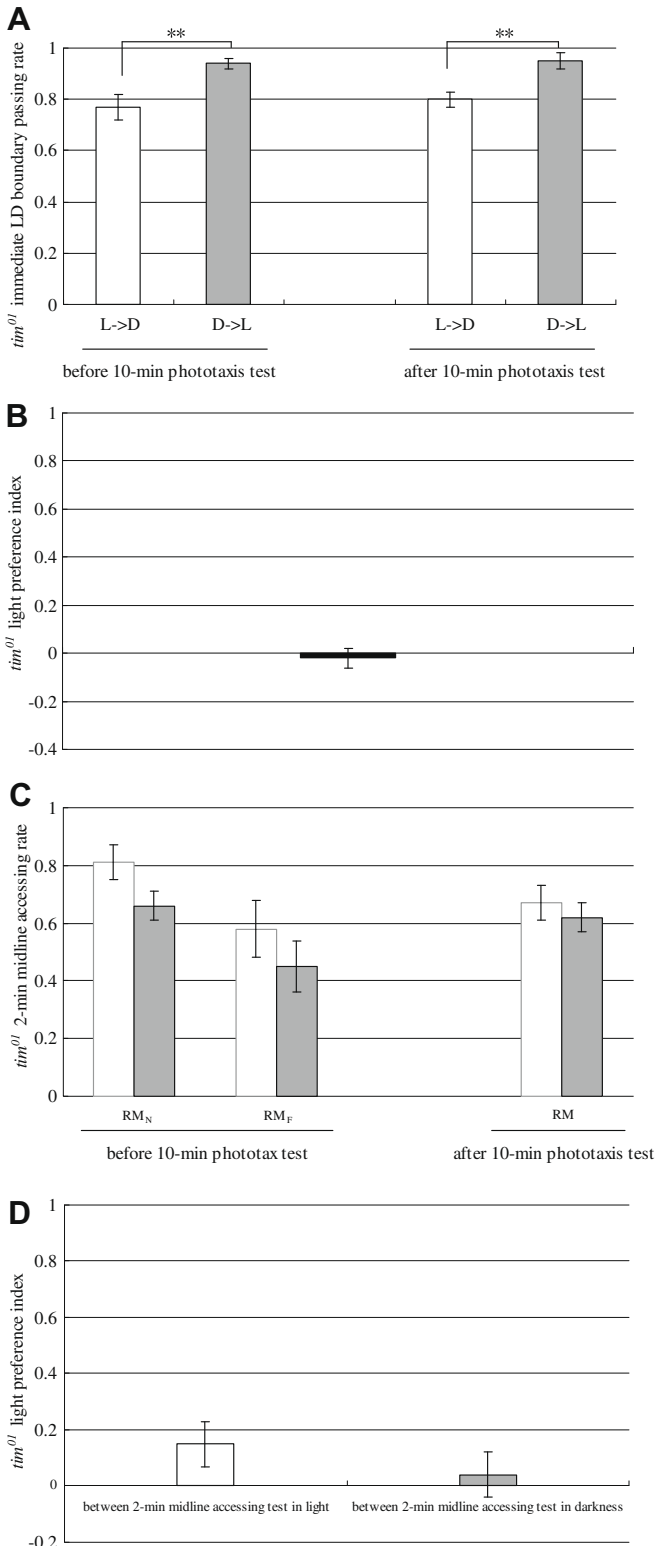
Because both factors, the immediate light/dark choice and the motility alteration, contribute to the final distribution of larvae, a question that arose was if these two factors were correlated.

Larvae of the strain *tim<sup>01</sup>*, which is a mutant allele of the circadian gene *timeless* and had been reported to be defective in light avoidance [9], were then tested. Larval distribution equilibrium was observed by the end of the 10-min phototaxis test. As shown in Fig. 3A, in the immediate LD boundary passing test, *tim<sup>01</sup>* larvae had an approximately 80% likelihood of passing from light to dark. The likelihood was, however, about 95% in the opposite direction, before and after the 10-min phototaxis test. This suggested that *tim<sup>01</sup>* larvae actually preferred light to darkness in the immediate LD boundary passing test. Meanwhile, the motility of *tim<sup>01</sup>* larvae did not change significantly after the 10-min phototaxis test (Fig. 3C).

In the accompanying 10-min phototaxis test, the *tim<sup>01</sup>* larvae exhibited no light avoidance or preference, which was consistent with the previous report [9]. Eqs. (3) and (4) in this case were then checked against the data in Fig. 3, to obtain roughly the following values:



**Fig. 3.** *tim<sup>01</sup>* larval light responses before and after the 10-min phototaxis test. (A) Immediate LD boundary passing rates of *tim<sup>01</sup>* 3rd-instar larvae before and after the 10-min phototaxis test. The LD boundary passing rates in the L → D direction are significantly lower than those in the D → L direction both before ( $0.77 \pm 0.05$  vs.  $0.94 \pm 0.02$ ,  $p < 0.01$ ) and after ( $0.80 \pm 0.03$  vs.  $0.95 \pm 0.03$ ,  $p < 0.01$ ) the 10-min phototaxis test. Open bar, LD boundary passing rate in direction of light to dark (L → D), gray bar, LD boundary passing rate in direction of dark to light (D → L). (B) Neutral performance of the same larvae in the 10-min phototaxis test carried out between the immediate LD boundary passing tests ( $-0.02 \pm 0.04$  vs. 0,  $p > 0.05$ ). (C) 2-min midline accessing rates of *tim<sup>01</sup>* 3rd-instar larvae in light and dark conditions before and after the 10-min phototaxis test.  $RM_F$  and  $RM_N$  before the 10-min phototaxis test as well as the RM after the 10-min phototaxis test in both light and dark conditions are shown. The midline accessing rates in the light condition are not significantly different from those in dark condition (before the 10-min phototaxis test, for  $RM_N$ ,  $0.81 \pm 0.06$  vs.  $0.66 \pm 0.05$ ,  $p > 0.05$ ; for  $RM_F$ ,  $0.58 \pm 0.10$  vs.  $0.45 \pm 0.09$ ,  $p > 0.05$ ; after the 10-min phototaxis test, for RM,  $0.67 \pm 0.06$  vs.  $0.62 \pm 0.05$ ,  $p > 0.05$ ). Open bars represent tests carried out in the light condition, gray bars represent tests carried out in the dark condition. (D) Neutral performance of the same larvae in the 10-min phototaxis tests carried out between the 2-min midline accessing tests in the light and dark conditions (for the 10-min phototaxis test between midline accessing tests performed in the light condition,  $0.15 \pm 0.09$  vs. 0,  $p > 0.05$ ; for the 10-min phototaxis test between midline accessing tests performed in the dark condition,  $0.04 \pm 0.08$  vs. 0,  $p > 0.05$ ; average preference index, 0.10). \*\* $p < 0.01$ .  $n = 10$  for all groups.



$$N_L = 9; \quad N_D = 11; \quad P_{LD} = 0.8; \quad P_{DL} = 0.95$$

Thus, the left part of Eq. (4) is

$$(N_D \times P_{DL}) / (N_L \times P_{LD}) = (11 \times 0.95) / (9 \times 0.8) = 1.45$$

On the right side of Eq. (4),  $P_L/P_D = 0.67/0.62 = 1.08$ , which is not particularly different from the left side of Eq. (4). Thus, the validity of the mathematical model was confirmed in *tim<sup>01</sup>* larvae.

So, *tim<sup>01</sup>* larvae were photophilic in the immediate LD boundary passing test and did not show motility alteration during the 10-min phototaxis test. Based on the results with *w<sup>1118</sup>* and *tim<sup>01</sup>* larvae, the immediate light/dark choice and the motility alteration seemed to be independent of each other.

## Discussion

*Drosophila* larval phototaxis is generally considered as a simple behavior. Here, larval phototaxis in a 10-min test was shown to be affected by a fast phase factor of immediate light/dark choice and a slow phase factor of motility alteration. These two factors seemed to be uncorrelated.

Our behavioral analysis was based on the distribution equilibrium equation. However, owing to technical reasons, it is difficult to directly measure all equation parameters. The experimental data provided just a rough estimation of the theoretic values. For example, the motility of larvae was approximately estimated with the parameter “midline accessing rate”. Also, the midline accessing rates before and after the 10-min phototaxis test were not strictly comparable, since they were measured in technically different ways. However, they could be compared to obtain a rough idea of the difference in larval motility owing to the 10-min phototaxis test.

The results with *w<sup>1118</sup>* and *tim<sup>01</sup>* larvae suggested that larval preference to light or darkness measured by the 10-min phototaxis test and immediate LD boundary passing test were not always consistent. This can be explained as follows: the larvae first made an immediate choice between light and dark conditions, but the outcome was unable to be sustained if larval motility was not changed correspondingly thereafter. In the case of *w<sup>1118</sup>* larvae, the light preference was first decided by the immediate LD boundary passing rate. Such initial preference was maintained or enhanced by subsequent motility decrement in dark

condition to achieve a final photophobic behavioral outcome. If the motility alteration did not help to maintain the initial preference, as in the case of *tim<sup>01</sup>* larvae, the larvae might appear to prefer the light condition at first when passing the LD boundary. Ultimately, however, they demonstrated neutral performance in phototaxis tests.

As larval phototaxis involves two underlying factors, the immediate light/dark choice and motility alteration, it is possible that some other factors not investigated are also involved. In addition, the immediate light/dark choice and motility alteration could be further analyzed to disclose the underlying mechanisms.

## Acknowledgments

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