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Phloxine B affects glycogen metabolism in larval stages of *Ceratitis capitata* (Diptera: Tephritidae)

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ABSTRACT

Xanthene dyes have been tested as photoinsecticides on different dipteran species. Phloxine B ingested by *Ceratitis capitata* larvae showed low toxicity under dark conditions and acute light-dependent toxicity when the insects were exposed to light during the dispersion stage before pupariation. In this study we show that sub-lethal concentrations of Phloxine B generated subtle changes in weight gain and in the accurately regulated jumping behavior of metamorphosing larvae. These changes are correlated with a strong accumulation of glycogen in fat body, intestine and muscles, as well as with an inhibition of the glycogen phosphorylase activity. These results suggest that some of the non-phototoxic effects of Phloxine B might be caused by an alteration of the glycogen catabolism, which can eventually affect the viability of the flies.

1. Introduction

Xanthene derivatives and other photosensitizers undergo rapid activation when exposed to light, leading to the formation of singlet oxygen and superoxide anions [1]. Therefore, when insects ingest a photosensitizer and are then exposed to light their detoxifying systems become overwhelmed and they consequently die. Xanthene dyes represent a family of photosensitizers that have been extensively tested as photoinsecticides on several dipteran adults [1–3]. The light-dependent phototoxic effect of Phloxine B (PhB[2]), a tetrabromide tetrachloride fluorescein, alone or in combination with uranine has been tested in laboratory and field experiments in the formulation of baits specifically designed for adult fruit flies [4–9]. PhB has been found to be strongly phototoxic when ingested by aquatic mosquito larvae [10,11], *Musca autumnalis* dung larvae [12], and *Ceratitis capitata* fruit larvae [13].

Cyclorrhapha Diptera present three larval instars. Most food intake is used to expand the cell and body size of first and second instars. However, in addition to an increase in body size, during the third instar the bulk of storage proteins, lipids and glycogen are synthesized and incorporated into fat body and other tissues [14]. At the end of this last instar, determined by a peak of 20-OH-ecdysone [15], Cyclorrhapha larvae III generally crawl away from the food source and initiate their dispersal phase (wandering stage), seeking a dry substratum to pupariate. *C. capitata* larvae exhibit a peculiar dispersion behavior, as after wandering briefly on the food surface most insects jump away using a unique elastic mechanism [16,17].

We previously studied the effects of PhB on immature stages of *C. capitata* under laboratory conditions [13]. In these experiments, the ingestion of PhB under a regime of constant darkness caused no significant mortality during the larval stages unless a concentration of 7 mM was reached in the food [13], indicating that PhB had a very low intrinsic chemical toxicity. Furthermore, we demonstrated that PhB has an acute light-dependent phototoxicity during the period of larval dispersion, 8 h before puparium formation (bpf), producing a concentration-dependent mortality of insects during the stages within the puparium. In this study we show that sub-lethal concentrations of PhB produce a delay in weight gain and slight alteration in the jumping behavior of *C. capitata* larvae that is correlated with a decrease in glycogen phosphorylase activity and a 9.3-fold increase in tissue glycogen.

2. Materials and methods

2.1. Insects

Wild-type *C. capitata* stocks (“Arg-17” strain) were reared in a new pumpkin based medium [18] in a Conviron chamber CMP 3244 (Canada) at 23 °C, 50–60% RH, under a photoperiod of

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[2] *Abbreviations used:* bpf, before puparium formation; E64, L-trans-epoxysuccinyl-leucylamide-(4-guanidine)-butane; PhB, phloxine B; PMSF, phenylmethanesulphonyl fluoride.

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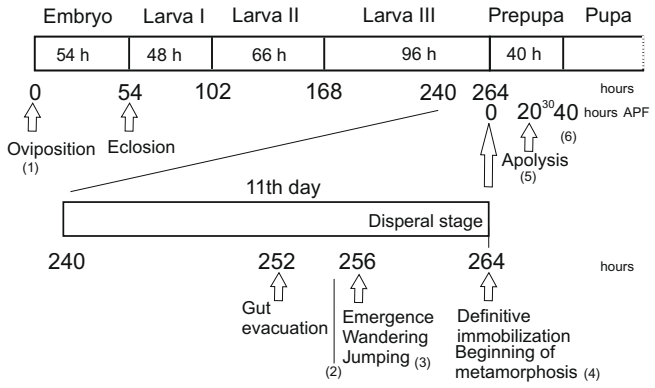

Fig. 1. Duration and events of late larva III instar. The timing of events is referred to life cycle and to larvae that emerge and jump off the food the day 11. (1) Oviposition; (2) ecdysone pulse; (3) after emerging and wandering shortly on the food surface the larvae disperse by jumping; (4) definitive immobilization (zero time of metamorphosis); (5) larval–pupal apolysis; (6) beginning of the pupal stage. APF: after puparium formation.

16:8 h (L:D). Under these conditions the span of embryo and larval stages is 11 days (264 h, see Fig. 1). The age of larvae III is expressed in hours before puparium formation (bpf), beginning at the point of definitive immobilization of the third instar larvae previously defined as "zero time" [16,19]. Adult flies were maintained with sugar: dry yeast (3:1) and 1% agar as food and water sources, respectively. These insects were used to lay eggs that represented the standard laboratory population.

2.2. Toxicity experiments

Small experimental dishes were prepared six days before starting the experiments. Pumpkin-based larval food (25.0 g per dish) was blended with 9 ml standardized peach juice (Baggio Juice, RPB S.A) per dish containing PhB disodium salt (D & C red N° 28, Warner Jenkinson, USA). Final PhB concentrations ranged from 0.25 mM to 5 mM. Control food contained peach juice without the dye. The dishes were maintained at 4 °C. Eggs were collected on plastic fruits during a three-h period and then washed, counted, and placed on the surface of the food (batches of 100 eggs per treatment). Both control and treated larvae were maintained under completely dark conditions during embryo and larval development until day 11.

2.3. Duration of larval development

To test the delay of larvae III leaving the food and beginning their jumping behavior, each dish was placed in a larger tray containing sterilized sand. For a period of ten days, individual larvae jumping off the cultures were immediately collected from the sand, washed with water, carefully blotted-dry on paper and weighed on a precision balance (Mettler Ae-240, OH, USA).

2.4. Larvae III dispersal activity

We started the experiment employing larvae III collected immediately after emergence of the food, starting the expected day of jumping (day 11). To do this, the culture dishes with the emerging larvae were subjected to 15 min illumination. Illumination was performed using 15 W white fluorescent lamps (Philips F15T8/0, made in Chile), and the light intensity at the level of the food surface was 47 photon μmoles/m^2/s (Quantum Meter model QMSW-SS, UT).

To test the jumping activity of larvae III during the dispersal stage, triplicate groups of six larvae were placed on a filter paper (58 × 58 cm) and the number of jumps per larvae was registered during a period of 5 min. Any larvae not ready to emerge early on day 11 eventually emerge the following days, following a 24 h cycle.

2.5. Tissue isolation

Larvae were washed thoroughly with distilled water before dissection. To remove larval fat body, muscles and intestines, the body was cut between the second and third anterior segments and the contents were squeezed out by applying pressure to the body wall into a drop of insect Ringer's solution (146 mM NaCl, 3.4 mM KCl, 2.7 mM $CaCl_2$, 0.5 mM $NaHCO_3$, pH 7.2) containing 20% sucrose and *N*-phenylthiourea (PTU) crystals. The different tissues were cleared away from the other organs and washed with Ringer's solution.

2.6. Tissue location of Phloxine B

Microscopic examination of larval tissues was performed on jumping larvae (8 h bpf) reared under constant darkness. These larvae emptied their gut 12 h bpf (Fig. 1). After dissection, the tissues were immediately examined by laser scanning confocal fluorescence microscopy (Zeiss LSM 510, motorized Axiovert 100 Microscope, Jena, Germany) using 488 nm excitation and visualizing the emission with bandpass filter (515–565 nm) to detect PhB (Maximum PhB excitation is at 535 nm and maximum emission is at 560 nm).

2.7. Quantification of total sugars in the hemolymph

To obtain hemolymph samples, larvae were punctured twice through the cuticle with an insect pin and immediately placed on a nylon mesh attached on top of a 1.5 ml eppendorf tube loaded with a PTU crystal. After centrifugation (350*g* for 2 min at 4 °C), the hemolymph was collected and centrifuged at 900*g* for 2 min at 4 °C to eliminate hemocytes. Total sugars were determined using the anthrone method [20].

2.8. Glycogen purification and quantification

Glycogen extraction was performed as described in Tolmasky et al. [26]. Samples were digested at 100 °C for 15 min in the presence of 0.9 ml of 33% KOH. The supernatant was separated and three volumes of 96% ethanol were added to precipitate the glycogen. After 30 min at 4 °C, the samples were centrifuged at 5000*g* and the pellets were re-suspended in 0.1 ml of water. The amount of purified glycogen was measured as previously described by Krisman [21].

2.9. Lipid purification and quantification

Larvae were homogenized in 0.2 ml of 2% Na_2SO_4. A solution of 1.3 ml chloroform: methanol (1:2) (v/v) was added to the homogenate, mixed, and centrifuged (5200*g*) for 10 min at room temperature. Lipids were quantified using the vanillin-reagent method [20]. A sample of the chloroform:methanol (1:2) extract was completely evaporated under N_2 stream and the lipids were suspended in 0.3 ml of sulfuric acid and hydrolyzed at 100 °C for 10 min. A 30 μl aliquot was reacted with 270 μl of vanillin reagent for 30 min. Total lipid content (mainly triglycerides) was estimated measuring absorbance at 490 nm in a microplate reader spectrophotometer (BioRad 3550, USA). Triolein was used as the standard (Sigma, St. Louis, MO).

2.10. Preparation of homogenates

Whole-insect crude extracts were prepared using batches of 200 liquid nitrogen frozen larvae. Isolated tissues dissected as above described were frozen at −70 °C until used. Homogenization was performed with 50 mM glycine/NaOH buffer, pH 8.6, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, 12 μM E64 and 1 mM PMSF (buffer A). After 20 strokes in a Teflon-glass tissue grinder, the homogenates were centrifuged at 25,000*g* for 30 min at 4 °C. The supernatants were then centrifuged at 150,000*g* for 2 h at 4 °C and the resulting supernatants were used as enzyme sources. Protein content was measured according to a Bradford Protein Assay commercial kit (Sigma, MO, USA). BSA was used as the reference standard.

2.11. Glycogen phosphorylase activity

Activity was measured as previously described by Hayakawa and Chino [22]. One unit of phosphorylase was defined as the activity generating 1 nmol/ml of NADPH for 30 min under experimental conditions. Due to the presence of AMP (adenosine monophosphate), the activity measured represents the total amount of phosphorylase present in the extract.

2.12. Statistical analysis

All the results are the mean of not less than three replicate experiments. Statistical significance is indicated in the legends to figures. GraphPad Instat software V2.02 (1993) was used for the analysis of variance (ANOVA). A one-way ANOVA with a Bonferroni's multiple comparison *post hoc* test was used to analyze data plotted in the figures. Separation of significant means was carried out with the Student–Newman–Keuls method program.

3. Results

We took advantage of the possibility of maintaining well-standardized culture conditions at 23 °C (L/D; 16/8 h) to analyze the impact of PhB ingestion on immature stages of *C. capitata*. In these conditions, embryonic development lasts 54 h, larval stages last 210 h (larva I, 48 h; larva II, 66 h and larva III, 96 h, approximately) and the puparium stages last 288 h (Fig. 1). In the wild, larval development occurs within a fruit where the penetration of light is limited. Under our standard laboratory conditions (L/D regime), food opacity is similar to that of a small fruit and darkness predominates within the food. Notwithstanding, in this study we maintained full darkness conditions to the end of the larvae III stage, when the dispersal stage begins (Fig. 1). No difference in the span of larval stages was found when comparing experimental controls (dark conditions) with the laboratory stock population subjected to a L/D regime of 16/8 h. Culture synchronization provided a good record of the occurrence of different physiological events during the last hours of the 3rd larval stage (Fig. 1, bottom). This could also be corroborated backwards since the "zero time" of metamorphosis was determined with an accuracy of ±8 min [16,19]. At the end of the third instar (i.e. 252 h after oviposition = 12 h bpf), the larvae that attained an optimum weight stopped feeding, evacuated their gut content, and four hours later left the food source (256 h after oviposition = 8 h bpf) to initiate the dispersing behavior (Fig. 1) [23].

The span of the *C. capitata* dispersion behavior (by "jumping" and/or crawling), ending with the definitive immobilization of the larvae, is highly variable. On the average it lasts 8 ± 2 h (Fig. 1). In the wild, this is the only period in which larvae are directly exposed to sunlight without the protection of a colored cuticle.

3.1. Effects of Phloxine B ingestion on mortality

No significant difference in mortality was registered between controls (11.1% ± 2.3 mortality) and larvae fed on different concentrations of PhB for any of the larval stages (from egg eclosion to the start of pupariation) (Fig. 2, inset). This indicates that the dye has a low intrinsic toxicity under dark conditions.

3.2. Larvae III weight

Fig. 2 shows that control larvae attained an apparent optimal weight on day 10 of the life cycle (13.76 ± 0.22 mg) but did not emerge from the food source until day 11. The larvae that do not leave on day 11 postponed their emergence to the following day. Interestingly, the average weight of the 11 day-old larvae leaving the food (12.82 ± 0.91 mg) was undistinguishable from those remaining in the food (12.74 ± 0.31 mg) (Fig. 2).

A delay in gaining the apparent optimal weight was observed in larvae fed on 3.0 mM PhB (day 9) and 5.0 mM PhB (days 9 and 10) (Fig. 2). In both, controls and insects exposed to different concentrations of PhB, the first larvae emerged from the food on day 11 and were similar in weight (Fig. 2). Furthermore, no significant differences in weight were found between emerged and non-emerged insects on day 11 (Fig. 2).

3.3. Effects of Phloxine B ingestion on behavior

Larvae fed on PhB showed a delayed pattern of emergence (Fig. 3). The proportion of control larvae leaving the food on the first day of jumping (day 11) was 53.5 ± 7.0% and was significantly different (see legend to Fig. 3) from larvae fed on 3 mM PhB (19.8 ± 0.8%) or 5 mM PhB (21.6 ± 1.9%). Most of the remaining control larvae (40.7 ± 5.3%) emerged during the second day (day 12) to a cumulative percentage of 94.2% (inset to Fig. 3). However, the bulk of the larvae exposed to 3 mM (62.6 ± 4.7%) and 5 mM (40.9 ± 2.3%) emerged during the second day (day 12) (Fig. 3).

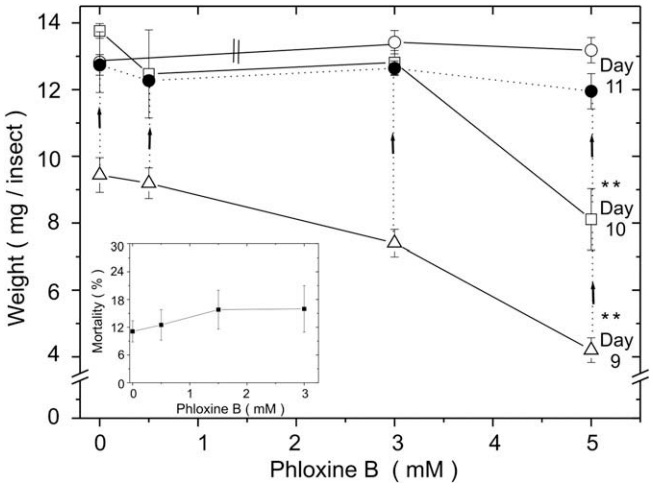

Fig. 2. Effect of Phloxine B ingestion on larval weight gain during the last three days of larval development. (△) Larvae within the food on day 9; (□) larvae within the food on day 10; (○) larvae within the food on day 11; (●) larvae outside the food and jumping on day 11. Arrows indicate the day by day evolution of weight. Each value represents the average of 6–11 individuals. Comparison of controls with Phloxine B concentrations for each day showed significant differences with $p < 0.001$ (**) for 5 mM. For each concentration, the weight in day 9 was always significantly different from the one in days 10 and 11 ($p < 0.001$). For 5 mM the weight is different in day 10 compare to day 11 in both conditions with a $p < 0.001$. No significant differences in weight were found between emerged and non-emerged insects, day 11. (Inset) PhB-dependent mortality during larval development in complete darkness (no statistical differences).

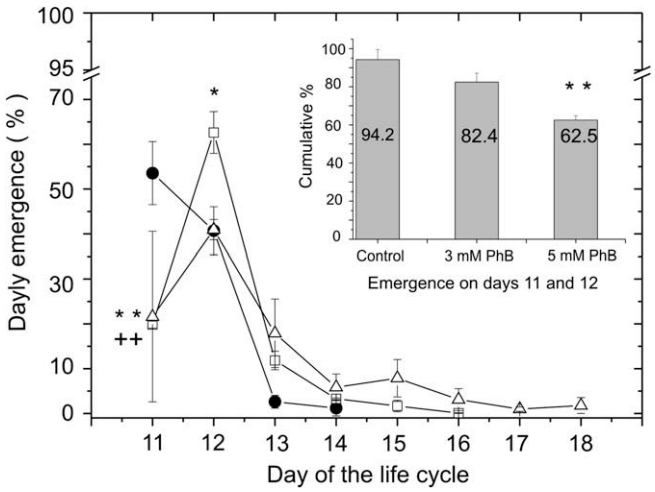

Fig. 3. Effect of Phloxine B concentration on the rate of larvae III emergence from food. (●) Control larvae (0 mM PhB); (□) 3 mM PhB added to the larval media; (Δ) 5 mM PhB added to the larval media. The inset shows the cumulative larvae III emergence from food. About 3–7 independent experiments were analyzed. Controls were compared with 3 mM PhB ($^{*}p < 0.05$ and $^{**}p < 0.001$) and with 5 mM PhB ($^{++}p < 0.001$). The inset shows the cumulative larvae III emergence from food. **Stands for $p < 0.001$.

The cumulative emergence was 82.4 and 62.5% for 3 and 5 mM PhB, respectively (inset to Fig. 3). During the 3rd day of emergence (day 13), the proportion of emerging flies was 2.6 ± 1.4% for controls, 9.1 ± 1.2% for 3 mM PhB and 17.9 ± 7.7% for 5 mM PhB treated flies. Thus, the emerging flies reared in PhB required more time to reach their physiological threshold and abandon the food source (Fig. 3 and inset).

Approximately 28% of the control larvae and 44% of the larvae reared with 3 mM PhB crawled during the entire dispersion time and never jumped. Control larvae performed 1–30 jumps during the first 5 min of illumination immediately after emerging from the food (mean 5.7 ± 1.0 jumps/5 min). However, larvae exposed to PhB never jumped more than 10 times (1 mM PhB) or seven times (3 mM PhB) (3.4 ± 0.8 jumps/5 min).

3.4. Accumulation of Phloxine B in the gut

Confocal microscopy images showed that PhB was mainly retained in the midgut of larvae fed with 1 mM PhB (Fig. 4A and B), with lower levels detected in the foregut and hindgut (Fig. 4A). Malpighian tubule cells were also highly stained (Fig. 4C). No fluorescence was detected in fat body, central nervous system, salivary glands and muscles, even with food concentrations of 3 mM (data not shown), indicating that the amount of PhB retained in these tissues was below the threshold of fluorescence detection. *In vivo* observations under a binocular microscope showed that a substantial fraction of the dye was absorbed through the intestine wall and that a strong excretion flow of the dye through the Malpighian tubules occurs.

3.5. Phloxine B-dependent changes in metabolism

The slight PhB-dependent delay in larvae emergence from food (eventually related to the delay in weight gain), the decrease in the number of jumping larvae, and in the average number of jumps, might be indicating some sort of impairment in the storage of energy-rich molecules. We therefore analyzed the content of the main energy-storage molecules in jumping larvae.

As shown in Table 1, no significant differences in the total content of lipids, proteins, and hemolymph sugars were observed between control and low PhB jumping larvae. A slight decrease in protein content was found in larvae exposed to 3 mM PhB (Table 1).

3.5.1. Glycogen levels and tissue distribution

A three and ninefold increase in glycogen content was registered in larvae reared with 1 mM and 3 mM PhB, respectively

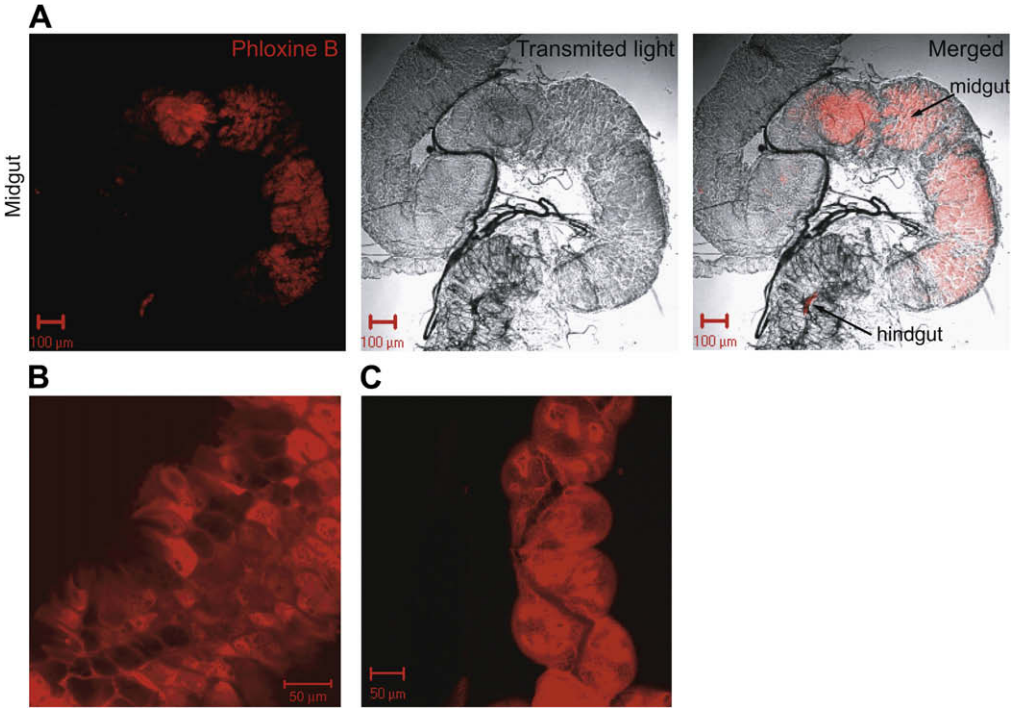

Fig. 4. Detection of fluorescence in larval tissues (8 h BPF) of insects treated with 1 mM Phloxine B. (A) Midgut and hindgut location of PhB. (B) PhB-stained cytoplasm of midgut cells. (C) Malphigian tubule.

Table 1
Metabolic parameters measured in larvae III just emerged from the food (mean + SD). At least 10 individuals were measured for each point. The only significant ($^{*}p < 0.01$) difference (Bonferroni test) was protein with 3 mM phB.

Parameters	Units	Phloxine B (mM)			
		0 (control)	0.5	1	3
Total lipid	μg/insect	1235 ± 50	1213 ± 52	1210 ± 31	1126 ± 77
Total protein	μg/insect	667 ± 14	657 ± 8	660 ± 6	585 ± 33*
Total hemolymph sugars	μg/ml	29.1 ± 6.6	3.6 ± 5.4	31.2 ± 6.2	30.4 ± 5.2

(Fig. 5A and indicated amounts in Fig. 5B). Excess glycogen was present in all tissues but the intestine and other tissues accumulated more glycogen than fat body and muscles. In control larvae, 42.4% of the glycogen was accumulated in larval fat body, 30.3% in muscles, 15.1% in the digestive tract and 12.1% in other tissues (Fig. 5B). In 3 mM-treated larvae the percentage of distribution among tissues changed (Fig. 5B). Glycogen content dramatically increased in the gut, representing 43.3% of total glycogen. Fat body glycogen was only 21.9% and muscles glycogen was also reduced to 10.5% (Fig. 5B).

3.5.2. Decrease in glycogen phosphorylase activity

Glycogen phosphorylase plays a crucial role in energy metabolism because it catalyzes the limiting step in glycogenolysis. Fig. 5A shows that the activity of this enzyme decreased in larvae III reared on PhB in a concentration-dependent manner. The activity of control larvae was 106 units/ng protein and was reduced by 36% in larvae reared on 3 mM PhB.

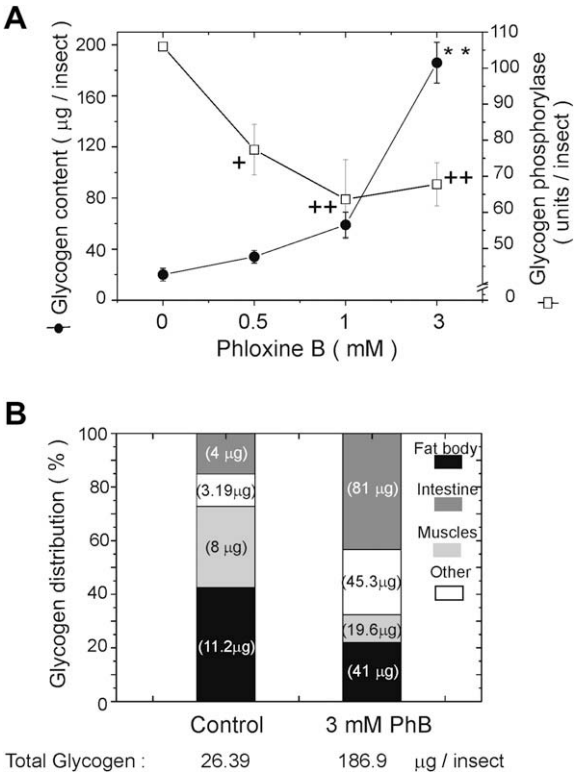

Fig. 5. (A) Phloxine B-dependent correlation between total glycogen content and glycogen phosphorylase activity in larvae III just emerged from the food. The glycogen content was measured in at least nine samples for each concentration (mean ± SD). The enzymatic activity represents the average of 4–8 measurements. Controls were compared with PhB data. Significance (Bonferroni Test, see Section 2) is $^{**}p < 0.001$ for glycogen and $^{+}p < 0.01$ or $^{++}p < 0.001$ for glycogen phosphorylase. (B) Phloxine B-dependent changes in the relative distribution of glycogen in larval tissues. The numbers indicate the amount of glycogen (mean + SD) in each tissue.

4. Discussion

As expected, PhB ingested during larval stages only showed mild toxicity under dark conditions, with no significant increase in death rate detected up to 7 mM PhB (Fig. 2 inset and [13]).

In order to detect the non-phototoxic PhB-dependent changes in metabolism and/or behavior during the commitment to metamorphosis, we focused our study on the last two days of the larval III stage. During the "skipping" behavior (jumping to find a suitable place to pupariate), glycogen must be used to provide muscles with energy. Our results suggest that an alteration of glycogen catabolism might be involved in the non-phototoxic effects of Phloxine B.

We found that a necessary but not compelling condition for larvae to emerge from the food on day 11th and disperse is to attain an adequate weight on the 10th day after oviposition (Fig. 2). Larvae feeding on low PhB (up to 3 mM) also reached an optimal weight on day 10 but showed a delay in weight gain on day 9 (Fig. 2). Despite this, intoxicated "early" larvae III always left the food on day 11. Larvae not ready to emerge delayed this behavior for 24, 48 or more h, (Fig. 3 and Fig. 3, inset), indicating the presence of a circadian clock control that is active even under dark conditions [24].

As shown in Fig. 3, the ingestion of PhB delayed the beginning of the dispersal behavior. Surprisingly, although the weight of larvae fed 3 mM PhB is indistinguishable from control larvae on day 10 (Fig. 2), a higher proportion of treated larvae emerge on days 12 and 13 (Fig. 3). This delay was accompanied by a reduced mean number of jumps in PhB-treated insects. The overall data reflect a low but significant level of PhB toxicity under dark conditions, impinging on the dispersion behavior of the insects and eventually causing an impact on other unknown fitness components.

PhB was mainly concentrated within the cells of the digestive system and Malpighi tubules, indicating that the digestive system represents a barrier (Fig. 4). Yoho et al. [25] observed that other xanthenes (Erythrosine B and Rose Bengal) were also concentrated in the midgut of adult *Musca domestica*. Carpenter et al. [11] showed that the same dyes present a similar location in mosquito larvae. In this study, no PhB fluorescence was observed in muscles, fat body, or suboesophagic ganglion in larvae reared in the presence of 3 mM PhB. However, the high presence of PhB in Malpighi cells and our *in vivo* observations indicated that the dye was constantly filtered from hemolymph.

No changes in lipids, proteins and hemolymph sugars were measured in treated larvae up to 3 mM PhB. Glycogen accumulation in third larval instars usually occurs in fat body, intestines and muscles and is mobilized during the dispersal period [26]. We here report that glycogen content in PhB-treated larvae increased 3- and 9-fold in larvae exposed to 1 and 3 mM PhB, respectively (Fig. 5A). Although the glycogen content increased in all the tissues, more than 40% of the PhB-dependent glycogen accumulation occurred in the gut and other tissues rather than in fat body and muscles (Fig. 5B). This correlates with the retention of PhB observed in the gut and Malpighi tubules (Fig. 4).

The huge increase in glycogen accumulated in the intestine indicates a misregulation of the glycogen metabolism whereas

the less active locomotion suggests that the larvae were unable to use this source of energy. An analysis of the glycogen phosphorylase activity confirmed that PhB was somehow inhibiting this enzyme, thus decreasing the degradation of glycogen. Preliminary assays on amylase activity also showed a decreased activity (data not shown).

Several insecticides decrease glycogen reserves in insects [27–29]. Rezg et al. [30] found in rats that malathion favors glycogen storage in liver and decreases glycogen phosphorylase activity by 50%. A similar scenario was observed in glycogenosis type V or McArdle's disease in humans [31].

The significant increase in the proportion of *Ceratitis* larvae that emerge "late" from the food (i.e. day 12 and later) cannot be attributed to a particular target, although we know that the neural system is usually the most sensitive to insecticides [32]. The slight decrease in the average number and range of jumps might be attributed to a slightly inefficient muscle activity due to the muscle itself or to neuromuscular coordination. This is in agreement with the previously reported PhD-dependent phototoxic malfunction of longitudinal muscles during pupariation and ecdysis [13]. All the aforementioned changes are correlated with the above discussed drastic accumulation of glycogen in larval organs. The accumulation in fat body and muscles can at least be explained by the partial inhibition of the glycogen phosphorylase activity. Thus, in addition to eventual damages to motoneurons innervating larval muscles, our results suggest that glycogen catabolism may play a significant role in non-photic xanthene toxicity. Further research is required to understand the non-phototoxic effect of PhB on glycogen phosphorylase activity.

It is interesting to note that our results show that, even under dark conditions and low PhB toxicity, some threshold of an unknown oscillator-dependent mechanism controlling the emergence from food is affected, in addition to the previously assumed threshold on weight gain.

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