Photochemistry and Photobiology, 2010, 86: 639–644

Photosensitizing Effect of Hematoporphyrin IX on Immature Stages of *Ceratitis capitata* (Diptera: Tephritidae)

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Received 2 October 2009, accepted 14 December 2009, DOI: 10.1111/j.1751-1097.2009.00704.x

ABSTRACT

Immature stages of *Ceratitis capitata* were tested as a model for hematoporphyrin IX (HP IX) phototoxicity. The lethal concentration 50 (LC_{50}) of HP IX in the food was determined during postembryonic development until adult emergence as 0.173 mM (95% CI: 0.138–0.209). The corresponding HP IX LC_{50} during the dispersal period alone was 0.536 mM (95% CI: 0.450–0.633). HP IX toxicity was compared against Phloxine B (PhB) (0.5 mM). HP IX elicited a mortality of 90.87%, which was mainly concentrated during prepupal and early pupal stages. PhB mortality was much lower (56.88%) and occurred mainly during the adult pharate stage. A direct correlation between light-dependent HP IX mortality, evidence of reactive oxygen species (ROS) and lipid peroxidation (conjugated dienes and thiobarbituric acid reactive substances) was established in *C. capitata* larvae. ROS were found to be very significant in both the brain and in the gut.

INTRODUCTION

Several photoactivatable substances are relatively safe for vertebrates and many invertebrates, having a low impact on the environment (1,2). Rebeiz *et al*. (3,4) proposed the use of porphyrins as photosensitizing agents against insects. These substances appear to be particularly promising because they exhibit an intense absorption band in the blue spectral region, the most intense component of the sunlight at midday, and also in the red region predominant at dawn and sunset (wavelengths >600 nm). Hence, they are prone to exhibit an efficient photoexcitation (1,5). Different porphyrins and substituted porphyrins were found to be toxic against adults of several dipteran species such as *Ceratitis capitata*, *Bactrocera oleae*, *Stomoxys calcitrans* (6–8), *Liriomyza bryoniae* (2,9), *Drosophila melanogaster* (10) and *Eretmapodites quinquevittatus* (11). Porphyrins were also highly phototoxic against the aquatic larvae of mosquitoes, like *Culex pipiens* (12), *C. quinquefasciatus*, *Aedes aegypti* and *A. stephensi* (13). Salama *et al*. (12) demonstrated that hematoporphyrin IX (HP IX) produced important histopathological effects on the midgut, epidermis, fat body and muscles of *C. pipiens* aquatic larvae. However, there is scarce information on the effects of porphyrins against immature stages of terrestrial diptera. More specifically, there are no reports on HP IX toxicity in Cyclorrhapha larvae that grow in fresh fruits, animal dung and animal tissues. As these larvae generally expose themselves to sunlight at the time of pupariation, we decided to study the effects of HP IX during postembryonic development of *C. capitata*, based on our well-established laboratory-conditioned life cycle (14–16).

In this study we show that HP IX is a light-dependent fast larvicide against *C. capitata*. We report toxicity parameters as well as biochemical evidence of reactive oxygen species (ROS) and lipid peroxidation when larvae were exposed to light. HP IX was compared to Phloxine B (PhB), which has been assayed in the field as an adult insecticide (17).

MATERIALS AND METHODS

Insects. Wild-type *C. capitata*, "Arg-17" strain, were reared in a Conviron chamber CMP 3244 (Canada), at 23°C, 50–60% RH, under a 16:8 h (L:D) photoperiod. Adult flies were maintained in flasks with free access to sugar:dry yeast (3:1) and 1% agar as sources of food and water, respectively. Eggs laid in artificial plastic fruits during a 3-h period were collected to start fly cultures. Larvae were reared in 30 mL flat containers on pumpkin-based semisolid larval food (18), and kept in the dark until the larvae dispersal period. This period begins when larvae emerge from the food in a circadian cycle-dependent behavior (19). After wandering briefly over the food surface, *C. capitata* larvae jump off using a peculiar elastic mechanism (20).

Sensitizer uptake and irradiation studies. The bioassay protocol was as described previously (21). Batches of 100 eggs per treatment were placed on the surface of the food containing no (controls) or 0.1–1.5 mM active HP IX (50% pure; Sigma-Aldrich Inc., St. Louis, MO) (Figs. 1–3). The effect of PhB disodium salt (92% pure, D&C Red No. 28; Warner Jenkinson, St. Louis, MO) and HP IX on the profile of mortality during metamorphosis was compared at 0.5 mM concentration (Fig. 2B). Four replicates were performed for each treatment. The required amount of photoactivatable substance was dissolved in a small volume of 0.1 mM NaOH (HP IX) or water (PhB) and mixed with the semisolid larval food under red light. After adjusting the pH to 4.5, 30 mL of food fraction was disposed in each container. Jumping third instar larvae (8 h before puparium formation) were collected from each treatment after the first jump, placed in plastic petri dishes and exposed to light trials for 8 h or left in the dark. The insects then continued their development until imago ecdysis in the dark. Mortality was recorded as a function of HP IX (or PhB) concentration. Larvae were considered dead when they were completely immobilized (no response to mechanical stimuli occurs) and remained elongated. Illumination was achieved using 15 W white cold fluorescent lamps (Philips F15T8/0, Chile), placed 7.5 cm above the petri dishes. The spectral distribution of the lamp is between 400 and 670 nm, with a sharp emission peak of 440–450 nm and a wide peak between 550 and 650 nm. The larvae received a light intensity of 47 photon μmol m^{-2} s^{-1} (Quantum Meter model QMSW-SS, UT).

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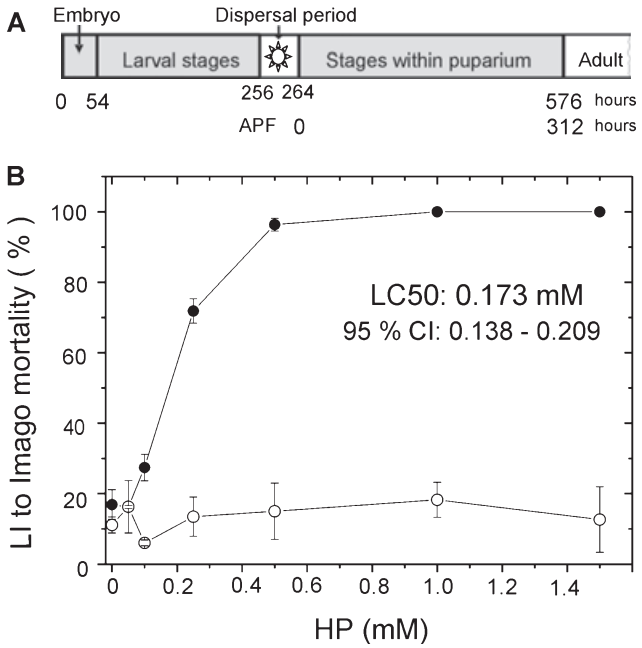

Figure 1. *Ceratitis capitata* mortality during postembryonic development until adult ecdysis. (A) Experimental design indicating the span of the different stages and the 8 h dispersal period subjected to light exposure. Postembryonic development takes 576 h since egg deposition. Metamorphosis takes 312 h since the definitive immobilization of the third instar larvae. (B) Hematoporphyrin (HP) IX-dependent mortality. Black circles indicate larvae subjected to direct white light (see Materials and Methods). Open circles represent insects kept in the dark. Bars are standard errors of the mean.

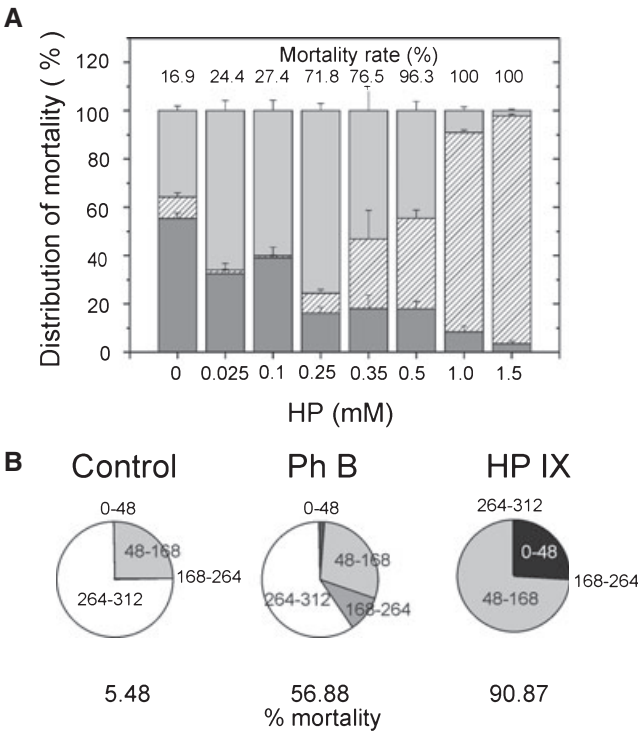

Figure 2. (A) Distribution of mortality during the postembryonic development. Insects were kept in dark during larval stages and stages within the puparium, and exposed to light only during the dispersal period. Dark gray indicates larval stages; stripes indicate the 8 h dispersal period; light gray indicates stages within the puparium. (B) Distribution of mortality during the stages within the puparium (pre–pupal, pupal and pharate adult). Black indicates the prepupal stage (0–48 h after puparium formation, APF); light gray indicates pupal stage (48–168 h APF); dark gray indicates early pharate adult stage (168–264 h APF); white indicates the late pharate adult (264–312 h APF).

To study the effect of the duration of light exposure on survival, jumping third instar larvae reared with 0.25 or 0.5 mM HP IX or no HP IX were exposed to light for different periods of time (0–480 min), and then maintained under darkness until adult emergence. Three replicates were performed for each treatment.

In situ indirect detection of ROS. Image-iT^{TM} LIVE Green Reactive Oxygen Species Detection Kit from Molecular Probes (Invitrogen, CA) was used to detect *in vivo* intracellular production of ROS in larval tissues. Before dissection, the jumping larvae were subjected to the aforementioned light treatment. All other subsequent procedures were carried out under a red security light. Insects were washed thoroughly with distilled water. To remove larval fat body, muscles, brain ganglia and intestine, insect bodies were cut off between the second and third anterior segments and the contents were squeezed out by pressing the body wall in a drop of Hank's buffered salt solution. Tissues were rapidly separated, washed and incubated with the fluorescent probe 5-(and-6), carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H_2-DCFDA). ROS oxidize the probe producing carboxy-DCF (green fluorescence). Fluorescent detection in larval tissues was performed with an Olympus BX60 Upright Microscope (excitation 495 nm/emission 529 nm).

Chemiluminescence. After 1 h of exposure to light or dark conditions, groups of 10 live jumping larvae reared with or without 0.50 mM HP IX were placed in glass vials and maintained for 30 min in the dark before counting light emission (22). Chemiluminescence was recorded in a Wallac 1214 Rackbeta LKB liquid scintillation counter. The number of light flashes is recorded as counts per minute (cpm), as the difference between the cpm registered on the vial with larvae and the background cpm of the vial alone.

Measurement of conjugated dienes (CD). Larvae reared with or without 0.5 mM HP IX were exposed to 0 or 30 min of light during the dispersal period. Larvae were immediately frozen with liquid N_2 and stored at −80°C until biochemical determination. CD of unsaturated fatty acids were determined by analyzing the second derivative spectrum of lipid extracts (23) (see the inset in Fig. 5B). Four groups of five larvae each were ground to a fine powder under liquid nitrogen and then homogenized with 1 mL of chloroform:methanol (3:2, vol/vol) in a tissue grinder. After centrifugation for 10 min at 13 600 ***g***, the lipid extract was dried under a nitrogen stream at

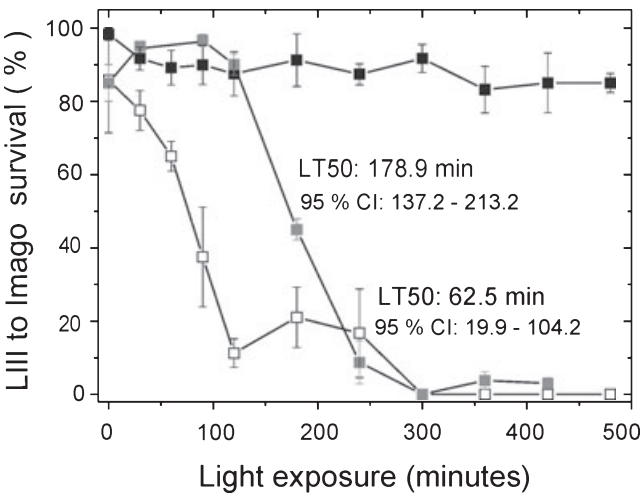

Figure 3. Effects of the duration of light exposure during the dispersal period of third instar larvae. Survival was registered on larvae reared with 0.25 mM (gray square), 0.5 mM (open square) or no (black square) hematoporphyrin IX in the larval media. Bars are standard errors of the mean.

40°C, and dissolved in 0.5 mL of cyclohexane. The absorbance was measured between 300 and 200 nm (JASCO V-550 UV–VIS spectrophotometer) and the height of the signals, with a minimum at approximately 230 and 236 nm in the second derivative spectrum, were measured and added together. We used the arbitrary units $d^2A/d\lambda^2$ that represent the measurement from minima to adjacent maxima at the higher wavelength (24). Cyclohexane was used to set the instrument baseline. The values obtained in each repetition were standardized before being analyzed.

Thiobarbituric acid reactive substances (TBARS) assay. The assay was performed according to Uchiyama and Mihara (25), with some modifications. Larvae reared with or without 0.5 mM sensitizer were exposed to 0 or 30 min of light during the wandering period. After irradiation, each larva was individually homogenized in 150 μL of 1.15% KCl and the homogenate was centrifuged at 13 600 ***g*** for 20 min at 4°C. A 100 μL aliquot of the supernatant was transferred into a tube along with 600 μL of 1% phosphoric acid and 200 μL of 0.6% thiobarbituric acid. The mixture was heated at 100°C for 45 min, and after cooling, 800 μL of *n*-butanol was added. The phases were then separated by centrifugation (13 600 ***g***, 20 min, 0°C) and the absorbance of the butanolic phase was measured at 532 nm. Protein content was measured according to a modification of the Lowry's method (26). A deoxycholate-trichloroacetic acid precipitation of insoluble protein was performed prior to colorimetric protein content determination. Bovine serum albumin (Sigma, MO) was used as reference standard.

Statistical analysis. Probit analysis with PoloPlus 2.0 (LeOra Software 2002–2007) was used to analyze toxicity data, determine LC_{50} and TL_{50} values, and also for testing the hypothesis of parallelism and equality of regression lines. The likelihood ratio test of parallelism tests whether slopes of the regression lines are the same. The test of equality determines whether slopes and intercepts are significantly different. If the hypotheses are rejected, the effects of the treatments are significantly different. Lipid peroxidation data (chemiluminescence, malondialdehyde [MDA] and CD) were analyzed by a two-way analysis of variance (ANOVA) using the Infostat software (27).

RESULTS

Under normal laboratory conditions (16:8 h L/D photoperiod throughout the life cycle), the mortality from egg hatching to adult emergence from the puparium was 15.16 ± 3.28%. The mortality of the insects kept in the dark during the whole life cycle or subjected to the experimental light treatment (Fig. 1A) was 11.26 ± 5.11% and 16.93 ± 4.20%, respectively (not significantly different from normal conditions, $P > 0.05$).

In order to study the toxicity of HP IX during postembryonic development, *C. capitata* larvae were administered food with different concentrations of the photosensitizer. Larval development was maintained in complete darkness until third instar larvae emergence from the food (Fig. 1A). At this timepoint (256 h after egg eclosion, AEE), no significant differences in mortality were detected between control groups and those fed up to 1.5 mM HP IX ($P > 0.05$, data not shown). Newly emerged third instar larvae were exposed to light for 8 h (dispersal period) while control groups were maintained in the dark during the same period. All groups (264 h AEE = 0 h metamorphosis) were then kept in the dark throughout metamorphosis until adult emergence (Fig. 1A).

Figure 1B shows that the mortality of the groups kept in the dark during their entire development was low, even at the highest HP IX concentration used, *i.e*. 1.5 mM. Low HP IX concentrations produced slightly higher mortality rates compared to controls due to phototoxic effects in third instar larvae and puparium stages, *i.e*. 7.5% at 0.025 mM and 10.5% at 0.1 mM HP IX (Fig. 1B). Figure 1B also shows that the groups that were photoexcited during the dispersal period suffered a concentration-dependent linear lethality up to 0.5 mM, and that 96.34 ± 1.81% of the individuals died. The calculated lethal concentration 50 (LC_{50}) of HP IX from egg hatching to adult ecdysis was 0.173 mM (95% CI: 0.138–0.209). Lethal concentration (LC_{50}) calculated for the 8 h dispersal period alone was 0.536 mM (95% CI: 0.450–0.633).

Figure 2A shows that in controls without HP IX (low mortality rate) more than half of the deaths occurred during the larval stages, while the remaining deaths (around 35%) occurred during the stages within the puparium. When the concentrations of HP IX in the larval medium were lower than the calculated LC_{50} (*i.e*. 0.025 and 0.1 mM), the distribution of mortality showed a higher proportion of deaths within the puparium (Fig. 2A). However, when the concentration of HP IX was higher than the LC_{50}, a threshold of fast lethality was apparently attained as the HP-dependent mortality during the period of light exposure increased linearly up to 82.6% and 94.4% for insects reared with 1.0 and 1.5 mM HP IX, respectively (Fig. 2A).

HP IX proved to be a stronger and faster lethal agent than PhB (Fig. 2B). Following light exposure during the dispersal stage, 5.48% of the control insects died within the puparium (Fig. 2B). The equivalent mortality of insects exposed to 0.5 mM PhB increased 10-fold (56.88%). Among these insects, 28.8% died during the pupal stage (48–168 h after puparium formation [APF]) and the rest during the pharate adult stage (10.1% during the 168–264 h APF and 59.3% during 264–312 h APF) (Fig. 2B). The 90.87% of the insects exposed

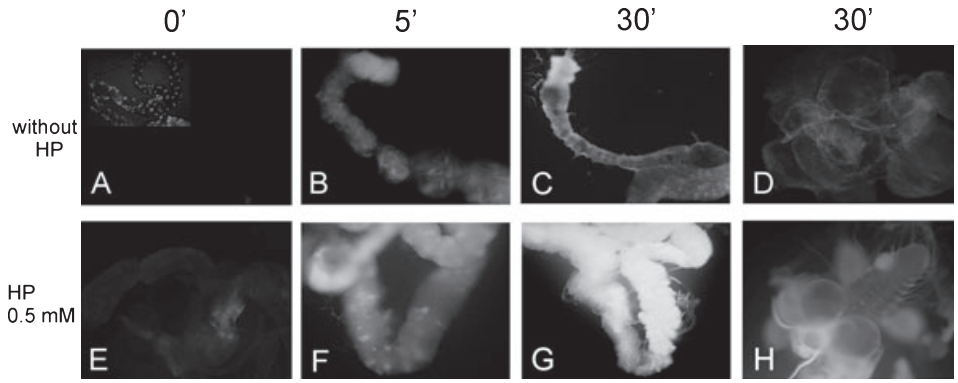

Figure 4. Detection of reactive oxygen species in larval tissues of treated with 0.5 mM hematoporphyrin (HP) or untreated (controls). Larvae that jumped off the food and began the dispersal period were exposed to light during the indicated time. A–C and E–G, gut; D and H, brain.

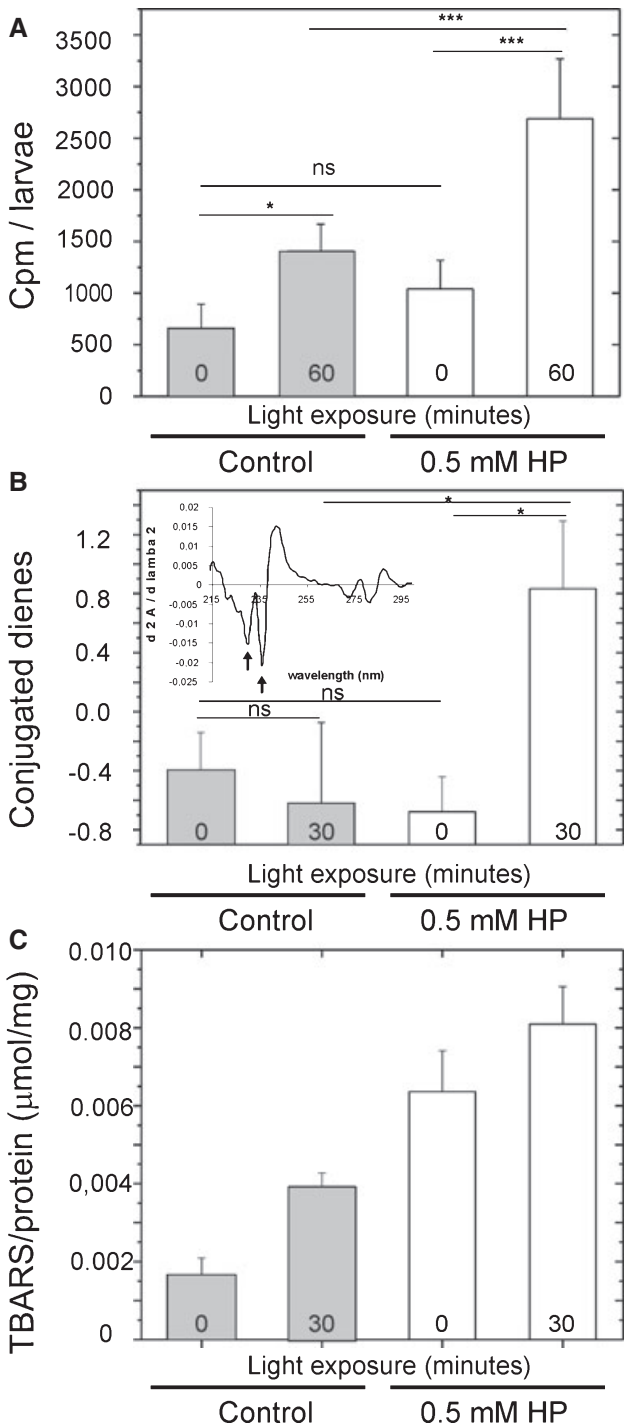

Figure 5. Indirect evaluation of reactive oxygen species formation. (A) Biological light emission of *Ceratitis capitata* jumping larvae reared without or with 0.5 mM hematoporphyrin (HP) IX and exposed to light for 1 h or maintained in the dark. *In vivo* chemiluminescence of third instar larvae was determined as the difference between the cpm registered on the vial with larvae and the counts per minute (cpm) background of the vial alone. (B) Conjugated dienes (CD) in larvae reared as above and exposed to light for 30 min. Inset: Lipid extract (0.25 mM HP IX, 15 min) absorbance spectrum to show CD of peroxidized lipids. The arrows indicate the minima at 230 and 236 nm, ascribed to *cis*, *trans* and *trans*, *trans* CD isomers, respectively (22). (C) Malondialdehyde formation in larvae reared as in (B). Bars are standard errors of the mean. ns = no significant differences. Significant differences: $*P < 0.05$; $***P < 0.001$.

to 0.5 mM HP IX died during early metamorphosis. Of these, 26.4% of the insects died during the prepupal stage (0–48 h APF) and the rest (73.6%) died during the pupal stage (48–168 h APF) (Fig. 2B).

To study the effect of the duration of light exposure on survival, we subjected jumping third instar larvae (reared with 0.25 or 0.5 mM HP IX) to different periods of light treatment. As expected, Fig. 3 shows that the survival of newly emerged adults was dependent on the span of light exposure during the dispersal period and on the concentration of the sensitizer (no significant light-dependent differences in survival were observed in the absence of HP IX). Lethal exposure time (LT_{50}) for 0.5 mM HP IX larvae was 62.5 min (95% CI: 19.9–104.2) and 178.9 min (95% CI: 137.2–213.2) for larvae reared with 0.25 mM HP IX. The hypothesis of equality (equal slopes and equal intercepts) was rejected at $\alpha = 0.05$ ($\chi^2 = 312$, d.f. = 2, $P = 0.000$), as was the hypothesis of parallelism (equal slopes) ($\chi^2 = 17.51$, d.f. = 1, $P = 0.000$), demonstrating that the effect of the duration of light exposure on survival depends on the concentrations of HP IX.

To indirectly detect *in vivo* ROS, larval organs were incubated with carboxy-H_2DCFDA, a fluorogenic marker that reveals the generation of ROS by oxidation producing green fluorescence (Fig. 4). No fluorescence was detected in the gut of control larvae reared without HP IX and maintained in the dark (Fig. 4A). However, after 5 and 30 min of light exposure, a basal background of fluorescence was observed in the gut of controls (Fig. 4B,C). Practically no signal was observed in the gut of larvae reared with 0.5 mM HP in the dark (Fig. 4E) whereas the digestive tract of larvae reared with HP IX and exposed to light was highly reactive (Fig. 4F,G). Elevated levels of fluorescence were also observed in the brain ganglia of individuals subjected to 0.5 mM HP IX and exposed to light (Fig. 4H), but no fluorescence was observed in the absence of HP IX (Fig. 4D). No differences in fluorescence intensity were registered in fat body and muscles of HP IX larvae with respect to the controls (data not shown).

The global chemiluminescence of larvae grown with 0.5 mM HP IX and subsequently exposed to light was increased compared to the controls (Fig. 5A). To confirm that oxidative processes were taking place, we measured the contents of CD and MDA, an intermediate and a final product of lipid peroxidation, respectively. The inset in Fig. 5B shows the second derivative of the UV spectrum of lipid extracts from groups of larvae treated with HP IX and exposed to light for 30 min. Figure 5B shows that the amount of CD was significantly higher in larvae treated with 0.5 mM HP IX and exposed to light (30 min) than in controls or larvae with HP IX kept in the dark ($P < 0.05$).

Changes in MDA were studied by measuring TBARS. TBARS levels were significantly increased in control larvae reared without HP IX exposed to light, compared to the controls kept in the dark ($P < 0.05$) (Fig. 5C). This shows that there is a natural oxidative response in *C. capitata* larvae exposed to light. When treated with 0.5 mM HP IX, both the unexposed and light-exposed larvae had significantly greater amounts of TBARS than controls ($P < 0.05$) (Fig. 5C). Thus, HP IX significantly enhanced the oxidative processes already triggered by the light.

DISCUSSION

This study shows that HP IX seems to be a strong and fast photolarvicide against *C. capitata*. To our knowledge, this is the first time that HP IX has been evaluated on immature stages of Cyclorrapha flies. Ben Amor *et al.* (7) reported that 1.2 mM HP IX in the *C. capitata* adult bait produced a mortality of nearly 80% during 72 h exposure to light. When 4.38 mM was applied to the bait, 90% mortality was registered after 24 h irradiation. Moreover, these authors showed that meso-(di-*cis*[4*N*-methyl-pyridyl]-*cis*-diphenyl-porphine) ditosylate, a dicationic porphyrin, produced high levels of mortality when 0.85 mM was administered in the bait and *C. capitata* adults were exposed to light for 1 h (6). However, these authors did not explore porphyrin toxicity on immature stages. In our experiments, HP IX showed an LC_{50} value of 0.171 mM for *C. capitata* postembryonic development when activated by light during the dispersal period (Fig. 1B), nearly three times less than that previously registered by Berni *et al.* (21) with PhB.

Different phototoxic levels of HP have been reported in other insects. Luksiene *et al.* (28) chose the hematoporphyrin dimethyl ether (HPde) as a representative of dicarboxylic porphyrins, which was more effective and chemically homogeneous than the clinically established agent photofrin. Nearly all *L. bryoniae* adults were killed after 24 h of light exposure when 25 mM HPde was administered in the bait. HP was found to be highly toxic for the aquatic larvae of mosquitoes. When 0.1 mM HP IX was applied to the fourth instar of *C. pipiens*, 100% mortality was recorded after 30 min of light exposure (12). Dondji *et al.* (13) determined an HP IX LD_{50} of 3.224 mg L^{-1} for the fourth instar of *C. quinquefasciatus*.

Our results showed that once the LC_{50} dose was surpassed, mortality due to phototoxic effects increased from more than five-fold at 0.25 mM HP IX up to 8.3-fold at 1.0 mM (Fig. 1B). Mortality was quick during the 8 h period of light exposure of jumping third instar larvae (Figs. 2 and 3). This result has great practical importance because it is the only period (until imago ecdysis) when larvae are directly exposed to light in field conditions.

Interestingly, the HP IX-treated larvae (0.5 mM) that survived light treatment and were able to pupariate died during the first 72 h APF (Fig. 2A,B, death within puparium). At the same concentrations, PhB produced deaths at the pharate adult stage, 168 h APF (Fig. 2B and Ref. 29). Thus, phototoxic effect of HP IX is stronger and faster than PhB. We also observed that dying HP IX-treated insects first became melanized in the anterior segments of the pupa (data not shown), indicating cell death and an antero-posterior polarity of toxicity.

Larvae chemiluminescence was measured to estimate the overall generation of ROS (Fig. 5A). It has been postulated that this light emission is due to the generation of free radicals of short life, and that excited states are produced by reactions associated with lipid peroxidation products such as carbonyl triplets (22,30). Our results indicate that the levels of CD and MDA formation increased in larvae exposed to HP IX and light (Fig. 5B,C). However, the levels of peroxidation produced by HP IX in the dark did not affect the survival of flies (Fig 1A). As demonstrated previously by Recknagel and Glende (31) and Girotti (32), one of the main consequences of ROS formation is the generation of CD and MDA in polyunsaturated fatty acids. Grossweiner *et al.* (33) also showed that the light-dependent lysis of phospatidylcholine liposomes was sensitized by incorporation of HP IX.

Using a probe that crosses plasma membranes and is deacetylated by intracellular esterases, to form dichlorofluorescein, we showed that HP IX increased ROS generation in brain ganglia and the gut (Fig. 4). Although light alone slightly increased fluorescence in the gut, ROS-dependent fluorescence was strongly induced as early as 5 min after exposure to light in the gut, and became evident in the brain after 30 min (Fig. 4). Ben Amor *et al.* (7) showed that the greatest damages were found in *C. capitata* imago gut, Malpighian tubes, adipose tissue and cuticle, especially at the level of membranes, owing to the lipophilic nature of HP IX. Helleck and Hartberg (34) also showed that photofrin II accumulates and fluoresces in the alimentary canal, Malpighian tubes and anal papillae of *E. quinquevittatus* larvae. Midgut gastric epithelium was the most affected.

The observation of anterior acute toxicity (see above) and the generation of ROS in the brain of *C. capitata* larvae suggest that the photodamaging effects of HP IX were prominent at the level of the nervous system. This is in agreement with the fact that many of the puparing larvae, prior to death, were unable to retract their anteriormost segments (data not shown). This seems to be caused by impairment of longitudinal muscle activity, probably due to neuromuscular damage. Similar phototoxic effects caused by PhB were observed in *C. capitata* larvae (21,29).

Administration of porphyrins to Tephritidae larvae is not a practical issue, because they feed exclusively within fruits and vegetable tissues. However, our *C. capitata* model suggests that the use of HP IX as photolarvicide might be promissory against animal pest flies like *M. domestica* and blood-sucking flies like *Haematobia irritans*, since their larvae grow in dung that could eventually be spiked with photosensitizers (35). At present there are no practical solutions for administering photosensitizers to different species of dung larvae due to technical or economic constraints. Although treating dung in poultry farms is easy, bleaching by exposure to light and the lack of penetration make HP IX a difficult option. Protection of cattle in feedlots and dairy farms from blood-sucking flies (stable flies and horn flies) is an important issue. However, low digestion capsules or pellets that could deliver photosensitizers to dung are not available yet. In this respect, stud, feedlot and dairy farms as well as the fine leather industry require an environmentally friendly solution to protect valuable animals and could eventually afford above average costs for photoinsecticidal treatments.

Acknowledgements—We thank an anonymous reviewer for helpful suggestions and critical reading and editing of the manuscript, Dr. M.C. Ríos de Molina (FCEyN-University of Buenos Aires) for help with the chemiluminiscence measures, Dr. J. Berni for preliminary PhB experiments, and Dr. Raúl Alzogaray and the CIPEIN (CONICET) for help in data analysis. This study was funded by CONICET, the AMPYCyT-FONCyT and the University of Buenos Aires, Argentina. L.M.P.-L. is a research fellow of CONICET. L.A.Q.-A. is a Full Professor at the Biological Chemistry Department, FCEyN, University of Buenos Aires. A.R. and L.A.Q.-A. belong to the Scientist Career of the CONICET.

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