Your article proof 2205 from Journal of Economic Entomology is available for download
=====
Dear Author,

This letter describes how to access and proof your article 2205 slated for an upcoming issue of Journal of Economic Entomology. If you have any questions, contact the Journal of Economic Entomology Issue Manager noted below. Do not reply to this e-mail.

Please refer to this URL address:

http://rapidproof.cadmus.com/RapidProof/retrieval/index.jsp

Login: Your e-mail address
Password: ----

This site contains one file. You will need to have Adobe Acrobat Reader software to read this file. This is free software that is available for user downloading at
http://www.adobe.com/products/acrobat/readstep.html

This file contains the following:
1) ESA Publishing Agreement (copyright transfer form)
2) Reprint Order Form
3) Proofreading marks for your reference
4) Page proofs of your article and author queries, if applicable

After printing the PDF file, please read the page proofs carefully and limit any changes to those that alter scientific meaning, such as ambiguous sentences or errors in tables.
* Mark clearly legible corrections in red directly on the page proof, preferably in the margins;
* Answer all author queries, indicated as AQ:1, AQ:2, AQ:3, etc., in the margins of the proofs and listed on the last page of the proofs; and
* Verify that tables, figures, and figure captions are correct and in proper sequence.

Authors are entirely responsible for marking all errors.

Any changes you make that are not the result of editor or printer error or response to a query will be billed to you at a cost of $6.00 per affected line of text. Changes in figures will be charged at $50.00 per figure. Color proofs of color figures are available upon request.

Publishing Agreement
Your article will not be published unless this form is completed and returned with your page proof.

Ordering Reprints
If you wish to order reprints, please use the Reprint Order Form. You can order paper reprints, a PDF reprint, or both. A PDF reprint is a PDF file of your article that is part of the online journal article and is freely accessible to anyone. You may immediately obtain the PDF file of your article from the ESA server and post it on your home page and on other publicly accessible servers. You may also use it to send your article to colleagues as an e-mail attachment and to make as many "electronic reprints" of your article as you wish. Reprint orders for articles must be received before the issue goes to press and should be mailed to the address on the form at the same time you return your page proofs.

Within 48 hours, please mail the page proofs to the address below. Also include the completed Publishing

Agreement and Reprint Order Form, otherwise publication of your article will be delayed.

Thank you for publishing with ESA. We hope that your experience has been positive. If you have any questions, contact the Journal of Economic Entomology Issue Manager noted below.

Lathifah Mohamed-Ibrahim
Issue Manager
Journal of Economic Entomology
Cadmus Professional Communications
940 Elkridge Landing Road
Linthicum, MD 21090
Phone: 410-691-6998
Fax: 410-691-6929
m-ibrahiml@cadmus.com

Journal of Economic Entomology

Reprint Order Form

Use the form to order paper reprints, PDF reprints, or both. The order form should accompany the return of your page proofs to ensure that your order is produced on time. Late orders for paper or PDF reprints will be billed at much higher prices. Be sure to fill out the order form completely and sign your name where indicated under Billing Information. Reprint prices are subject to change without notice. Paper reprints are shipped within 2 weeks of the journal issue's mailing date.

1 Article Information (Please type or print clearly)

Manuscript number 2205 Number of pages

Title

Author(s)

2 Billing Information

For all international orders, a valid credit card number is required for prepayment.

❑ Visa ❑ Mastercard

Account No. Exp. Date

Cardholder Signature

Cardholder Name

Cardholder Address

City State/Province

Postal Code Country

Telephone Fax

❑ Check/Money Order (payable to Entomological Society of America)

❑ Purchase Order

Purchase Order must:

- accompany reprint form
- list Entomological Society of America as vendor
- include manuscript number
- include journal title, article title, and author's name

3 Reprint Prices ($US) (Domestic shipping included)

Paper Reprints (B&W)					PDF Reprints
	Quantity				Quantity
Pages	100	200	300	400	Unlimited
1-2	$ 49	53	57	63	36
3-4	83	94	102	112	62
5-8	161	178	197	217	120
9-12	231	260	291	321	172
13-16	300	339	381	423	225
Add'l 1-4 pages	75	87	98	108	56
Covers*	108	122	135	147	N/A
Paper Reprints (Color)					**PDF Reprints**
1-16	173	285	398	510	Use B&W prices
17-32	263	375	488	600	Use B&W prices

* Covers stating the title, authors, and journal issue are printed in black ink on heavy weight gray paper.

4 Reprint Information

Two kinds of reprints are offered. **B&W Paper Reprints** are printed on high-quality, uncoated paper, trimmed, and stapled at the fold. Color reprints are overruns of the printed journal pages, trimmed, and side stapled. **A PDF Reprint** is a PDF file of your article that is posted with the online journal issue and is freely available in unlimited quantities.

Paper Reprints (quantity) _____ $ _____

PDF Reprints $ _____

Covers (quantity) _____ $ _____

Total reprint cost $ _____

5 Shipping (International Orders Only) $ _____

All orders in the United States are shipped UPS, unless otherwise specified, and shipping is included in the price of the reprints. **Orders shipped outside the United States must have Expedited Shipping. Add 40% of the reprint cost for Expedited Shipping for each address. For addresses in Canada and Mexico add 20%.** Expedited Shipping provides delivery in 5-9 days and includes package tracing and proof of delivery.

6 Total Reprint Amount $ _____

7 Shipping Information (No PO Boxes allowed)

Ship _____ copies to:

Name

Address

City State/Province

Postal Code Country

Telephone Fax

Ship _____ copies to:

Name

Address

City State/Province

Postal Code Country

Telephone Fax

8 Complete and send at once with your page proofs to:

Issue Manager, Journal of Economic Entomology
Cadmus Professional Communications
940 Elkridge Landing Road
Linthicum, MD 21090
Fax: 410-691-6929 Phone: 410-691-6998
e-mail: m-ibrahiml@cadmus.com

Proofreader's Marks

MARK	EXPLANATION	EXAMPLE
	TAKE OUT CHARACTER INDICATED	 Your proof.
	LEFT OUT, INSERT	 Your proof.
	INSERT SPACE	 Yourproof.
	TURN INVERTED LETTER	Your proof.
	BROKEN LETTER	Your proof.
	EVEN SPACE	A good proof.
	CLOSE UP: NO SPACE	Your proof.
	TRANSPOSE	A proof good
	WRONG FONT	 Your proof.
	LOWER CASE	Your proof.
	CAPITALS	Your proof.  Your proof.
	ITALIC	Your proof. Your proof.
	ROMAN, NON ITALIC	 Your proof.
	BOLD FACE	Your proof. Your proof.
	LET IT STAND	Your proof.  Your proof.
	DELETE, SEE COPY	She Our proof.
	SPELL OUT	 Queen Eliz.
	START PARAGRAPH	 read. Your
	NO PARAGRAPH: RUN IN	marked. Your proof.
	LOWER	 Your proof.

MARK	EXPLANATION	EXAMPLE
	RAISE	 Your proof.
	MOVE LEFT	Your proof.
	MOVE RIGHT	Your proof.
	ALIGN TYPE	Three dogs. Two horses.
	STRAIGHTEN LINE	Your proof.
	INSERT PERIOD	Your proof
	INSERT COMMA	Your proof
	INSERT COLON	Your proof
	INSERT SEMICOLON	Your proof
	INSERT APOSTROPHE	Your mans proof.
	INSERT QUOTATION MARKS	Marked it proof
	INSERT HYPHEN	A proofmark.
	INSERT EXCLAMATION MARK	Prove it
	INSERT QUESTION MARK	Is it right
	QUERY FOR AUTHOR	Your proof read by
	INSERT BRACKETS	The Smith girl
	INSERT PARENTHESES	Your proof 1
	INSERT 1-EM DASH	Your proof.
	INDENT 1 EM	Your proof
	INDENT 2 EMS	Your proof.
	INDENT 3 EMS	Your proof.

ENTOMOLOGICAL SOCIETY OF AMERICA
Publishing Agreement

Manuscript Number: 2205 Journal: Journal of Economic Entomology

Manuscript Title: _____

_____

Author(s): _____

1. I hereby transfer to the Entomological Society of America (ESA) full and complete ownership of any copyright I may have with respect to the work named above. If there is more than one author, I also certify that I am the duly authorized agent for any or all other authors of the work. (If the work is written as part of official duties as an employee of the United States or Canadian governments, check the box below.)

❑ This certifies that the work named above was prepared by an employee of the United States or Canadian governments as part of official duties and therefore cannot be copyrighted by ESA.

2. I understand that ESA assesses editorial review charges that are based upon the total number of pages published, reprint charges for any number of reprints I order, special charges for any color reproduction I require, and alteration charges for any changes deemed as author alterations made to page proofs of the article named above.

   Editorial review charges are billed to the senior or corresponding author within one month of publication at rates established by the ESA Governing Board. **Current rates are listed below and are applied based on an authorís membership status at the time of publication.** ESA members should ensure that their dues are up to date at the time of publication. Charges are made in full-page increments only, regardless of how much type appears on the last page of the article. Credit is given to members only when ESA is able to produce an article from a correctly formatted disk.

ESA Member Rate for articles prepared on disk:	$48.00 per published page
ESA Members:	$60.00 per published page
Nonmembers:	$75.00 per published page

3. I agree to be responsible for the payment of all appropriate and relevant publication charges associated with the production of the above named article. **All publication and reprint charges are due upon receipt of invoice.** Unpaid balances more than 30 days past due are subject to a finance charge of 1.5% per month (18% per annum). Authors with unpaid balances of more than 90 days will lose their right to publish with ESA until the outstanding balance is settled. There is a $25 fee for returned checks.

4. I have obtained written permission to use any quotations or excerpts from another work or from anotherís property not in the public domain or covered by fair use provisions of the U.S. Copyright law. Proper acknowledgment has been given in the article for the use of such material.

(OVER)

5. I authorize the editor to modify the manuscript as necessary to prepare the article for publicationó including changes in title, style, and format to conform to editorial usage, journal format, and ESA editorial style. Minor stylistic changes may be made right before publication to meet ESA editorial requirements. I understand that I may be asked to update material that appears in the article before publication.

6. I understand that I will receive page proofs of the above named article that will also show how the figures will be reproduced.

7. I agree to review and return the page proof within 48 hours of my receipt of it and to mark on the page proof any corrections that need to be made to the article. I understand that I am responsible for extra charges for any changes that are not a result of a printer or editor error or query.

8. I agree to refer to ESA all requests to republish or reprint the whole or any part of this article, including my own requests. Although ESA allows published articles to be posted on the authorís web site two years after publication, ESA still holds the copyright on the original article and should be contacted for permission to republish or reprint it. ESA will respect the wishes of the author as to the use of the article or any material appearing in the article.

9. I have read the ethics in publishing statements that appear on the ESA web site (http://www.entsoc.org/pubs/publish/ethics.html) and affirm that this article is in compliance.

10. I understand that ESA will invoice me for the article after the issue of the journal in which the article appears is mailed and that I will secure complete payment upon receipt of the invoice.

11. I hereby provide a billing address for the article mentioned above.

Name _____

Address _____

_____

_____

_____

Telephone number_____ Date _____

Signature_____ Printed name _____

Author acting as agent for all other authors

A valid credit card number is required for international payments, including Canada and Mexico. Failure to do so will delay the publication of your paper.

❑ VISA ❑ Mastercard

Card number_____ Expiration date _____

Signature_____ Printed name _____

ECOTOXICOLOGY

Phloxine B Effect on Immature Stages of the Mediterranean Fruit Fly, *Ceratitis capitata* (Diptera: Tephritidae) (Wiedemann)

JIMENA BERNI, ALEJANDRO RABOSSI, AND LUIS A. QUESADA-ALLUÉ[1]

Instituto de Investigaciones Bioquímicas-Fundación Campomar CONICET and FCEyN University of Buenos Aires, Patricias Argentinas 435 (1405), Buenos Aires, Argentina

J. Econ. Entomol. 96(3): 000–000 (2003)

ABSTRACT A laboratory bioassay was developed to determine both the chemical toxicity and the phototoxicity of the xanthene dye, phloxine B (D&C red N°28), to the immature stages of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). An additional goal was to find out which main tissues are affected first. A low, but significant, level of toxicity was observed when the insects were maintained in the dark: at the point of adult ecdysis, the LC_{50} was 11.03 mM. As expected, after 8-h exposure of late larva III to light, a high level of mortality was produced (LC_{50} at ecdysis: 0.45 mM) as a dose-dependent function of dye concentration. At sublethal concentrations of the dye, the surviving insects showed a number of physiological abnormalities. Phloxine B appeared to mainly affect the larval longitudinal muscles as well as the abdominal muscles of ecdysing adults, giving rise to abnormal puparia and failed adult ecdysis, respectively. Moreover, a significant phloxine B-dependent delay in the jumping of surviving larvae for dispersal was documented. This could be attributed to a delay in attaining a threshold weight for jumping and/or to abnormalities in neuromuscular coordination, thus reinforcing the idea of pleiotropic effects of the dye.

KEY WORDS *Ceratitis capitata*, Mediterranean fruit fly, phloxine B, metamorphosis, photoinsecticides

XANTHENE DERIVATIVES ARE photosensitizing agents that undergo rapid activation when exposed to sunlight or strong artificial light, leading to the formation of singlet oxygen and superoxide anion (Heitz and Wilson 1978). Certain xanthene derivatives, such as phloxine B and other halogenated eosin analogues, are effective pesticides that can be used to control populations of several insect species. The toxic effect of these photoactivated dyes on insects was first studied by Barbieri (1928) and then by Schilmacher (1950). More recently, the photoinsecticidal efficacy of xanthenes was investigated in detail by Heitz (1987, 1997) and others (see Ben Amor and Jori 2000, for review). As expected, the degree of phototoxicity depends on the chemical structure, rose bengal (a tetraiodine, tetrachloride derivative of fluorescein) being the most toxic. Interest is growing in the use of some of these photoinsecticides, which are environmentally friendly, because they are relatively safe for mammals (Lipman 1995) and are photodegraded by sunlight in aqueous media (Licudine et al. 2002), and therefore do not persist in the environment.

The light-dependent phototoxic effects of xanthenes, and most particularly of phloxine B (a tetrabromine, tetrachloride derivative of fluorescein), have been studied in adults of several insects: ants (Broome et al. 1975, David and Heitz 1978); boll weevil (Callaham et al. 1975); cockroaches Ballard et al. 1988); and grasshoppers (Capinera and Squitier 2000). Several species of dipterans have also been shown to be susceptible to these photosensitizers. Experiments with adult were carried out with the house fly *Musca domestica* (Yoho et al. 1971, 1973; Pimprikar et al. 1980) and the face fly *Musca autumnalis* (Fondren and Heitz 1978). Because they must be ingested to be effective, carefully designed bait formulations should assure the necessary insect selectivity (Liquido et al. 1995a).

Phloxine B is considered to be one of the less toxic xanthenes, because concentrations shown to be lethal for insects were not found to be toxic in the case of human contact and ingestion (FDA 1982, Lipman 1995). It has also been assumed that ingested phloxine B is nontoxic in the absence of exposure to light, although nonphototoxic effects have been described in ants (David and Heitz 1978).

In particular, phloxine B (red) alone or a mixture of phloxine B and uranine (yellow), commercially known as Sure Dye, has been extensively tested in the formulation of baits specifically designed for adult fruit flies. This showed acute toxicity in the Mexican fruit fly *Anastrepha ludens* (Mangan and Moreno 1995, 2001; Moreno and Mangan 1995; Moreno et al. 2001), in the West Indian fruit fly *Anastrepha oblicua*

[1] E-mail: lualque@iib.uba.ar.

0022-0493/03/0000–0000$04.00/0 © 2003 Entomological Society of America

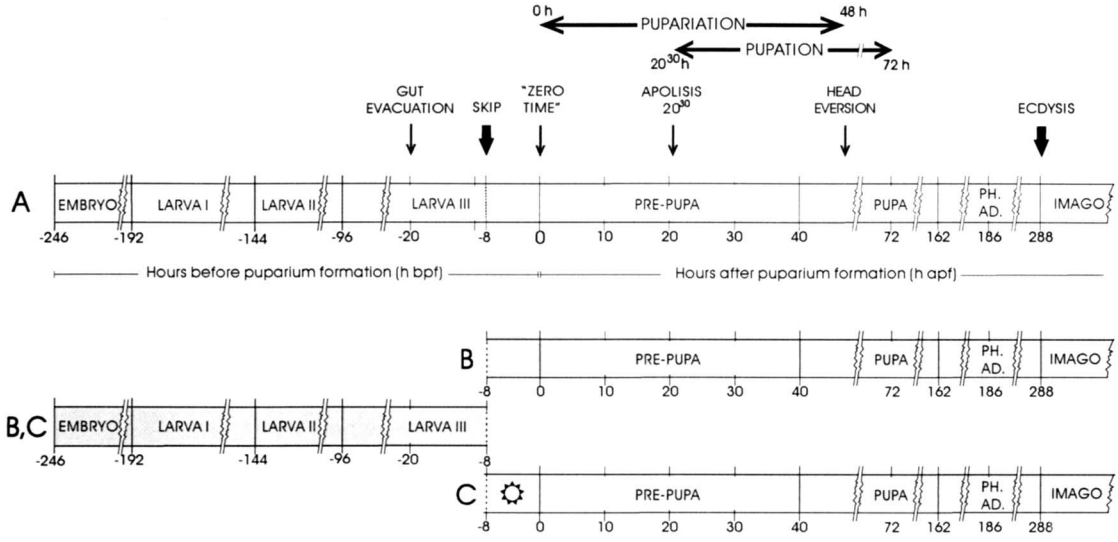

Fig. 1. (A) Main developmental events in the *C. capitata* life cycle under our well-synchronized laboratory standard conditions at 23°C and with a photoperiod of 16:8 (L:D). The age of larvae is expressed in hours bpf. Age within the puparium is expressed in hours apf starting at zero time (Rabossi et al. 1991). The arrows show the beginning of events or morphological changes. (B, C) Experimental scheme. (B) Batches of 100 insects were maintained in the dark throughout the life cycle until emergence of the imago, in the presence or absence of phloxine B. (C) Equivalent groups of insects were subjected to direct white light (see Materials and Methods) during a key developmental period: from 8 h bpf to 0 h. SKIP: refers to the first jump out of the food. PH. AD.: pharate adult.

(Moreno et al. 2001), in the Oriental fruit fly *Bactrocera dorsalis* (Liquido et al. 1995b), and the medfly *Ceratitis capitata* (Licudine et al. 2002, Liquido et al. 1995a, Moreno et al. 2001, Peck and McQuate 2000, Vargas et al. 2001).

Few studies have examined the effect of xanthene derivatives on immature stages of dipterans. Most of the experimental work was done in aquatic larvae of several mosquitoes (*Culex pipiens quinquefasciatus*, *Anopheles superpictus*, *Anopheles maculipennis*, *Aedes aegypti*, and *Aedes triseriatus*) using erythrosin B, fluorescein, octabromofluorescein, phloxine B, and rose bengal (Barbieri 1928; Carpenter and Heitz 1980, 1981; Pimprikar et al. 1979; Schilmacher 1950). Susceptibility of larvae to erythrosin B was also demonstrated in the house fly, *M. domestica* (Pimprikar et al. 1980) and in the face fly, *M. automnalis*, with rose and erythrosin B (Fairbrother et al. 1981).

When studying the larva to adult transition in the Mediterranean fruit fly, *C. capitata* (Rabossi et al. 1991, 1992), in detail, we became interested in determining the effect of phloxine B on immature stages, first to distinguish chemical toxicity from phototoxicity and then to establish sublethal conditions allowing further studies on the main targets at tissue and molecular levels.

The objectives of this study were to establish the degree of phloxine B toxicity in immature stages of *C. capitata* maintained in the dark, and to confirm the expected strong toxicity after exposure to light during a short developmental window encompassing the jumping period of the larvae. We also wanted to describe the macroscopic physiological symptoms of phloxine B toxicity at sublethal doses, to infer which tissues are primarily affected.

Materials and Methods

Insects. Wild-type *C. capitata* stocks (strain Arg-17) were reared in a Teran's carrot-based medium, as described by Quesada-Allué et al. (1994). Larval and adult flies were maintained in a Conviron chamber CMP 3244, at 23°C, 50–60% RH, with a photoperiod of 16:8 (L:D). Under these conditions, the span of larval stages is 10 d and the stages within the puparium last 12 d. These insects were used to lay eggs representative of the standard laboratory population.

Feeding Trials. To test for toxic effects, we used different concentrations of phloxine B disodium salt (D&C red N° 28; Warner Jenkinson, St. Louis, MO). We developed a bioassay protocol (Fig. 1, B and C) based on the well-established life cycle at 23°C under our standard laboratory conditions (Fig. 1A). As control insects showed no or negligible differences in developmental time or occurrence of developmental landmarks, the age of individual insects was assigned using the previously described markers of the abovementioned *C. capitata* life cycle (Rabossi et al.1991, 1992) (see Fig. 1A). AQ: B F1

Six days before starting the experiments, small experimental dishes were prepared. Carrot-based larval food (25.0 g per dish) was blended with 9 ml per dish of standardized orange juice containing phloxine B (final volume 30 ml). Final concentrations of the dye from 0.1 to 10 mM were prepared and maintained at 4°C until use. Control food contained orange juice

without phloxine B. Batches of eggs oviposited during a 2-h period were collected in plastic fruits, washed, and counted. For each concentration tested, batches of 100 eggs were placed on small circles of filter paper (Whatman 1) located on the surface of the food. The dishes containing the small *C. capitata* colonies were covered with light-proof lids and maintained under experimental conditions, as described in the legend to Fig. 1 and in Results and Discussion. The light-promoted toxicity experiments were performed on groups of jumping larvae (8 h puparium formation (bpf)) illuminated until immobilization (0 h) with 15 W white fluorescent lamps (Philips F15T8/0, made in Chile) mounted at a height of 7 cm above the petri dishes. The light intensity at the level of flies was 47 photon micromoles/m^{-2}/s^{-1} (measured with a Quantum Meter model QMSW-SS, UT). After immobilization, the prepupae were returned to dark conditions (Fig. 1C). Light-independent experiments were carried out on groups of insects held in the dark during the entire time course of the experiment (Fig. 1B).

To test the delay in jumping behavior, we placed each dish in a tray containing sterilized sand. This tray was also covered, as described above. Individual jumping larvae from parallel cultures were immediately collected and chilled at 4°C, weighed on a Mettler Ae-240 precision balance, and measured with a Vernier caliper (±0.02 mm).

Statistical Analysis. GraphPad Instat software V2.02 (1993) (San Diego, CA) was used for analysis of variance (ANOVA). Separation of means was determined by the Student-Newman-Keuls test. The concentration dose–response relationship, LC_{50} values, and 95% fiducial limits were determined by probit analysis (PROC LOGISTIC, SAS System, Stokes et al. 2000).

Results and Discussion

The times of the occurrence of the main developmental events of the *C. capitata* standard life cycle in the laboratory, under our well-synchronized conditions, are indicated in the scheme in Fig. 1A. All of the results below refer to time-dependent events occurring at 23°C, before or after puparium formation (bpf, apf), as shown in Fig. 1.

Two experimental protocols (Fig. 1, B and C) were designed to determine the light-dependent and light-independent effects of phloxine B during development. As shown in Fig. 1B, batches of 100 insects were maintained in the dark throughout the life cycle until emergence of the imago, in the presence or absence of phloxine B. Equivalent groups of insects were subjected to direct white light (see Materials and Methods) during a key developmental period: from 8 h bpf to 0 h (Fig. 1C). At the beginning of this period, the larva III starts to jump off the food, to find a suitable dry substrate for pupariation (skip behavior) (see first thick arrow, Fig. 1A). After reaching a definitive pupariation site, the larva shortens the body to adopt an ovoid shape and reaches an irreversible stage of immobilization (at zero time, according to Rabossi et al. 1991).

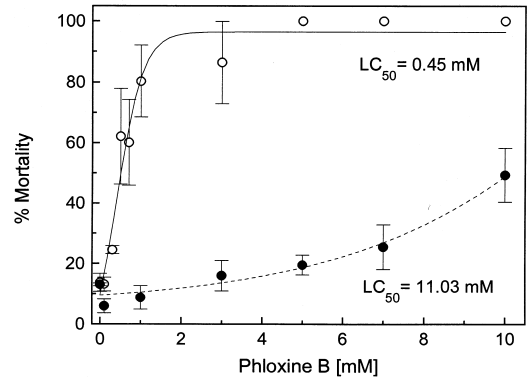

Fig. 2. Dose-dependent mortality (±SEM) of *C. capitata* from egg eclosion to adult ecdysis, when maintained in dark (●) or subjected to 8-h illumination (○).

As expected, the rate of noneclosed eggs (10.28 ± 0.85%) in our control (no dye) series of experiments was similar to that of the much larger, wild-type population, Arg-17, maintained permanently in our insectary under the L16/D8 regime shown in Fig. 1A (9.97 ± 1.80%). Moreover, embryo development did not appear to be affected when up to 3 mM phloxine B was present in the food (nonsignificant, $P = 0.2$).

Effect of Phloxine B in the Dark

We were interested in determining the intrinsic chemical toxicity of phloxine B in larvae and stages within the puparium of the Mediterranean fruit fly. Figure 2 shows the phloxine B dose-dependent rates of accumulated mortality from egg eclosion to adult ecdysis. A low level of toxicity of phloxine B was found in insects not exposed to light: the mortality at adult emergence was 15.98 ± 5.03% with 3 mM phloxine B and 49.48 ± 8.94% with 10 mM phloxine B. The LC_{50}, in completely dark conditions, based on the number of ecdysed insects, was 11.03 mM (6.78–15.19, 95% CL; slope 3.59 ± 0.61) (Fig. 2). As far as we know, this is the first observation of chemical toxicity of phloxine B, under controlled conditions, for larval stages of cyclorraphan flies.

Phototoxicity of Phloxine B

An important phototoxic lethal effect occurred in flies exposed to light during the 8-h jumping period. The rates of mortality from egg eclosion to adult ecdysis were 100% at concentrations of phloxine B in the food >3 mM (Fig. 2). The mortality rate was directly dependent on concentrations up to 1 mM phloxine B. The LC_{50} at ecdysis, after 8-h exposure to light, was 0.45 mM (0.41–0.49, 95% CL; slope 1.36 ± 0.05) (Fig. 2), which is ≈25 times more toxicity than that obtained under dark conditions. The toxicity rapidly became evident during the 8-h light period and persisted during the stages within the puparium (see below).

rich3/ec-eco-ent/ec-eco-ent/ec0303/ec2205d03a | meadel | S=6 | 3/31/03 | 21:20 | Art: | Input-1st disk, 2nd 2205

The postulated mechanism of eosin (fluorescein) toxicity involves the absorption of visible light energy by the ingested dye, apparently leading mainly to the formation of intracellular singlet oxygen and superoxide anions (Carpenter and Heitz 1980). Singlet oxygen is highly reactive and may attack and oxidize many cellular components, including small metabolic intermediates, proteins, and membranes (Krinsky 1979). This occurs because of the long lifetime of singlet oxygen, particularly in hydrophobic media. In turn, superoxide anions can be converted to very reactive and cytotoxic species, such as the hydroxyl radical and hydrogen peroxide (Bensasson et al. 1983). The main factors related to the photosensitivity of insect tissues are the reactivity of the dye, the dose, and the light intensity.

Toxicity Within Stages of Development

To determine the levels of sensitivity to phloxine B of the different stages, we analyzed mortality within larval stages (246 h to 8 h bpf), during the jumping to immobilization period (8 h bpf to 0 h) and within the stages in the puparium (0–288 h apf) separately. Because no exposure to light occurred during the larval stages (Fig. 1, B and C) under our experimental conditions, both experimental series of larval groups were identical.

F3 **Larval Stages.** Fig. 3A shows that there is a dose-dependent increase in mortality (slope 3.21 ± 0.49, $r^2 = 0.85, P < 0.001$) during the larval stages from 1 mM phloxine B (no significant mortality) up to 10 mM phloxine B concentrations when 43.36 ± 4.60% mortality was obtained. As expected, the rate of unsuccessful larvae (that do not reach the jumping stage) in the absence of phloxine B (9.21 ± 1.95%) was identical with that of the standard population in the laboratory (~8.0%). Therefore, the resulting difference of ≈35% in larval death at 10 mM phloxine B should be attributed to the chemical toxicity of this dye ($P < 0.001$). This result might be useful in the control of other dipterans such as the horn fly, *Hematobia irritans*, and the stable fly *Stomoxys calcitrans*, in which all of the immature stages of development occur within the manure, which, eventually, could be produced containing phloxine B. This idea was first explored by Pimprikar et al. (1980), who tested the effect of erythrosin B in *M. domestica* by spraying plots of manure, and by Fairbrother et al. (1981), who demonstrated strong toxicity for the face fly, *M. automnalis*, in the manure of cattle fed with eosin B. It is noteworthy that, because of its presumptive absence of toxicity at low concentrations, phloxine B is used in human drug coatings and in cosmetics (FDA 1982). Therefore, in theory, low levels of phloxine B might be added to the food or water of poultry and other farm animals, to control immature stages present in the manure, the only inconvenience being the red stain produced by the dye.

Larva III Jumping Period. Almost no mortality was observed in insects not exposed to light during the critical jumping period, from 8 h bpf to 0 h (Fig. 3B).

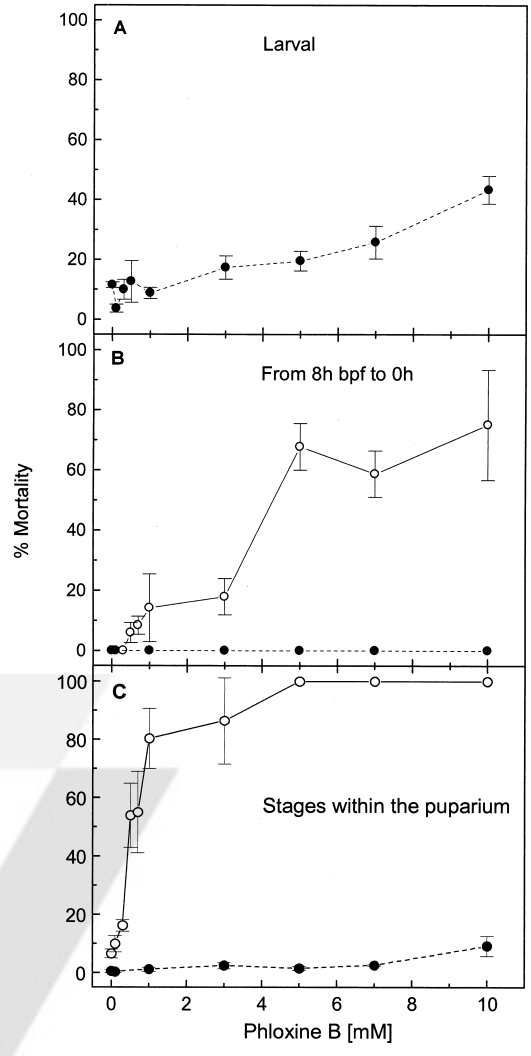

Fig. 3. Percent mortality (±SEM) within developmental stages of *C. capitata* fed with phloxine B. Insects maintained in dark conditions (●), and exposed to light during the jumping immobilization period (○) are compared. (A) Mortality of larval stages up to 8 h bpf larva III. (B) Mortality during the jumping immobilization period (8 h bpf to 0 h). (C) Mortality from 0 h to adult ecdysis.

This suggests that the period after initial jumping is more refractory to the chemical, nonphototoxic effect of phloxine B, because the absence of feeding and the preparation for immobilization are accompanied by a slowing of the metabolism. In contrast, starting at a concentration of 0.5 mM phloxine B, the larvae exposed to light during this jumping period showed highly dose-dependent mortality (slope 0.23 ± 0.02, $r^2 = 0.92, P < 0.001$) during the 8 h of exposure to light (Fig. 3B). Mortality was 17.94 ± 6.04% at 3 mM and 75.05 ± 18.37% at 10 mM of phloxine B.

We noticed that most of the mortality seemed to occur during the last 2 h of this short illumination period (data not shown). One of the most relevant

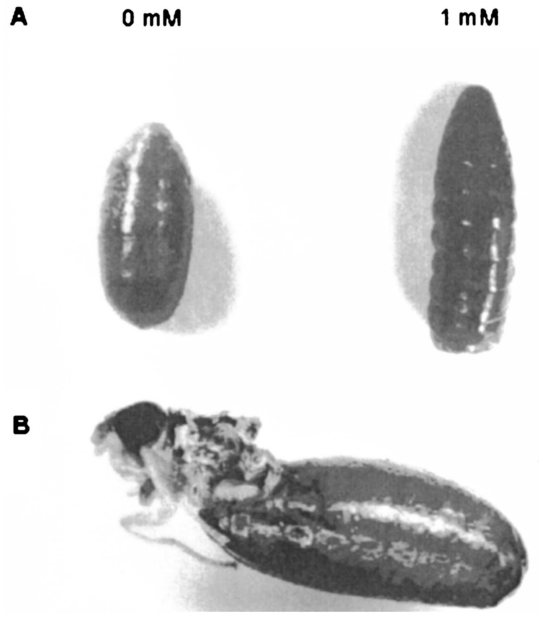

Fig. 4. (A) Effect of phloxine B on puparium formation. Control insects (0 mM) showed the typical barrel shape. Some larvae treated with 1 mM phloxine B were unable to retract the three first segments to acquire the typical body shape. (B) Adult unable to complete ecdysis after photosensitization with 1 mM of phloxine B.

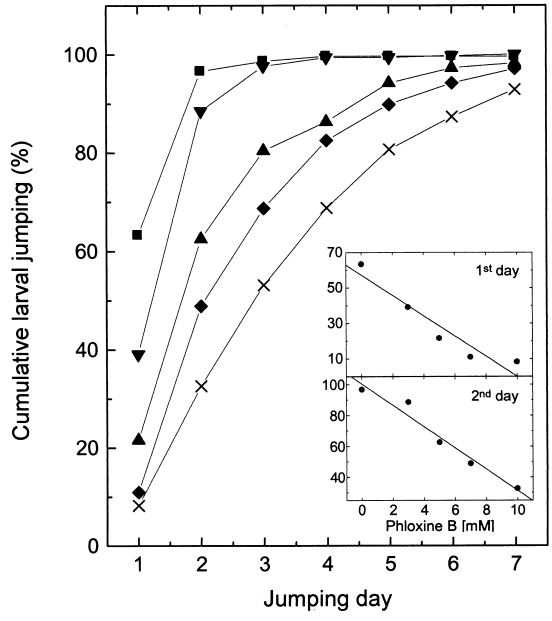

Fig. 5. Percentage of cumulative larval jumping after ingestion of phloxine B; (■) 0 mM, (▼) 3 mM, (▲) 5 mM, (♦) 7 mM, (X) 10 mM. Inset: Relationship between cumulative jumping (%) and phloxine B concentration on the first day (top) and on the second day (bottom). Regression slopes are -5.72 ± -1.05 and -6.89 ± -0.81, respectively.

effects was that, at the end of the 8-h period, observation of the dead larvae revealed that they had been unable to retract the anterior part of the body (Fig. 4A, right) to acquire the typical barrel shape (Fig. 4A, left). Few of the nonretracted larvae were able to initiate cuticle sclerotization and coloration to give rise to an elongated tanned puparium (Fig. 4A, right). This points to possible rapid phototoxic damage of all or some of the muscles involved in the retraction of the first three segments. It is possible that muscle mitochondria were affected, thus impairing muscle performance. Preliminary analysis of these organelles isolated from insects maintained under the same experimental conditions supports this idea (not shown).

Stages Within the Puparium. Fig. 3C shows that, again, in dark conditions, negligible chemical toxicity of phloxine B was detected in stages within the puparium, because mortality was only detected at very high (10 mM) concentrations of the dye.

The phloxine B dose-dependent phototoxic effect was linear (slope 73.28 ± 10.04, $r^2 = 0.93$, $P < 0.01$) in the corresponding groups up to 1 mM phloxine B, and full mortality was reached at concentrations of phloxine B >3 mM (Fig. 3C). As also shown in Fig. 2, this concentration seems to represent a full mortality threshold. Most of the nonecdysed insects died during the pharate adult stage (data not shown) and, significantly, many of those that survived until the end of the pharate adult stage were unable to complete the last steps of ecdysis. Figure 4B shows that, after the opening of the operculum and the beginning of emergence from the puparium, in many cases ($12.15 \pm 6.26\%$ and $15.47 \pm 4.2\%$, respectively, of 0.5 and 1 mM phloxine B-treated insects) ecdysis was interrupted, because the emerging adults remained trapped. Again, this seems to indicate damage in the muscles commanding the required peristaltic movements for emergence from the puparium.

Sublethal Effect of Phloxine B on Skip Behavior

From the above results, mortality caused by phloxine B phototoxicity seems to result from pleiotropic toxic effects because of multiple targets of singlet oxygen and other reactive species generated by the dye (see above). Under sublethal conditions and, in general, in surviving insects, the main targets first affected seem to be the muscular and/or neuromuscular systems. However, nothing is known about the target(s) of the chemical toxicity (i.e., in the absence of light) described in this work. To study this, we focused on the skip behavior of larvae emerging from the food, which, according to our previous experience, seems to be a sensitive health indicator.

When no phloxine B was present in an experimental population synchronized at oviposition, >60% of the larvae ($63.81 \pm 4.58\%$) jumped during the first day, and almost all of them ($97.14 \pm 0.73\%$) were off the food on the second day (Fig. 5). This is close to the normal behavior in light/dark conditions (laboratory standard population), which has around 75% jumping on the first day, thus indicating that the eventual

rich3/ec-eco-ent/ec-eco-ent/ec0303/ec2205d03a | meadel | S=6 | 3/31/03 | 21:20 | Art: | Input-1st disk, 2nd 2205

desynchronization of the circadian clock caused by the lack of light during the first 238 h does not produce more than a 15% deviation from the normal jumping pattern. Figure 5A shows that the skip behavior of larvae was affected by phloxine B in a concentration-dependent manner. Analysis of the correlation of the delay of jumping with phloxine B concentration during the first day (Fig. 5, inset; slope -5.72 ± -1.05, $r^2 = 0.91$, $P < 0.01$) and during the second day (Fig. 5 inset; slope -6.89 ± -0.81, $r^2 = 0.96$, $P < 0.01$) showed a clear dose-dependent pattern; when exposed to 3 mM phloxine B, <40% (39.07 ± 14.74%) of the surviving larvae (see Figs. 2 and 3) jumped the first day and 100% was only reached on the fourth day. The phloxine B effect was more evident at concentrations of 5 mM or higher. Cumulative jumping rates at this concentration were ≈22% (21.60 ± 18.99) on the first day, 65% (62.55 ± 17.66%) on the second day, and 80% (80.44 ± 10.05%) on the third day, and jumping continued until the sixth day (Fig. 5). However, it should be taken into account that, because of toxicity, the number of surviving insects was constantly being reduced. The weight and size of the 3 mM phloxine B-surviving jumping insects (11.9 ± 0.5 mg and 8.07 ± 0.37 mm, respectively) were found to be similar to those of controls (12.1 ± 0.5 mg and 8.26 ± 0.34 mm, respectively; the difference was not statistically significant, $P = 0.22$ and $P = 0.19$, between the treated insects and the control), thus suggesting that some kind of weight threshold is required for jumping, under conditions of unlimited food availability.

The delay in jumping behavior is probably a reflection of the significant chemical toxicity described above, occurring when a concentration of 3 mM phloxine B or more is present in the food. Therefore, possible reasons for the skip delay are the above postulated damage to muscles, in turn provoking slow mobility, as well as observed damage to midgut cells (not shown) that might lead to a lower rate of nutrient absorption. If this were the case, the surviving larvae may require more days to reach the threshold jumping weight, but the possibility that neural damage delays jumping cannot be discarded.

In conclusion, the present contribution shows a low, but significant chemical toxicity of phloxine B, as measured in *C. capitata* insects maintained in the dark from egg oviposition until adult ecdysis. As far as we know, this is the first time such an observation has been documented in dipterans and only the second report in insects, following the published data on ants (David and Heitz 1978). However, light-independent toxicity of the more reactive rose bengal has been previously documented in the house fly and in other insects (Heitz and Wilson 1978). The documented delay in jumping (Fig. 5), in otherwise apparently normal flies, is also a consequence of the toxicity produced in the dark. This result points to a greater sensitivity of the gut required for food intake and/or of the neuromuscular system for jumping, probably reflecting pleiotropic toxic effects of the dye. More investigations should be performed to infer which molecular species are responsible for the toxicity observed in the dark and to identify the main tissue and cellular targets. As expected, we found that the immature stages within the puparium were very sensitive to phototoxicity when exposed to artificial light for a relatively short 8-h period. Most of the significant phototoxicity during this period seems to target the anterior muscles directly or indirectly, because most of the dying larvae, as well as some survivors, were unable to contract the first three segments to achieve the typical barrel shape of the puparium (Fig. 4). In normal flies, this change of shape is provided by alternated retractions and extensions of the anterior muscle segments (Zdarek 1985). Interestingly, the surviving uncontracted larvae were able to tan the larval cuticle, thus indicating that this process, triggered by the molt hormone 20-OH-ecdysone and mediated by catecholamines, is probably unaffected by phloxine B. Furthermore, we observed a similar lack of performance of the abdominal muscles at the time of ecdysis, when many flies were unable to leave the puparium and remained trapped (Fig. 4), probably because of a lack of proper contraction of these muscles. This is in agreement with similar results obtained with erythrosin B in the face fly (Fairbrother et al. 1981).

Experiments are underway to determine the extent and rate of mitochondrial damage, if any, caused by phloxine B, under both dark and illuminated conditions.

Acknowledgments

We thank R. L. Mangan for his valuable advice and for supply of phloxine B, and D. S. Gitlin for detailed statistical analysis. We are grateful to the University of Buenos Aires, CONICET, and ANPCyT for funding this work. J. B. is a fellow from UBA, A.R. is a fellow from ANPCyT, and L.A.Q.-A. is a Career Investigator from the Argentine Research Council (CONICET).

AQ: F

References Cited

Barbieri, A. 1928. Sensibilizadores fluorescentes como larvicidas: acción fotodinámica de la luz. Rev. Malariologia vii, n°. 4: 456–463.

AQ: G

Ballard, J. B., A. D. Vance, and R. E. Gold. 1988. Light-dependent and independent responses of populations of German and brownbanded cockroaches (Orthoptera: Blattellidae) to two photodynamic dyes. J. Econ. Entomol. 81: 1641–1644.

Ben Amor, T., and G. Jori. 2000. Sunlight activated insecticides: historical background and mechanisms of phototoxic activity. Insect Biochem. Mol. Biol. 30: 915–925.

AQ: H

Bensasson, R., C. Land, and R. G. Truscott. 1983. Exited states and free radicals in biology and medicine. Oxford University Press, New York.

Broome, J. R., M. F. Callaham, L. A. Lewis, C. M. Ladner, and J. R. Heitz. 1975. The effects of rose bengal on the imported fire ant, *Solenopsis richteri* (Forel). Comp. Biochem. Physiol. 51C: 117–121.

Callaham, M. F., J. R. Broome, O. H. Lindig, and J. R. Heitz. 1975. Dye-sensitized photooxidation reactions in boll weevil, *Anthonomous grandis.* Environ. Entomol. 4: 837–841.

Capinera, J. L., and J. M. Squitier. 2000. Insecticidal activity of photoactive dyes to American and migratory grass-

rich3/ec-eco-ent/ec-eco-ent/ec0303/ec2205d03a | meadel | S=6 | 3/31/03 | 21:20 | Art: | Input-1st disk, 2nd 2205

hoppers (Orthoptera: Acrididae). J. Econ. Entomol. 93: 662–666.

Carpenter, T. L., and J. R. Heitz. 1980. Light-dependent latent toxicity of rose bengal to *Culex pipiens quinquefasciatus.* Environ. Entomol. 9: 533–537.

Carpenter, T. L., and J. R. Heitz. 1981. Light-dependent and independent toxicity of erythrosin B to *Culex pipiens quinquefasciatus.* Environ. Entomol. 10: 953–957.

David, R. M., and J. R. Heitz. 1978. Toxicity of an imported fire ant bait based on phloxine B (D + C red 27). J. Agric. Food Chem. 26: 99–101.

Fairbrother, T. E., H. W. Essig, R. L. Combs, and J. R. Heitz. 1981. Toxic effects of rose bengal and erythrosin B on three life stages of the face fly, *Musca autumnalis.* Environ. Entomol. 10: 506–510.

(FDA) Food and Drug Administration. 1982. D&C Red No. 27 and D&C Red No. 28; confirmation of effective date. Fed. Regist. 47: 53343.

Fondren, J. E., Jr., and J. R. Heitz. 1978. Xanthene dye induced toxicity in the adult face fly, *Musca autumnalis.* Environ. Entomol. 7: 843–846.

Heitz, J. R. 1987. Development of photoactivated compounds as pesticides, pp. 1–21. *In* J. R. Heitz and K. R. Downum (eds.), Light activated pesticides: ACS symposium series 339. American Chemical Society, Washington, DC.

Heitz, J. R. 1997. Historical perspectives of xanthene dyes as pesticides. Photochem. Photobiol. 65S (abstr.) TPM-D1.

Heitz, J. R., and W. W. Wilson. 1978. Disposal and decontamination of pesticides, pp. 35–48. *In* M. V. Kennedy (ed.), ACS symposium series 73. American Chemical Society, Washington, DC.

Krinsky, N. L. 1979. Biological role of singlet oxygen, pp. 597–641. *In* H. H. Wasserman and R. W. Murray (eds.), Singlet oxygen. Academic, New York.

Licudine, J. A., G. T. McQuate, R. T. Cunningham, N. J. Liquido, and Q. X. Li. 2002. Efficacy and residues of phloxine B and uranine for the suppression of Mediterranean fruit fly in coffee fields. Pest Manag. Sci. 58: 38–44.

Lipman, A. L. 1995. Safety of xanthene dyes according to the U. S. Food and Drug Administration, supplement, pp. 35–53. *In* J. R. Heitz and K. R. Downum (eds.), Light-activated pest control: ACS symposium series 616. American Chemical Society, Washington, DC.

Liquido, N. J., G. T. McQuate, and R. T. Cunningham. 1995a. Light-activated toxicity of phloxine B and uranine to Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (Diptera: Tephritidae), adults, pp. 81–106. *In* J. R. Heitz and K. R. Downum (eds.), Light-activated pest control: ACS symposium series 616. American Chemical Society, Washington, DC.

Liquido, N. J., G. T. McQuate, and R. T. Cunningham. 1995b. Light-activated toxicity of phloxine B and fluorescein in methyl eugenol to Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), males, pp. 107–114. *In* J. R. Heitz and K. R. Downum (eds.), Light-activated pest control: ACS symposium series 616. American Chemical Society, Washington, DC.

Mangan, R. L., and D. S. Moreno. 1995. Development of phloxine B and uranine bait for control of Mexican fruit fly, pp. 115–126. *In* J. R. Heitz and K. R. Downum (eds.), Light-activated pest control: ACS symposium series 616. American Chemical Society, Washington, DC.

Mangan, R. L., and D. S. Moreno. 2001. Photoactive dye insecticide formulations: adjuvants increase toxicity to Mexican fruit fly (Diptera: Tephritidae). J. Econ. Entomol. 94: 150–156.

Moreno, D. L., and R. L. Mangan. 1995. Responses of the Mexican fruit fly (Diptera: Tephritidae) to two hydrolyzed proteins and incorporation of phloxine B to kill adults, supplement, pp. 257–279. *In* J. R. Heitz and K. R. Downum (eds.), Light-activated pest control: ACS symposium series 16. American Chemical Society, Washington, DC.

Moreno, D. S., H. Celedonio, R. L. Mangan, J. L. Zavala, and P. Montoya. 2001. Field evaluation of a phototoxic dye, phloxine B, against three species of fruit flies (Diptera: Tephritidae). J. Econ. Entomol. 94: 1419–1427.

Peck, S. L., and G. T. McQuate. 2000. Field tests of environmentally friendly malathion replacements to suppress wild Mediterranean fruit fly (Diptera: Tephritidae) populations. J. Econ. Entomol. 93: 280–289.

Pimprikar, G. D., B. R. Norment, and J. R. Heitz. 1979. Toxicity of rose bengal to various instar of *Culex pipiens quinquefasciatus* and *Aedes triseriatus.* Environ. Entomol. 8: 856–859.

Pimprikar, G. D., J. E. Fondren, Jr., and J. R. Heitz. 1980. Small- and large-scale field test of erythrosin B for house fly control in caged layer chicken houses. Environ. Entomol. 9: 53–58.

Quesada-Allué, L. A., A. Rabossi, and P. Wappner. 1994. La mosca del mediterráneo: Guía de laboratorio. *In* L. A. Quesada-Allué (ed.). YPF S. A. Press, Buenos-Aires, Argentina.

Rabossi, A., G. L. Boccacio, P. Wappner, and L. A. Quesada-Allué. 1991. Morphogenesis and cuticular markers during larval-pupal transformation of the Medfly *Ceratitis capitata.* Entomol. Exp. Appl. 60: 135–141.

Rabossi, A., P. Wappner, and L. A. Quesada-Allué. 1992. Larva to pharate adult transformation in the Medfly, *Ceratitis capitata.* Can. Entomol. 124: 1139–1147.

Schilmacher, H. 1950. Über Photosensibilisierung von Stechmüskenlarven durch fluoresizierende farbstoffe. Biol. Zentralbl. 69: 468–477.

Stokes, M., C. S. Davis, and G. G. Koch. 2000. pp. 235–345. Categorical data analysis. SAS Institute, Cary, NC.

Vargas, R. I., S. L. Peck, G. T. McQuate, C. G. Jackson, J. D. Stark, and J. W. Armstrong. 2001. Potential for areawide integrated management of Mediterranean fruit fly (Diptera: Tephritidae) with a braconid parasitoid and a novel bait spray. J. Econ. Entomol. 94: 817–25.

Yoho, T. P., L. Butler, and J. E. Weaver. 1971. Photodynamic effect of light on dye-fed house flies: preliminary observations of mortality. J. Econ. Entomol. 64: 972–973.

Yoho, T. P., J. E. Weaver, and L. Butler. 1973. Photodynamic action in insects. 1. Levels of mortality in dye-fed light-exposed house flies. Environ. Entomol. 2: 1092–1096.

Zdarek, J. 1985. Regulation of pupation in flies, pp. 301–318. *In* G. A. Kerkut and L. I. Gilbert (eds.), Comparative insect physiology: biochemistry and pharmacology, vol. 3. Pergamon, New York.

Received for publication 8 July 2002; accepted 13 February 2003.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES **1**

A: Should 'red' be changed to 'Red'? Please see throughout.

B: Please verify location for Warner Jenkinson.

C: Please verify change to 'photon micromoles/m^{-2}/s^{-1}.'

D: Please include city in UT in which Quantum Meter is located.

E: Please define 'CL' throughout, if necessary.

F: Please define 'UBA' and 'ANPCyT.'

G: Please verify journal title, and clarify information following title.

H: Please verify change to 'Amor.'

I: Please provide volume, page number and publisher and its location.

J: Please include book title following editor.

K: Please include article/chapter title prior to page numbers, followed by editor(s).