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Evaluation of phloxine B as a photoinsecticide on immature stages of the horn fly, *Haematobia irritans* (L.) (Diptera: Muscidae)

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Abstract The use of photoactive substances for controlling adult or immature stages of insect pests is an attractive alternative to chemical insecticides. Phloxine B is an environmentally friendly xanthene derivative that is safe for mammals but toxic for dipterans. In this study we tested the effect of phloxine B as a phototoxic larvicide against immature stages of the blood-sucking horn fly, *Haematobia irritans* (L.). The mortality rate of phloxine B was very low in the dark during the larval stage (100 h) unless a 0.5-mM dye concentration was used. However, a high mortality rate was attained when larvae III were transferred to containers exposed to 5000 lux during the last 2 h before pupariation. This was concentration-dependent up to 0.1-mM phloxine B. After a 2-h larval exposure to light the phloxine B 50% lethal concentration was 0.043 mM. These results indicate that *H. irritans* larvae are very sensitive to this dye, which in turn seems a promising component for larvicide formulations to control horn flies.

Key words larvicide, photosensitising agents, xanthene.

INTRODUCTION

New strategies of insect population control are required to implement integrated pest management policies for controlling fly pests. The use of photosensitising agents (such as halogenated xanthenes, certain porphyrin intermediates and lactones) as potential biopesticides is an interesting alternative to conventional synthetic pesticides (Ben Amor & Jori 2000).

Different halogenated xanthenes have proven to be effective photoinsecticides against at least two-dozen insect species (Heitz 1995). Diptera are particularly susceptible to the photodynamic action of dyes (Heitz 1995; Ben Amor & Jori 2000). More specifically, the photoactive dye phloxine B (PhB), one of the less-toxic xanthenes, is considered an environmentally friendly substance that is innocuous to mammals (Lipman 1995). PhB has been shown to be toxic against adult specimens of several fruit flies such as *Ceratitis capitata* (Wiedemann), *Anastrepha ludens* (Loew), *Rhagoletis pomonella* (Walsh) and *Bactrocera dorsalis* (Hendel), among others (Liquido *et al.* 1995; Mangan & Moreno 1995; Licudine *et al.* 2002). Additionally, PhB was also toxic when ingested by *Ceratitis* larvae (Berni 2002; Berni *et al.* 2003).

The horn fly *Haematobia irritans* (L.) is an external obligate bloodsucking parasite of cattle that generates major economic and health concerns on world livestock production. In Argentina, the populations of *H. irritans* increase, under normal weather conditions, from early spring (October) to the beginning of fall (late March) (Guglielmone *et al.* 2001, 2002; Tarelli 2004); and are distributed over 60% of the country, which comprises approximately 90% of the cattle development territory (Torres *et al.* 2002). *Haematobia irritans* is mostly host-specific and oviposits in cattle dung where its immature stages develop. This substrate provides food and a favourable microenvironment for the larvae (Bruce 1964; Stanley & Kunz 1996; Perotti *et al.* 2001; Perotti & Lysyk 2003).

When *H. irritans* was introduced in Australia (*H. i. exigua*, de Meijere, in the mid-nineteenth century) and in South America (*H. i. irritans*, early twentieth century) they lacked the natural enemies present in their native region. Therefore, horn fly control has been primarily based on chemical insecticides (Lysyk & Colwell 1996). However, this has led to the development of resistance to many commercial insecticides

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(Schnitzerling *et al.* 1982; Casida & Quistad 1998). Horn fly resistance to pyrethroid and organophosphate insecticides has been reported in most countries (Sparks *et al.* 1990; Kunz *et al.* 1995; Sheppard & Torres 1998; Byford *et al.* 1999; Barros *et al.* 2001).

Phloxine B was found to be toxic to *H. irritans* adults when high concentrations were present in blood, with an 50% lethal concentration (LC_{50}) of 1210 mg/L (Gallardo Loera 2000). It would be ideal to stop fly proliferation using natural enemies and larvicidal substances to decrease the number of bite scars in hides, one of the main sources of economical damage.

Thus, to establish a proof of principle this study examines the toxicity of PhB when ingested by immature stages of *H. irritans*, under laboratory conditions.

MATERIALS AND METHODS

Collection of adult horn flies and eggs

Adult horn flies were collected in the field using 0.8-mm mesh entomological trapping nets and transferred to fly cages (15 × 15 × 25 cm, approximately 1500 flies). They were maintained at 30°C and fed with rags soaked in a solution of 0.5 g/L sodium citrate in bovine blood (70%) and NaCl 8.5 g/L (30%). Females were allowed to oviposit their eggs on pieces of cloth saturated with NaCl 8.5 g/L during 8 h. The cloth was then withdrawn from the adult cage and maintained wet in a 90% humidity chamber for 12 h at 30°C until egg hatching.

Larval growth in Bovine Faeces Extract

Urine-free bovine faeces were obtained immediately after deposition from Aberdeen Angus and Hereford cattle reared under natural grazing conditions.

The detailed procedure to obtain a semi-liquid larval medium will be published elsewhere. Briefly, fresh dung was processed as follows: 100 g of bovine faeces were suspended in 100 mL 8.5 g/L NaCI processed in a paddle blender and filtered. The filtered suspension was centrifuged (12 min at 8000 × g), and the pellet was suspended in 10 mL 8.5 g/L NaCl. This suspension constitutes a Bovine Faeces Extract (BFE) and contains most of the bacteria in the original bovine faeces. The bacterial content of 0.5 mL of 100% BFE was equivalent to 1 g of fresh bovine faeces. For the assays, fresh BFE was mixed with sterilised BFE to avoid the effect of potential toxicity as reported by Perotti *et al.* (2001). Optimal larval survival was obtained using a fresh: sterilised BFE proportion of 3:7. All processes were carried out at room temperature.

Plastic containers were lined with a 7-cm diameter circle of blotting paper (previously soaked in physiological solution). A double layer of starch-free, hospital-type cotton gauze (6 × 6 threads per cm^2) was placed over the wet blotting paper. Then, 5 mL of BFE was poured onto the gauze followed by a new double layer of gauze soaked with another 5 mL of BFE that was finally covered with a last double layer of gauze. This array allows larvae good access to fluid BFE without drowning them and at the same time minimises local desiccation. The containers were kept in a convection-free chamber at 30°C in the dark. The humidity of the chamber was close to dew point and maintained by an ultrasound vaporiser (5 L/min of water-saturated ultra fine fog). Any condensed water was drained out.

Efficiency of dung for larval development

The efficiency of fresh bovine faeces for larval development was compared with the efficiency of BFE. For this, 50-g fresh bovine faeces were placed in plastic containers and 50 newly hatched first instar larvae were seeded on the surface. The percentage of larvae development was assessed at 48, 72 and 100 h.

Phloxine B bioassays

Phloxine B disodium salt (PhB) (D&C Red N° 28, Warner Jenkinson, ST Louis, MO) was dissolved in bovine faeces or fresh: sterilised BFE (3:7) under red light and maintained at 30°C. Controls had no PhB.

The aforementioned liquid BFE medium and experimental array was used for the larvae development assays in most bioassays to favour a better homogenisation of the dye and hence increase reproducibility. After pre-warming the medium to 37°C, batches of 20 larvae I were placed on the top of the exposed gauze (under red light). The containers were placed in the culture chamber and maintained in the dark, at 30°C and 95–100% humidity, for 98 or 100 h after egg eclosion (AEE). Red lights were used when necessary. Under our synchronised culture conditions with liquid BFE, the larvae empty their guts and crawl out from the wet culture gauze to the dry periphery after 98 h AEE.

To determine the light intensity to be used in exposure experiments, the 98-h AEE larvae III were removed from the BFE medium and placed on a circle of filter paper (2.5-cm diameter) soaked with distilled water located in the centre of a 6-cm diameter plastic Petri dish. The dish was closed and exposed to 'cold light' illumination using 15 W white fluorescent tubes (Philips F15T8/0), located at a distance of no less than 15 cm. Light intensity was determined with a luxometer (Extex Instrument, Model 401036).

To determine the effect of time of exposure to 5000 lux, the 98-h AEE larvae III were removed from the BFE medium and placed in a Petri dish as above. Mortality was registered every 15 min for each concentration of PhB.

Phloxine B LC_{50} was determined in 98 h AEE larvae III subjected to 5000 lux as described above, during to 2 h until pupariation. To determine the larvicide effect of PhB in the dark, the 98 h AEE larvae were allowed to pupariate for two more hours without illumination.

Embryo and adult sensitivity to PhB

In order to test the permeability of the eggs to the dye, batches of 50 eggs were incubated in a soft agar containing 0.05-mM

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PhB with or without 0.2 g/L Nonidet P40, a neutral detergent, under continuous light (5000 lux). Controls were incubated similarly without PhB.

Batches of 150 adult flies were fed on rags soaked in bovine blood containing 0.5-mM PhB for 75 min in the dark. They were then permanently exposed to 5000 lux and the number of dead individuals was registered after 96 h. Controls were fed on heparinised blood without PhB.

Data analysis

A completely randomised experimental design was used for each assay, with a single factor, five replicates per treatment and 20 or 50 larvae per replicate. At least two series of experiments were carried out in duplicate. Analysis of variance (ANOVA) and Tukey's Honestly significantly different *post hoc* test were performed using the STATISTICA package for Windows (StatSoft Inc 1995).

RESULTS

Efficiency of dung extracts for larval development

Experiments were performed to determine the efficiency of bovine faeces and BFE for larval development. When 50 g of fresh bovine faeces (6 cm high) were seeded with newly hatched *H. irritans* first instar larvae, the percentage of larvae recovered 48 h AEE was 93.3%, whereas the pupariation rate at 100 h AEE (end of the third instar larva) was 69%. In order to design a reproducible system to study the effect of soluble compounds on *H. irritans* larvae, we replaced the fresh bovine faeces with BFE as a nutritional substrate. Based on preliminary growth experiments, we adopted the most reliable diet, a 3:7 proportion of fresh: sterile BFE. Survival rate after 48 h AEE was 86.5%, similar to that obtained using fresh manure ($\alpha < 0.05$). However, bovine faeces were significantly more efficient for larval development than BFE at 72 h and at the end of the third instar larva (100 h AEE) (data not shown). Despite this result the assays were carried out using BFE because the outcome was more reproducible and larval death was easier to determine. No larval development was obtained at 48 h AEE when fully sterilised faeces or BFE was used, thus confirming that living bacteria are essential to larvae development.

Effect of light intensity and time of exposure

Late larvae III (98 h AEE) were transferred to containers exposed to light during the last 2 h of larval development (98–100 h, Fig. 1). Different light intensities were tested. Flies fed on 0.1-mM PhB showed low mortality when exposed to 250–2000 lux (Fig. 1). However, when these larvae were exposed to 5000 lux, the mortality was 66.6% (SEM = 6.3), much higher compared with the controls without PhB (6.7%, SEM = 3.8) (i.e. 59.9% increase in mortality). When treated larvae were exposed to 12 500 lux a mortality of 83.0%

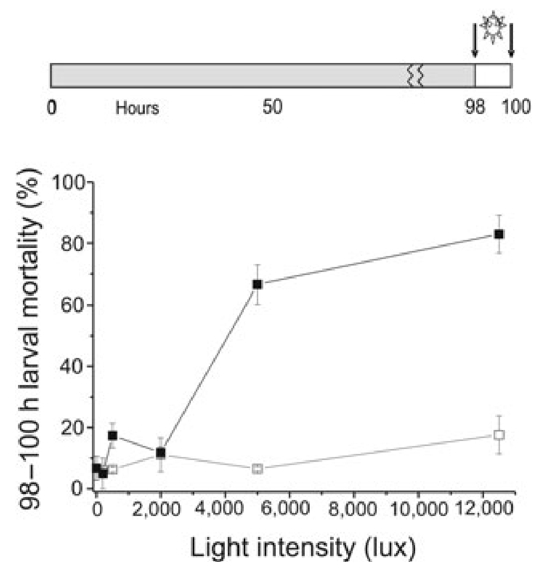

Fig. 1. Effect of light intensity on larvae fed on 0.1-mM phloxine B (closed squares) or not (open squares, controls). Larvae were grown during 98 h in the dark and then illuminated for 2 h (arrows).

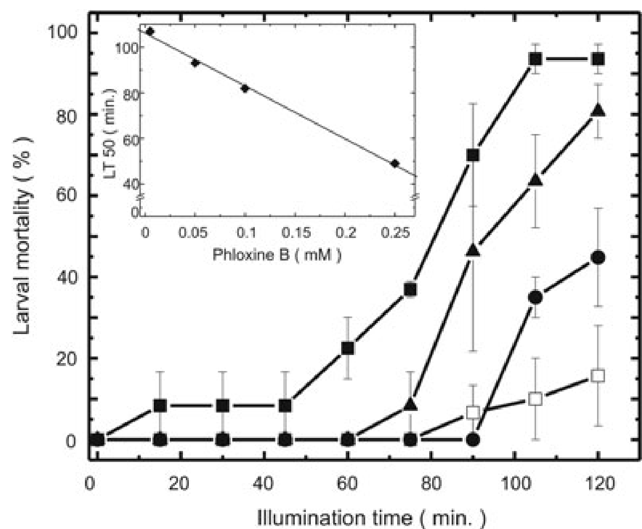

Fig. 2. Light-dependent progression mortality during the last 2 h of larval development. Larvae were grown during 98 h in the dark and then transferred to an illuminated container (5000 lux). Phloxine B concentrations were: (□) 0 mM, (●) 0.025 mM, (▲) 0.05 mM, (■) 0.1 mM. Inset: correlation between phloxine B concentration and LT_{50} values.

(SEM = 4.2) was registered compared with 17.6% for the controls (SEM = 6.2) (i.e. a 65.4% increase in mortality).

Time-dependent phototoxicity was tested on insects reared on different amounts of PhB. The progression of larval III death during the 2 h of exposition to light (5000 lux) at the end of the third instar larvae stage is shown in Figure 2. Surprisingly, the phototoxic effects on the horn fly larvae were fast. Most deaths occurred during the second hour of light exposure in all the concentrations analysed. When 0.1-mM PhB was

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present in the larval medium, a mortality rate of over 50% was reached after approximately 80 min (LT_{50} = 82 min) and almost all the larvae (93%) died after 120 min of light exposition. To attain a mortality of 50% with 0.05-mM PhB, the larvae had to be exposed 93 min and an 80% mortality rate was reached at 120 min. With 0.025-mM PhB, 55% of the flies survived and pupariated. As expected, the LT_{50} decreased linearly with PhB concentration (Fig. 2, inset).

Concentration-dependent mortality

Figure 3a shows the concentration-dependent, light-independent effect of PhB in BFE during larval development (100 h AEE). Small containers with 20 newly hatched first instar larvae were maintained in the dark throughout larval development until the beginning of pupariation (100 h at 30°C, 100% RH). Under these conditions toxicity was low as the apparent PhB LC_{50} for larval development was 0.35 mM. Thus, after 100 h, no difference in cumulated mortality was registered at the end of the period, (time of pupariation) between controls and larvae treated with up to 0.25-mM PhB (Fig. 3a). However, with a concentration of 0.5-mM PhB mortality increased to 83.75% (SEM = 8.54) (Fig. 3a). Therefore, even in the dark high concentrations of PhB were toxic for *H. irritans*.

An important concentration-dependent increase in lethality occurred under light conditions. When 98 h AEE larvae reared in the presence of PhB were transferred to containers exposed to 5000 lux 2 h before pupariation (98–100 h AEE, Fig. 3b), the rate of mortality was linearly dependent on concentration up to 0.1-mM PhB (Fig. 3b). The PhB LC_{50} during the 2 h of larval exposure to light was 0.043 mM (R^2 = 0.981), indicating that during this period *H. irritans* larvae are highly sensitive to the light excitation of the dye. On the contrary, controls showed low mortality (Fig. 3b).

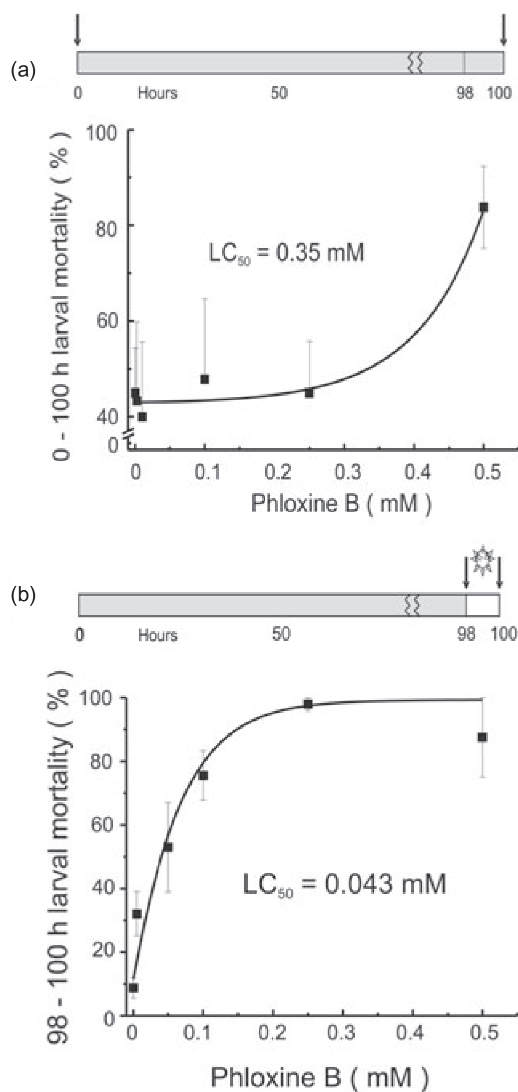

Fig. 3. Toxicity of phloxine B (PhB) during *H. irritans* larval development. The arrows indicate the period analysed to obtain the respective LC_{50}. (a) PhB-dependent mortality in the dark measured at the end of the larval stage (100 h). (b) PhB-dependent mortality after illumination (5000 lux) during the last 2 h of larval development (arrows).

Embryo and adult sensitivity to PhB

Different experiments showed that the rate of hatching was similar between batches of 50 eggs under continuous light (5000 lux) exposed to up to 0.05-mM PhB and controls without the dye. Survival were 73.6% with PhB (SEM = 3.3) and 73.7% in controls (SEM = 6.0). Furthermore, the rate of egg eclosion was similar in the presence of 0.2 g/L Nonidet P-40 (73.7% egg hatching in controls, SEM = 6; 65.9% with NP-40 alone, SEM = 10.4; and 64.6% eclosion using NP-40 and 0.05-mM PhB, SEM = 7.7). These results indicate that the chorion was not permeable to the dye even in the presence of a neutral detergent.

When wild adults were fed on blood supplemented with up to 0.5-mM PhB during 75 min in the dark and then exposed permanently to 5000 lux for 96 h, the death rates after feeding were 2% for controls and 6.6% for individuals treated with PhB. Therefore, the PhB ingested by adults did not seem to be activated, probably because of the opacity of the dark cuticle.

DISCUSSION

The control of Horn and Buffalo flies is becoming increasingly difficult because of insecticide resistance (Schnitzerling *et al.* 1982; Sparks *et al.* 1990; Sheppard & Torres 1998; Barros *et al.* 2001). In several countries, studies of alternative methods of control focus mainly on entomopathogenic fungi as the different developmental stages of horn flies are susceptible to these organisms (Sahagún Angel *et al.* 2005; Lohmeyer & Miller 2006). Therefore, we conducted a proof of principle study to examine PhB toxicity and phototoxicity on the larvae of wild *H. irritans irritans*. Variability because of dung quality was reduced (but not eliminated) using dung extracts as insect food. Experiments using bovine faeces have significant problems of reproducibility because the particulate

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nature of the faeces impedes proper homogenisation of the tested compound (in this case PhB). Additionally, the quality and performance of bovine faeces varies according to the time of year (Lysyk 1991; Lysyk & Colwell 1996) as well as the conditions of nutrition, physiology, health, age, etc. of the animal that produced it (Temeyer 1990), introducing further sources of variation.

The results of this study show that *H. irritans* larvae fed on PhB and exposed to enough light for a relatively short period of time are very sensitive to this phototoxic substance. When larvae III fed on PhB were exposed for 120 min to 5000 lux, the LC_{50} was 0.043 mM (35.6 mg/L) (Fig. 3b), almost 100-fold more toxicity than that registered for *C. capitata* under similar experimental conditions (LC_{50}: 4.7 mM) and 5-fold higher than for *D. melanogaster* (LC_{50}: 0.2 mM) under equivalent periods of time and conditions of development (Berni *et al.* 2003; Pujol-Lereis *et al.* 2006). From a practical point of view, it is noteworthy that under our experimental conditions the luminous flux density that killed significant numbers of *H. irritans* larvae in LC_{50} conditions was 5000 lux during 2 h, which is between 6.4 and 20 times less light than that registered during a sunny day (32 000–100 000 lux). This is important because under natural conditions the post-embrionary development of the horn fly occurs in cattle dung and the opacity of this substrate changes over time (the external surface becomes drier and darker). However, in natural conditions most of the pupariating insects are found in the periphery of the dung pats where it is thinner and more illuminated than the core.

Figures 1 and 2 indicate that death only occurs when certain threshold values of phototoxicity and derived levels of cellular damage are attained. This toxicity/mortality threshold may depend on the integration of pleiotropic cell oxidative stress caused by reactive oxygen species (ROS), probably singlet oxygen (Ben Amor & Jori 2000). In turn, the threshold values depend on the combinatorial values of illumination (lumens/m^2), exposition time and PhB concentration. Light alone generates ROS that are mostly controlled by the natural antioxidant cellular systems. As shown in Figure 2, an exposure of 80–90 min to light slightly increases mortality in control larvae during the last 2 h before pupariation. When the excited PhB generates extra ROS, these systems are probably overwhelmed (Girotti 1998). We know (from unpublished experiments with *C. capitata*) that most of the ingested PhB is retained by gut cells and that Malpighii tubules rapidly excrete the dye present in the hemolymph. Hence, in our experiments, the real concentration affecting tissue targets other than the gut was probably much lower than the ingested concentration because of this gut-filtration effect. Higher PhB toxicity levels (LC_{50} = 2.1 mg/L, i.e. 0.025 mM) were recorded in mosquito larvae (Carpenter *et al.* 1984); however, these are aquatic and the dye was dissolved in the water of the experimental tanks rather than mixed in with the food.

We believe that we have established a proof of principle for high phototoxicity in horn flies and that if a suitable method of administration to cattle is found PhB is a promising component for larvicide formulations. However, the efficient incorporation of this compound into manure still remains a challenge. Therefore, further experimentation is necessary to carry out field experiments with cattle to investigate delivery devices and try to enhance PhB toxicity with other photoactive substances such as those reported by Mangan and Moreno (1995), as well as to design useful formulations with other non-traditional co-insecticides such as the tailor-made anti-horn fly *Bacillus thuringiensis* toxin.

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