Entomologia
Experimentalis et Applicata

DOI: 10.1111/eea.12597

Poor geotaxis correlated with haematoporphyrin-induced peroxidation of brain lipids as a predictor of medfly longevity reduction

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Accepted: 17 February 2017

Key words: negative geotaxis, *Ceratitis capitata*, photosensitizer, lipid peroxidation, functional senescence, glycogen content, anticipated death, Diptera, Tephritidae

Abstract

Under illumination conditions, porphyrins generate cytotoxic radicals in cells. Our study evaluated the effects of haematoporphyrin IX (HP IX) in a laboratory population of male *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) during exposure to a low fluence rate (39 μE m^{-2} s^{-1}) of light. We found that exposing flies to HP IX for at least 5 days was sufficient to cause irreversible damage that led to anticipated death, as also provoked by chronic exposure to the same concentration. To identify early indicators of the accelerated senescence, we analysed both in vitro and in vivo parameters. The thiobarbituric acid reactive substances content in the heads of treated flies revealed a significant increase in lipid hydroperoxides at day 10, whereas this occurred several days later in controls. In addition, a significant decrease in glycogen content was observed at 15 days of age, 5 days before the reduction observed in the control group. This decrease has been associated with a decline in locomotor activity. Differences in the distribution of flies in the rearing flasks were observed, reflecting an impairment of the motility and climbing capacity of HP IX-treated flies. This finding was also corroborated by a geotactic response assay (a rapid iterative negative geotaxis or RING assay). The results presented here demonstrate that low-lethal oxidative stress can anticipate the senescence of flies, which can be predicted using a simple and fast behavioural test, such as the RING assay.

Introduction

A distinctive feature of ageing is progressive functional decline, which includes a loss of adaptive responses to stress with the passage of time (Yu & Chung, 2006). Studies have reported the effects of several environmental stresses on the survival of fly populations: cold or heat stress (Partridge et al., 1995; Sisodia & Singh, 2002; Helfand & Rogina, 2003), hypergravity (Le Bourg, 1999), UV irradiation, starvation, and population density (Carey et al., 1995; Gaskin et al., 2002). These and other environmental factors produce various degrees of oxidative stress, a condition under which increased production of free radicals, reactive species, and oxidant-related reactions occur, thereby generating cellular damage (Yu & Chung, 2006).

Ageing in insects is accompanied by a progressive functional decline in the behavioural performance of locomotion, flight, exploratory activity, learning, and circadian rhythmicity (Le Bourg, 1983; Minois et al., 2001; Grotewiel et al., 2005; Simon et al., 2006). Interestingly, these behaviours do not senesce at the same rate, indicating that the age-related declines are caused by functional deficits in specific organ systems (Cook-Weins & Grotewiel, 2002). The degree of senescence can be assessed by a number of biomarkers, but it is difficult to predict the overall functional senescence (the intrinsic age-related decline in the functional status of an organism; Grotewiel et al., 2005), which is not dependent on chronological age. There is considerable variability in the insect response to oxidative

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stress; consequently, the eventual changes in longevity parameters of a given population are dependent on individual responses. Thus, the measurement of non-lethal parameters could provide a useful insight into functional senescence and estimating the longevity of flies.

In recent years, the use of photosensitizers as tools to control insects has been explored (Ben Amor et al., 2000). When ingested, several porphyrins and substituted porphyrins were found to be toxic against dipteran species of various families (i.e., Tephritidae, Muscidae, Adromyzidae, Sarcophagidae, Culicidae) (Ben Amor et al., 1998; Helleck & Hartberg, 2000; Salama et al., 2002; Dondji et al., 2005; Buda et al., 2006; Luksiene et al., 2007; El-Tayeb et al., 2011). Under illumination conditions, porphyrins generate cytotoxic radicals in cells, such as singlet oxygen ($^1O_2$) (Thomas et al., 1987; Luksiene et al., 2007). In particular, Ben Amor et al. (1998) demonstrated that adults of *C. capitata* exposed to light after ingestion of haematoporphyrin IX (HP IX) showed decreased survival in a dose-dependent manner and that this photosensitizer appeared to accumulate mainly in the midgut and Malpighian tubules. We previously showed that HP IX is also a strong and fast light-dependent photo-larvicide against *C. capitata*, producing high levels of reactive oxygen species (ROS) in the midgut, Malpighian tubules, and brain (Pujol-Lereis et al., 2010), consistent with previous reports in *Culex pipiens* L. larvae (Salama et al., 2002). As in other animals, lipid peroxidation is an indicator of ageing in insects (McArthur & Sohal, 1982; Fleming et al., 1984; Simm & Johnson, 2010). The efficiency of mitochondria and the decline in several biochemical and behavioural parameters are excellent tools for assessing the effects produced by the administration of HP IX on adult survival of a laboratory strain of *C. capitata*.

Insect mobility decreases with age (Lane, 2003) and therefore can be used as a robust indicator of functional status. Negative geotaxis (NG) is a measure of locomotor behaviour that declines with age in both *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Pujol-Lereis et al., 2012) and *Drosophila melanogaster* Meigen (Gargano et al., 2005). The NG is the ability of flies to move vertically when startled. Rhodenizer et al. (2008) established that NG is a climbing behaviour that is independent of other forms of locomotion, such as jumping and flying. These authors also established that the decline of NG in *D. melanogaster* is primarily due to an age-dependent decrease in climbing speed but not to the latency to initiate climbing. The spontaneous distribution of the flies within flasks, as a measure of their dispersal or exploratory activities, has been associated with the insects' internal physiological state and local environment (Simon et al., 2011).

To the best of our knowledge, the effects of porphyrins on locomotor activity or other behavioural responses have not been studied. We assumed that the locomotion of flies expresses an integrated response to the changes that occur in biochemical and physiological processes after treatment with HP. Our aim was to demonstrate that the anticipation of death due to low lethal toxicity of HP IX can be predicted early by biochemical and behavioural parameters that are measured in vivo.

Materials and methods

Insects

The *C. capitata* wild-type strain 'Mendoza' was maintained in a climate chamber (CMP 3244; Conviron, Winnipeg, MB, Canada), at 23 °C, 50–60% r.h., and L16:D8 h photoperiod. Adult flies were maintained in 3.75-l flasks with free access to sucrose:dry yeast (3:1) and 1% agar as a source of food and water, respectively. Eggs that were laid in artificial plastic fruits during a 4-h period were collected to start synchronized fly cultures. The larvae were reared in pumpkin-based medium as described in Pujol-Lereis et al. (2006).

HP IX feeding trials

In each experiment, adult flies were collected after emergence from the puparium, sexed under CO_2 anaesthesia, and placed in flasks with food and drink as described above. Five days later, when the males had reached sexual maturity, 1% agar with or without HP IX 0.5 mM was administered chronically (that is, until day 20, which was the end of the experiment) or for a period of 5 days (days 5–10 after emergence). Low-intensity illumination was achieved using 15-W white cold fluorescent lamps (F15T8/0; Philips, Santiago, Chile) that were placed above the top of the flask. The flies received a light intensity of 39 $\mu E\ m^{-2}\ s^{-1}$ (LM-81LX light meter; Lutron, Taipei, Taiwan). HP IX (50% pure; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1 mM NaOH and mixed with 1% agar (at a temperature not higher than 40 °C, as this substance is heat-sensitive). Every 5 days, the vials were renewed with fresh medium with or without HP IX.

Lifespan assays

Control and both HP IX treatment groups (chronic and 5-day exposure) each consisted of 100 flies (n = 3). Dead flies were removed every day and counted.

Rapid iterative negative geotaxis (RING)

RING assays were performed as previously reported by Pujol-Lereis et al. (2012). Flies of each HP treatment and control groups of different ages were tested to evaluate

their ability to respond to a mechanical stimulus. Four replicates with 10 flies per group were forced to fall to the bottom of the tubes by gentle tapping. After 10 s, the flies that climbed up the wall of the tube were recorded using a digital camera (Sony DSC-W100, Minato, Tokyo, Japan). This test was repeated 8× for each sample, with 2-min intervals. The distances climbed by the flies were measured using Image v.4.0 software (Scion, Frederick, MD, USA). For each group, the average distances measured from the third to the seventh repetitions were compiled, as they represented the optimal behavioural performances. The first, second, and eight repetitions were discarded because they did not show a homogeneous response. The trials always began at 09:00 hours under the same illumination, at room temperature (23 ± 1 °C).

Effect of HP IX on the spontaneous distribution of flies in the rearing flask

In order to assess whether HP IX affects the distribution of flies in the flask, we determined the position of control and treated flies during the first 20 days of adulthood. The experimental flask to analyse the spontaneous distribution of flies was a 40-cm-high cylinder (2 l), and we considered three sections: bottom (10 cm), intermediate (20 cm), and top (10 cm). The food was positioned at the bottom or at the top (at 37 cm from the bottom). Newly emerging flies (50 males) were collected as described above. Three replicates each of the chronic HP IX group and control group were performed with the food at the bottom, and three with the food at the top. Dead flies were removed every day and counted. The distribution of flies was determined as an indirect indicator of mobility, exactly at 14:00 hours which is within the period of most intense activity.

Oxygen consumption

To determine whether HP IX ingestion affects the metabolism of the flies, we measured oxygen consumption. To achieve this, we generated an ad hoc respirometer that consisted of an airtight container with a screw cap (50-ml polypropylene tube) and a 1-ml micropipette inserted across the cap (Bochicchio, 2012). Flies were lightly anaesthetized under CO_2 and weighed, and 20 males were placed into the container. All of the tubes containing flies were immersed in water (23 °C) within an adiabatic chamber. The amount of oxygen consumed was recorded by absorbing the carbon dioxide released in a piece of cotton soaked with KOH (15% wt/vol). The pressure decrease was measured by the ingress of water in the pipette, which was directly proportional to the volume of oxygen consumed after a specific time interval. We recorded the volume of water every 5 min for 40 min of testing. The results were expressed as the oxygen consumption rate per fly. Curve fittings (linear regressions) were performed using Origin v.8.5 (OriginLab, Northampton, MA, USA). Three replicates were performed for each treatment and age.

Lipid and glycogen extraction and quantification

In order to ascertain whether HP IX ingestion affects the main energy reserve substances, we quantified lipids and glycogen. The insects were sampled at different ages using five specimens per replicate for each age. Flies were weighed and homogenized in 0.2 ml of 2% Na_2SO_4 and 1.3 ml of chloroform:methanol (1:2) was added to the homogenate, mixed, and centrifuged (5 200 ***g***) for 10 min. After centrifugation, aliquots of 0.5 ml of the supernatant were used for lipid determination using the vanillin-reagent method. The samples were evaporated to dryness, and the material was resuspended in 0.3 ml of H_2SO_4 and hydrolyzed at 100 °C for 10 min. An aliquot of 30 µl was reacted with the vanillin reagent (270 µl) for 30 min. The amount of total lipids per insect was measured as the absorbance at 490 nm in an ELISA-reader spectrophotometer (standard: triolein; Sigma-Aldrich).

Glycogen was extracted from flies of different ages as described by Tolmasky et al. (2001). The material was digested at 100 °C for 15 min in the presence of 0.9 ml of 33% KOH. The supernatants were separated and three volumes of 96% ethanol were added to precipitate glycogen. The solution was maintained at 4 °C, glycogen was obtained by centrifugation at 5 000 ***g***, and the pellet was then resuspended in 0.1 ml water. The amount of glycogen was determined by reacting 50 µl of the suspension with 250 µl of I_2/KI/$CaCl_2$ reagent (Krisman, 1962) and measuring the absorbance at 450 nm in an ELISA-reader spectrophotometer (standard: glycogen from rabbit liver; Sigma-Aldrich).

Lipid peroxidation

To estimate the age-dependent degree of oxidative stress, we measured the thiobarbituric acid reactive substances content (TBARS) to quantify the lipid hydroperoxides. We used the method described by Yagi (1987) and modified by Badcock et al. (1997). This adjustment involves butanol extraction of the TBARS adducts, followed by aqueous extraction (NaOH 1N). In this phase, the adducts were solubilized to measure malondialdehyde-TBAR. Thoraces or heads (2 mg of tissue each) were homogenized in 250 µl of 30 mM potassium phosphate buffer (pH 7.4, 120 mM KCl) containing a protease inhibitor cocktail [10 mM phenantroline, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF), final concentration] and 1%

butylated hydroxytoluene (BHT) as an antioxidant. The homogenates were centrifuged at 1 600 ***g*** for 10 min at 4 °C, the supernatants were transferred and the pellets discarded. Next, 200 μl was obtained from each supernatant and reacted with 25 μl of glacial acetic acid plus 25 μl of 0.7% TBA. The resulting mixture was stirred and incubated at 90 °C for 60 min on a hot plate. Next, the samples were cooled to room temperature, and 0.5 ml of butanol was added. After centrifugation at 10 000 ***g*** for 3 min, 0.4 ml of the pink organic upper phase was transferred to a new tube, and 0.5 ml of 1N NaOH was added. The solution was stirred and 0.4 ml of the aqueous NaOH inner phase was placed in a new tube. Next, 0.1 ml of phosphoric acid was added to each tube and stirred. The absorbance spectrum between 400 and 700 nm was recorded using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The reaction product MDA-TBA was absorbed at 532 nm. Simultaneously, standard curves were generated with 1,1,3,3 tetramethoxypropane (Sigma-Aldrich), ranging between 0 and 50 μM.

Statistical analysis

The WinModest Program (Pletcher, 1999) was used to determine the mortality model that best fit the data. The mortality parameters estimated for each subgroup were compared with likelihood ratio tests (Pletcher, 1999). ANOVA and Tukey's honestly significant difference (HSD) post hoc test were performed using Infostat 2010 Software (UNC, Córdoba, Argentina). Blocked ANOVAs were performed when necessary.

Results

Effect of haematoporphyrin IX ingestion on medfly survival

The survival curves of *C. capitata* male populations of controls and treated flies exposed to HP IX (0.5 mM) chronically or for 5 days under a L16:D8 photoperiod at a low fluence rate (39 μE m^2 s^{-1}) were determined (Figure 1). As expected, the mean and maximum lifespan of HP IX-treated adults were lower than those of controls (Table 1). The mean lifespan of control flies was 36.1 ± 1.92 days, which was significantly different from that of chronically HP IX-treated flies and that of flies treated for 5 days (28.9 ± 1.01 and 30.8 ± 0.5 days, respectively) ($F_{2,8}$ = 25.48, P = 0.0012). Control flies displayed a significantly higher maximum lifespan (81.7 ± 2.1 days) than did treated flies (52.0 ± 2.6 and 51.0 ± 1.7 days for chronic or 5 days of exposure, respectively) ($F_{2,8}$ = 190.63, P<0.0001). No differences in the mean and maximum lifespan between HP IX treatments were recorded (Table 1).

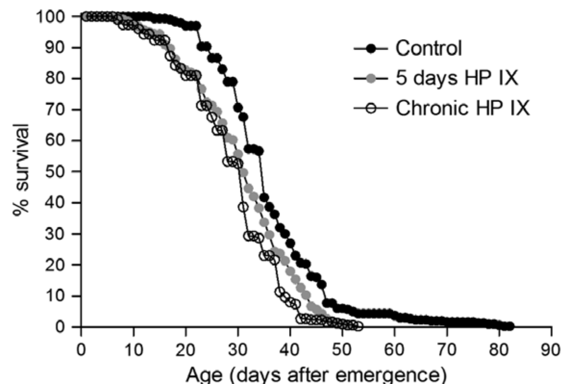

Figure 1 Survival curves of *Ceratitis capitata* populations exposed to 1% agar + 0.5 mM HP IX for 5 days or chronically, or to only 1% agar (control). Flies were maintained at 23 °C and L16:D8 photoperiod at a low fluence rate (39 μE m^2 s^{-1}).

The logistic model (characterized by three parameters: initial mortality rate, slope parameter, and deceleration of mortality rate) appeared to be the best model to describe the survival results. The initial mortality rate was significantly higher in both HP IX-treated populations than in the control, and no significant differences were found between the two HP IX treatments (Table 1). The age-dependent increase in mortality rate (i.e., the slope parameter) was also different between control and HP IX treatments (Table 1). Finally, the deceleration of the mortality rate at advanced ages was significantly lower for both treated populations than for the control (Table 1). These results indicated that the first 5 days of exposure to HP IX was sufficient to cause irreversible damage that determines the anticipated death of these flies (Figure 1, Table 1). Our results also indicated that the dose of HP IX used was sufficient to provoke oxidative stress in the flies and its toxic effects generated mortality only after 15 days of age. Therefore, all subsequent biochemical and behavioural studies were performed during the period when the flies were young (up to 20 days of age).

Effects of oxidative stress on biochemical parameters

The TBARS levels in the head (mostly the brain) were dependent on both the age of the insects and the chronic HP IX treatment: at 10, 15, and 20 days, the levels were higher in the treated flies (2-way ANOVA, age*treatment: $F_{3,16}$ = 26.316, P<0.001; Figure 2A). Ten-day-old HP IX-treated flies achieved similar lipid oxidation levels as that measured in controls at 15 and 20 days old (Tukey HSD post hoc 10HP vs. 15Cont P = 0.15, 10HP vs. 20Cont P = 0.08) (Figure 2A).

The TBARS content of thoracic homogenates (mostly flight muscles) also depended on the age of the flies and

Table 1 Demographic parameters (mean ± SD) of *Ceratitis capitata* populations exposed to 1% agar with 0.5 mM HP IX for 5 days or chronically, or only to 1% agar (control) and comparison with thermal stress parameters by Pujol-Lereis et al. (2012)

Parameters	Control (23 °C)	5-Day pulse HP IX (23 °C)	Chronic HP IX (23 °C)	Thermal stress (28 °C)
Life history				
Lifespan (days)	36.1 ± 1.9b	30.8 ± 0.5a	28.9 ± 1.0a	27.9 ± 2.2
Maximum lifespan (days)	81.7 ± 2.1b	51.0 ± 1.7a	52.0 ± 2.6a	64
Logistic				
Initial mortality rate (a)	0.00002	0.00189*	0.00127*	0.0015
Slope parameter (b)	0.290	0.122*	0.156*	0.18
Deceleration of mortality rate (s)	2.474	0.190*	0.466*	1.28

Means within a row followed by the same letter are not significantly different (Tukey HSD: P>0.05). Asterisks indicate significant differences compared to the control group [WinModest, compared by likelihood ratio (χ^2) tests; *P<0.05].

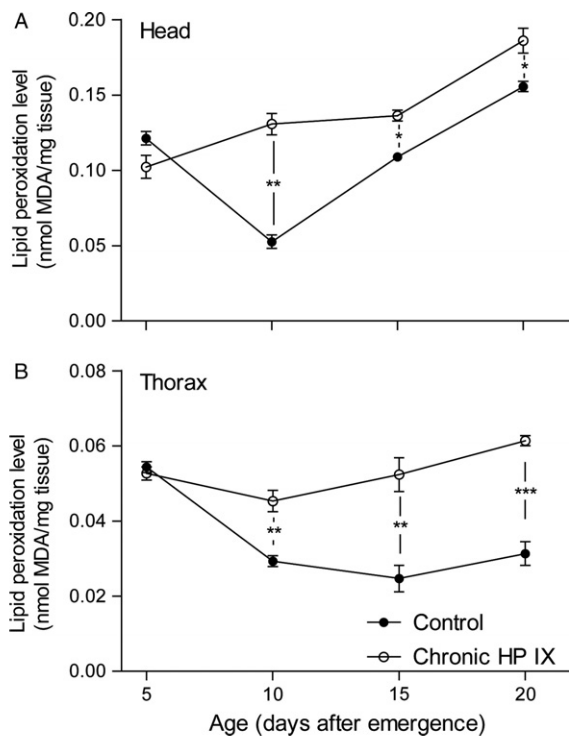

Figure 2 Mean (± SEM) lipid peroxidation levels (nmol MDA per mg tissue) of *Ceratitis capitata* during 20 days of male adulthood in (A) the head and (B) thoracic homogenates, after chronic exposure to 1% agar + 0.5 mM HP IX, or to only 1% agar (control). Asterisks indicate significant differences between treatments, within ages (Tukey HSD tests; *0.01<P<0.05, **0.001<P<0.01, ***P<0.001). Note the difference in scale on the vertical axes.

chronic HP IX treatment: the levels were higher in the treated flies at ages of 10, 15, and 20 days (2-way ANOVA, age*treatment: $F_{3,16} = 17.3$, P<0.001; Figure 2B). It was expected that gut tissues involved in digestion would be the first and most affected by the photochemical processes. This finding was confirmed in abdomens of 15-day-old flies permanently exposed to HP IX, demonstrating 28-fold higher TBARS levels compared with the controls (data not shown).

Importantly, the TBARS levels per mg of tissue registered in the heads were higher than in the thoraces. For instance, the mean level of 10-day-old HP IX-treated flies was 2.9-fold higher in the head than in the thorax, whereas in control flies, the mean level was 1.8-fold higher (Figure 2).

Lipids content was found to differ with age, but no significant effect of HP IX treatment on lipids content was detected, nor of the interaction of age and treatment (2-way ANOVA, age: $F_{3,16} = 9.552$, P = 0.0007; treatment: $F_{1,16} = 3.897$, P = 0.07; age*treatment: $F_{3,16} = 0.759$, P = 0.53; Figure 3A). Age, treatment, and their interaction had significant effects on the glycogen level of the flies (age: $F_{3,16} = 22.722$, P<0.001; treatment: $F_{1,16} = 5.327$, P = 0.035; age*treatment: $F_{3,16} = 12.565$, P = 0.0002). Glycogen level increased in both groups from adult emergence on day 1 until day 10 (Figure 3B). Next, at 15 days of age, a significant drop in glycogen content was observed in the HP IX group (Figure 3B). Five days later, the glycogen content had remained the same, both as the treatment level on day 15, and as the control level on day 20 (Figure 3B).

During the period analysed, exposure to HP IX did not affect oxygen consumption compared to the respective controls and the in vivo oxygen consumption rate did not significantly decrease with age (Figure 4) (2-way ANOVA, age: $F_{3,32} = 3.69$, P = 0.02; treatment: $F_{1,32} = 2.14$, P = 0.15; age*treatment: $F_{3,32} = 0.21$, P = 0.9).

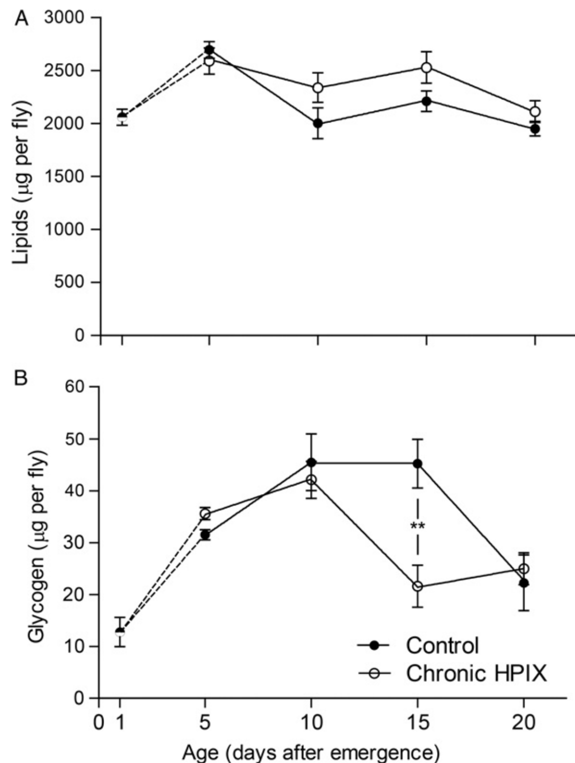

Figure 3 Mean (± SEM) (A) lipid and (B) glycogen content (µg per fly) of *Ceratitis capitata* during the first 20 days of male adulthood. The levels of both energy reserves were measured since the imago emergence (day 1). The experiment was initiated on day 5, when the adults were chronically exposed to 1% agar + 0.5 mM HP IX or only 1% agar (control). Statistical analysis was performed since the 5th day of age. Asterisks indicate significant difference between treatments, within ages (Tukey HSD tests; $^{**}P<0.01$).

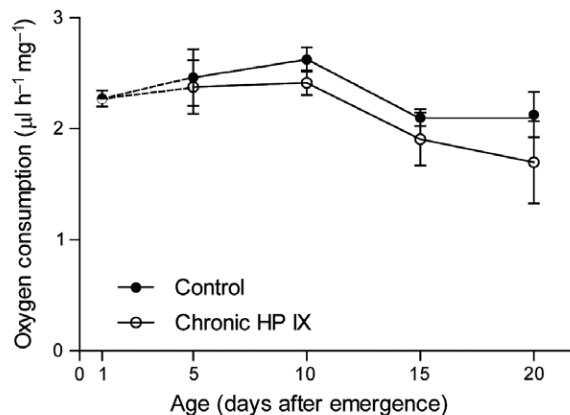

Figure 4 Mean (± SEM) oxygen consumption rate (µl O_2/h/mg fly) during the first 20 days of *Ceratitis capitata* adulthood. Oxygen consumption was registered since the imago emergence (day 1) in order to determine the baseline of metabolism. The experiment was initiated on day 5, when the adults were chronically exposed to 1% agar + 0.5 mM HP IX or only 1% agar (control). Statistical analysis was performed since the 5th day of age.

Effects of oxidative stress on general motor activity

In breeding flasks with food placed at the bottom, control and HP IX-treated 5-day-old flies occupied mostly the central section (section B) (Figure 5A). In subsequent days, the flies of both experimental groups moved more towards the bottom: the percentage of flies in the sections B and C (top) was reduced, that of flies in section A (bottom) increased (Figure 5). At 20 days of age, 50% of the control flies were located in section A, and 50% in section B, whereas 64% of the HP IX-treated group were located near the food.

In breeding flasks with food placed at the top (3 cm below the top of the cylinder; section C), control and HP IX-treated 5-day-old flies occupied mainly the central section B (Figure 5B). In the following days, the flies of the control group moved more towards the bottom of the flask; 46% of flies were located in section A at day 20 (a similar percentage as the registered in control group when the food was at the bottom). The rest of the control flies were distributed in section B (25%) and section C (29%). On the other hand, when food was at the top, flies of the HP IX-treated group did not show important changes in the distribution (from day 5 to the end of the experiment). At 20 days of age, 11% of the HP IX-treated flies were located in section A, 59% in section B, whereas 30% were located near the food in section C (Figure 5B). Thus, the HPIX-dependent decline in functional senescence can be inferred by the time-dependent increase of flies close to the food, both when located at the top and at the bottom.

When the food was placed at the bottom of the cylinder, survival of 20-day-old control and HP IX-treated flies was similar (71.3 and 71%, respectively), whereas when the food was placed at the top, survival was only 64 and 32.7%, respectively. This difference in survival reflects the toxic effects of HP IX, which affected the motility and climbing capacity of the flies and prevented them from reaching the food.

We performed a RING assay to improve the detection of HP-dependent intoxication (Figure 6). In the RING assay, the geotactic response appeared to be influenced by age, treatment, and their interaction (2-way ANOVA, age: $F_{3,35} = 30.70$; treatment: $F_{2,35} = 26.98$; age*treatment: $F_{6,35} = 5.45$, all $P<0.001$) (Figure 6).

On days 10 and 15 after emergence, the climbing performance of HP IX-treated flies (both chronically or with a 5-day pulse) declined relative to the same-age controls (Figure 6). The chronically intoxicated flies showed the

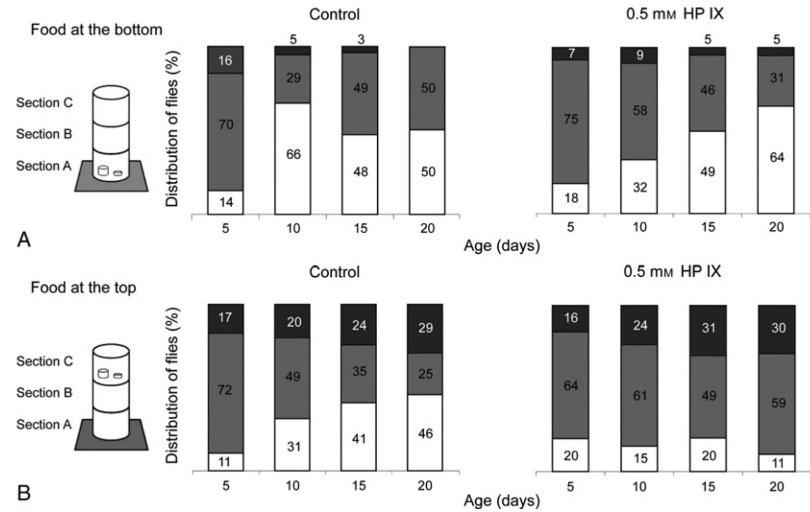

Figure 5 Spontaneous distribution of male *Ceratitis capitata* during adulthood (5–20 days of age) in vertical breeding flask divided in three sections with the food placed at (A) the bottom or (B) the top. Numbers indicate the percentages of flies present in sections A (white), B (grey), and C (black).

worst climbing capacity throughout the assay – on day 20 after emergence the geotactic response of the chronically HP IX-treated flies was significantly lower than that of the 5-day pulse intoxicated as well as the control flies (Figure 6). On day 20, the geotactic response of the 5-day pulse intoxicated and the control flies did not differ (Figure 6).

Discussion

In this report, we demonstrated that it is possible to predict anticipated senescence in populations of *C. capitata* males using behavioural tests. We used 0.5 mM HP IX at

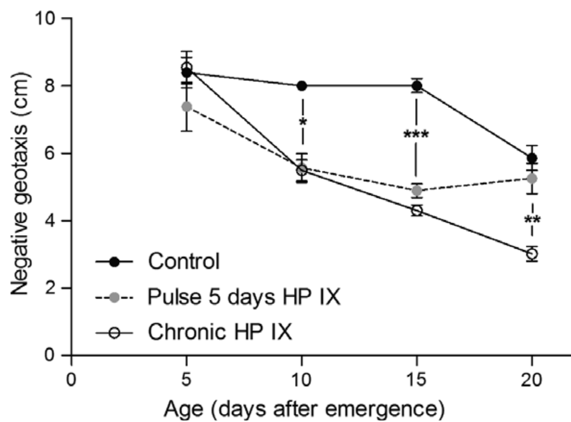

Figure 6 Negative geotaxis assay: effect of age on the mean (± SEM) distance climbed (cm) by *Ceratitis capitata* males treated chronically or for 5 days with 1% agar + 0.5 mM HP IX, or only 1% agar (control). Asterisks indicate significant differences between treatments, within ages (Tukey HSD tests; *0.01<P<0.05, **0.001<P<0.01, ***P<0.001).

39 μE m^{-2} s^{-1} fluence rate to induce photosensitization and oxidative damage, but prevented high mortality rates to anticipate senescence and to perform the analysis during this process. The effectiveness of HP IX as a photoinsecticide was previously demonstrated on adult flies by Ben Amor et al. (1998, 2000). HP IX (1.2 mM) was added to food under 72 h of light exposure at a fluence rate of 1 220 μE m^{-2} s^{-1}, which provoked nearly 100% mortality (Ben Amor et al., 1998). The photoactivation of HP IX has been reported to produce singlet oxygen (Bodaness & Chan, 1977; Thomas et al., 1987). Singlet oxygen species trigger reactions that generate peroxidative modifications of unsaturated phospholipids, glycolipids, and cholesterol (Girotti, 2001).

We first measured senescence and biochemical parameters to demonstrate the degree of intoxication generated by HP IX. The mean lifespan of medfly adults chronically exposed to HP IX was significantly lower than that of control flies. Interestingly, the HP IX-treated populations showed similar demographic parameters as observed with mild thermal stress at 28 °C in previously published studies (Pujol-Lereis et al., 2012). Medflies exposed to HP IX for only 5 days displayed a similar survival curve to chronically exposed flies and no statistically significant differences between the parameters of a logistic model were found. Thus, our results indicated that 5 days of exposure were sufficient to cause permanent damage to the flies.

Expectedly, our results indicated that the addition of HP IX to the drink caused a significant (28-fold) increase in lipid peroxidation in the abdomen, confirming that the gut was subjected to oxidative stress, which is consistent with the results obtained by Ben Amor et al. (1998). Salama et al. (2002) described the appearance of mitochondria with irregular morphology, in

which fragmentation of the Golgi body in small vacuoles and the breakdown of the rough endoplasmic reticulum in the midgut tissue of HP-treated larva of *C. pipiens* were observed. The increased lipid peroxidation observed in the head of 10-day-old flies indicated that HP IX crossed the gut and affected the head more strongly than the thorax. Similar results were reported by Schuck et al. (2015) who found that ethylmalonic acid-injected rats presented a marked increase in TBARS levels in both the cerebral cortex and muscle, and the registered levels in the former tissue were higher than in the latter tissue. As indicated by Rao & Balachandran (2002), the brain is particularly vulnerable to free radical damage because membrane lipids contain high levels of polyunsaturated fatty acid side chains, and consume large quantities of oxygen. The brain has also been shown to contain low to moderate levels of detoxifying enzymes, which play an important role in the metabolism of ROS (Matés, 2000). Thus, a misbalance of the detoxifying system may affect this organ.

Abdelsalam et al. (2014) proposed that an increase in peroxidized lipids in the thorax of flesh fly adults treated with HP-dihydrochloride (HPD) might be associated with perturbations in the activities of antioxidant enzymes. The activities of superoxide dismutase, catalase, and glutathione S-transferase decreased in HPD-treated flesh fly (Abdelsalam et al., 2014). If this also occurred in medflies, increasing peroxidized lipids in the head and later in the thorax might affect the detoxifying system with consequent accumulation of ROS, eventually affecting the proper functioning of mitochondria.

We determined the lipids and glycogen content to estimate the age and HP IX effects on energy reserves. Although no change in lipid content was observed, a 5-day anticipation of glycogen content reduction was registered. Our results were consistent with previous reports by Bui-Nguyen et al. (2015) in zebrafish exposed to the organophosphorus pesticide dichlorvos where oxidative stress alters carbohydrate homeostasis. Bui-Nguyen et al. (2015) demonstrated that dichlorvos exposure as other organophosphorus pesticides affected the expression of genes involved in glucose metabolism in the liver and that the glycogen stores were depleted. In response, the carbon flux is redirected into gluconeogenesis for energy and into the pentose phosphate pathway to generate reducing equivalents to mitigate oxidative stress. Bui-Nguyen et al. (2015) observed a reduction in the levels of transcripts for key enzymes involved in both glycogenolysis and glycogen synthesis. However, the underlying mechanism of how oxidative stress affects carbohydrate metabolism has not yet been elucidated. To analyse whether early damage to mitochondria might be predicted in vivo, we studied the oxygen consumption rate, which, as expected, decreased with age. However, the ages analysed and the significance of the data was not sufficiently strong to adopt respiration rate as a good predictor. Thus, lipid peroxidation and glycogen content were the most sensitive biochemical parameters affected by the oxidative stress generated by the low dose of HP IX employed.

We then switched our analysis to behaviour in order to obtain a good and simple senescence anticipation predictor. Functional senescence was defined as the intrinsic age-related decline in the functional status of an organism (Grotewiel et al., 2005). Among the various skills that age over time, locomotor activity is one of the most evident. To determine whether the above metabolic changes might affect the locomotor output of flies, the spontaneous distribution of the flies within flasks was examined first as an indirect measure of functional senescence status. This indirect measure of mobility has been associated with the insects' internal physiological state in concordance with the local environment (Simon et al., 2011). Clear differences were observed in the distribution of control and HP IX-treated flies with the food placed at the bottom vs. the top of the flask, and these differences resulted in a reduction of survival of HP IX-treated flies with the food placed at the top. These results reflected the toxic effects of HP IX, which affected the motility and climbing capacity of the flies to reach the food.

In order to better determine whether the ingestion of HP IX affects locomotion abilities, we analysed the negative geotaxis (an innate escape response in which flies ascend the wall of a container after being tapped to its bottom). This has been established as a sensitive method for early detection of neuromotor disabilities (Gargano et al., 2005). Consistent with our previous work (Pujol-Lereis et al., 2012), *C. capitata* adult control males displayed a close correlation between older age and decreased response to geotactic stimulus. Here, our results indicated that the geotactic response was early affected by the addition of HP IX to the drink, as shown by a rapid decrease in the climbing response at day 10 after emergence (after 5 days exposure to HP IX). This decline was similar to that recorded in controls on the 20th day and was thus consistent with the above biochemical data. Abdelsalam et al. (2014) demonstrated an important reduction in the detoxification activity of acetylcholine esterase in adult male flesh flies exposed to HPD. This reduction was generated by singlet oxygen and occurred via the oxidation of tryptophan amino acid in the active site of this enzyme (Michaeli & Feitelson, 1994). Furthermore, Williamson et al. (2013) found that motor function was altered by acetylcholine esterase inhibitors. Thus, we postulated

that low concentrations of HP IX crossing the gut are sufficient to cause early effects on the central and/or peripheral nervous system, provoking a decline in locomotive efficiency.

Our study revealed that the RING assay of geotaxis is an excellent in vivo predictor of longevity reduction in a medfly population, as even low concentrations of HP IX produced damage in flies, affecting their behavioural fitness. Thus, the functional senescence status of the population might be inferred with a simple and fast behavioural test.

Acknowledgements

This study was funded by CONICET and ANPCyT, Argentina.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic of the respirometer and adiabatic chamber used in the present work.