**ORIGINAL PAPERS**

# Fly into the light: eliminating *Drosophila melanogaster* with chlorophyllin-based Photodynamic Inactivation

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## Abstract

Fruit flies spoil crops in agricultural settings. As conventional pesticides may generate negative off-target effects on humans or the environment, existing treatment methods need eco-friendly and safe alternatives. Photodynamic Inactivation (PDI) is based on the photosensitizer-mediated and light-induced overproduction of reactive oxygen species in targets. We here explore the potential of PDI for the control of fruit fly pests. *Drosophila melanogaster* serves as well-established model organism in this study. Two distinct experimental approaches are presented: the feed assay, in which fruit flies are provided with sodium magnesium chlorophyllin (Chl, approved as food additive E140) along with sucrose (3%) as their food, and the spray assay, where the photosensitizer is sprayed onto the insects. We show that PDI based on Chl can induce moribundity rates of *Drosophila melanogaster* of more than 99% with 5 mM Chl and LED illumination (395 nm, 8 h incubation in the dark, radiant exposure 78.9 J/cm$^2$) with the feed assay. If the radiant exposure is doubled to 157.8 J/cm$^2$, 88% of insects are killed by PDI based on 1 mM Chl. The photoactive compound is also effective if presented on strawberries without addition of sucrose with somewhat lower moribundity (71% at 5 mM Chl). Spraying Chl onto insects is less effective than feeding the photosensitizer: 5 mM Chl resulted in 79.5% moribundity (drug to light interval 8 h, radiant exposure 78.9 J/cm$^2$), but if 5 h of sun light (532 J/cm$^2$) and overnight (14 h) dark incubation is used for activation of Chl, more than 95% of insects are killed. As conclusion, Chl serves as effective photoinsecticide against *Drosophila melanogaster* if a drug to light interval of 8 h is maintained. Feeding the photoactive compound together with sucrose is more effective than spraying it onto insects and increasing the radiant exposure allows for lowering the photosensitizer concentration. Photodynamic Inactivation might therefore represent an eco-friendly addition to the farmers armamentarium against (semi-transparent) insects.

**Graphical abstract**
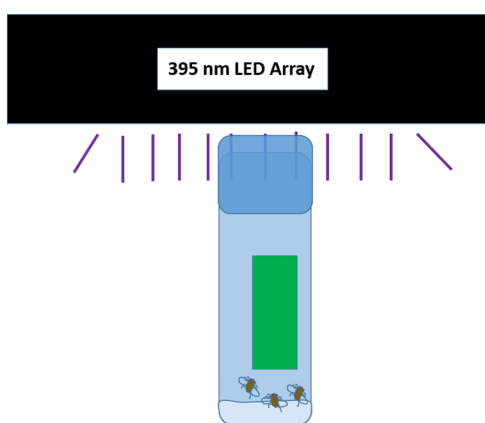
Extended author information available on the last page of the article

## Abbreviations

| | |
|---|---|
| Chl | Sodium magnesium chlorophyllin |
| $LC_{50}/LD_{50}$ | Dose at which 50% of test organisms die |
| PDI | Photodynamic Inactivation |
| PPP | Plant protection products |
| PS | Photosensitizer |
| ROS | Reactive oxygen species |

# 1 Introduction

The projected global population is expected to reach 9.7 billion by 2050 and 10.4 billion by 2100, as reported by the United Nations Department of Economic and Social Affairs in 2022 [1]. However, the availability of agricultural land remains constrained. Expanding agricultural activities represents a primary catalyst for the ongoing decline in biodiversity [2, 3] and must thus be prevented [4].

For yield increase without area expansion, pre- and post-harvest losses must be eliminated, as, for example, agricultural pests and pathogens can cause 50% yield losses in wheat and up to 80% yield losses in cotton in unmanaged agricultural systems [5]. For this purpose, different classes of plant protection products (PPP) are in use. Most economic compounds exhibit unintended off-target toxicity, thereby again compromising biodiversity [6]. Many natural approaches, such as the exploitation of natural predators, are not economic [7]. Organic, or near natural PPP are not free of risk either [8–11]. For example, the organic insecticide Neem oil with its active ingredient azadirachtin, causes malformation and reduced fertility in bumble bees (*Bombus terristris*) in field realistic doses, far below the maximum concentration used in the field (32 mg/l) [12]. In addition, EFSA has deemed azadirachtin very toxic to aquatic organisms [13].

On top, insufficient efficacy and rapid resistance development are common challenges in agriculture [14–16]. To break this vicious circle, affordable and effective strategies for pest management are required in order to feed the constantly growing world population while not compromising on the end of biodiversity.

Photodynamic Inactivation (PDI) has been demonstrated as a promising tool to tackle various microbial infections caused by fungal and bacterial pathogens both in the medical and the agricultural field [17–23]. For higher developed organisms the effect of light-activated photosensitizers (PS) has been tested against mosquito larvae [24, 25], snails [26, 27], honeybees (*Apis mellifera*) [28], African cotton leaf worm (*Spodoptera littoralis*) [29, 30], camel ticks (*Hyalomma dromedarii*) [31], western flower thrips (*Frankliniella occidentalis*) [32], Mediterranean fruit flies (*Ceratitis capitata*) [33] and olive fruit flies (*Bactrocera oleae*) [34]. Thus, studies on insect pests relevant in agriculture are still very sparse.

In its natural habitat *Drosophila melanogaster* as well as other *Drosophila* species increase sour-rot diseases in vineyards [35], which makes control of these flies necessary. This is usually done with regular pesticides, which fire development of resistance. In New York *D. melanogaster* evolved resistance to zeta–cypermethrin, acetamiprid, and malathion, leading to an outbreak of sour rot in a vineyard in 2018, as these insecticides are the go-to-solution to eliminate these flies [36]. Besides its agricultural relevance, *D. melanogaster* is a very well-studied standard test organism with a broad range of available toxicological data [10, 37–39].

Herein, we provide two reproducible and stringent experimental procedures for (1) the identification of lead PS candidates for targeting insect pests, (2) optimization of administration conditions, such as drug to light interval, concentrations, radiant exposure, dark incubation, concentrations, solvent systems or further adjuvants, and (3) determination of parameters relevant for risk assessment. This is achieved by establishing cost- and time-efficient protocols for rearing, photosensitizer administration and subsequent illumination of *D. melanogaster*. This assay enables quantification of photosensitizer efficacy based on fly lethality/moribundity as a direct readout while allowing for two different administration routes, namely spraying and feeding. In order to grant effective, eco-friendly and economic treatment of *D. melanogaster* sodium magnesium chlorophyllin (Chl), approved as food additive E140, serves as eco-friendly and economic PS in this study.

# 2 Material and methods

## 2.1 Fly rearing

*Drosophila melanogaster* wild type strain Oregon-R, obtained from Bloomington Drosophila Stock Center, was kindly provided by Nikolaus Bresgen from the Paris Lodron University Salzburg. The cultivation medium was prepared by dissolving 6 g of Formula 4–24® Carolina Instant Medium, Plain (Burlington, NC, USA) in 30 ml of $ddH_2O$ supplemented with dry yeast from the Formula 4–24 medium in a 175 ml breeding vial (Greiner Bio-One GmbH, Kremsmuenster, Austria). The vial was sealed with a foam plug (Greiner).

For transferring, flies were dazed with $CO_2$, mixed between different cultures and then separated into different vials. The culture vials were kept active (flies being used for experiments/new cultures) for up to four weeks in total darkness at 24 °C, 70% relative humidity.

## 2.2 Preparation of (photo-) insecticides

A stock solution of 3% sucrose (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) was prepared in ddH$_2$O. Ten mM Chl (Carl Roth GmBH + Co KG, Karlsruhe, Germany) stock solution was either prepared in ddH$_2$O or 3% sucrose solution and stored at − 20 °C in the dark until use. The chemical structure and absorption spectrum of Chl are shown in supplementary Fig. 1 and 2, respectively.

Primary solutions of the organic insecticide Solabiol Neem Bio-Schädlingsfrei (SBM Life Science Austria GmbH, Vienna, Austria) with the active ingredient azadirachtin (10.6 mg/ml), were prepared by dissolving 150 µl of the solution in 40 ml of either ddH$_2$O (for spray assays) or a 3% sucrose solution (for standard feed assays). This preparation yielded a final concentration of about 39.75 µg/ml of azadirachtin.

Substral Naturen Cuproxat Flüssig Pilzfrei (Evergreen Garden Care Österreich GmbH, Wals-Siezenheim, Austria), useable in organic agriculture, with its active ingredient tribasic copper sulfate (345 g/l) and an unknown amount 1,2-Benzothiazol-3-on was used as fungicide positive control. This resulted in a concentration of about 9.14 g/l of pure copper or 16.60 g/l copper sulfate.

The comprehensive experimental treatment configuration is illustrated in Table 1.

## 2.3 Illumination

Illumination was performed either by lab made LED arrays (480 diodes, diode type L-7113UVC, Kingbright Electronic Europe GmbH, Issum Germany, main emission wavelength 395 nm) from top of the vials or by sunlight. The LED array was placed 1 cm above the top of the vial and the irradiance measured at this point was 24.10 mW/cm$^2$. In addition, the irradiance was also determined at the bottom of the vial (distance to light source 10 cm). Here, the irradiance was 19.75 mW/cm$^2$. For calculating the radiant exposure of the

fruit flies, the average of the top and bottom measurement (21.93 mW/cm$^2$) was multiplied by the illumination period. Radiant exposure of sunlight was calculated by measuring light irradiance every minute with a LI-180 spectrometer (LI-COR Biosciences GmbH, Bad Homburg, Germany) for the duration of the illumination. Integration of the irradiance data points led to the final radiant exposures referred to later in the sunlight assay. To ensure identical illumination conditions with the sunlight assay all four biologic replicates were illuminated in parallel using different culture vials to guarantee four independent biologic experiments. Irradiance data of the sunlight assay are shown in supplementary Fig. 3.

## 2.4 Photodynamic Inactivation of insects

The treatment schematics can be seen in Figs. 1, 2, 3 and 4. A treatment setup consisted of four biologic replicates which consisted of two technical replicates each, resulting in about 240 flies tested per treatment.

## 2.5 Experimental procedure for the feed assay

At the bottom of an empty breeding vial an organic cotton pad (ebelin, dm Drogerie Markt, Wals, Austria) was moistened with 2 ml of dH$_2$O. A ~ 13 cm$^2$ piece of filter paper was fixed on the side of the vial with a piece of adhesive tape. On that filter paper 250 µl of the treatment solution, dissolved in 3% sucrose, was applied.

Directly after adding the treatment solutions, the alive flies in the vials were counted. After 1 or 8 h of dark incubation at room temperature (*i.e.,* drug to light interval) the flies were illuminated with a 395 nm LED array for 1 h (resulting in a radiant exposure of 78.9 J/cm$^2$) or 2 h (157.8 J/cm$^2$). Each experiment lasted for a total of 18 h (see Fig. 5).

The treatment scheme for the feed assay is shown in Fig. 1.

## 2.6 Experimental procedure for feed assay on strawberries

Organic strawberries were bought in a local supermarket (origin country: Slovakia, Veganis GmbH, St. Andrä am Zicksee, Austria). Ten ml of agar were poured into the breeding vials at around 55 °C to fix the strawberries in place to prevent flies being crushed by the strawberries. Treatment solutions contained the Cuproxat/Neem and Chl as described above but without sucrose. The strawberries were submerged in the solutions and directly afterward placed in the still liquid agar. The vials were kept in the dark for around 2 h to let the agar solidify and cool down.

Afterward, around 30 flies were transferred into a single vial. Assessment of moribundity was carried out directly after transferring flies into the treatment vial. The dark

**Table 1** Samples and controls in all experiments in this study

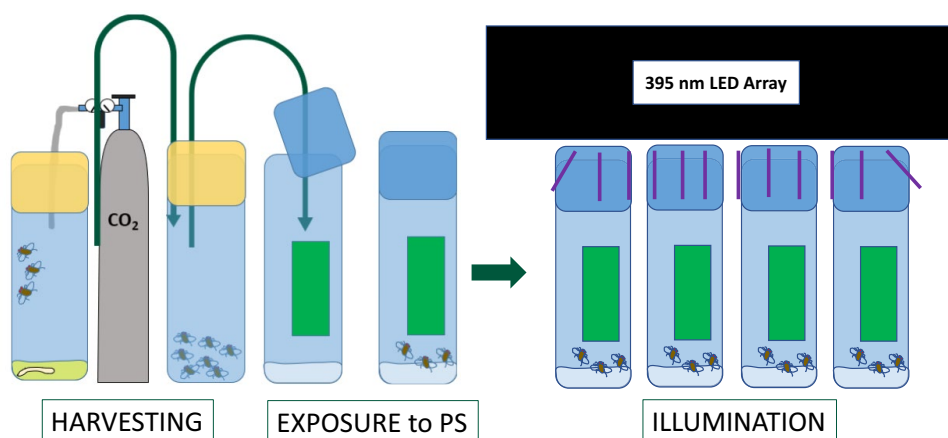| Sample | Concentration of active ingredient (g/l) | Illumination |
| --- | --- | --- |
| Control -/- (double negative control) | 0 | No |
| Light control | 0 | Yes |
| Neem (neem oil) | 39.750 | No |
| Cuproxat (cuproxat liquid) | 9.142 | No |
| PS 5 mM Chl (dark control) | 3.425 | No |
| PDI 0.1 mM Chl | 0.069 | Yes |
| PDI 1 mM Chl | 0.685 | Yes |
| PDI 5 mM Chl | 3.425 | Yes |

**Fig. 1** Treatment schematic of the feed assay setup to test photoinactivation of *Drosophila melanogaster*. After dazing the flies with $CO_2$ they were transferred into new breeding vials with a filter paper containing 3% sucrose and sodium magnesium chlorophyllin. Subsequent to 1 or 8 h incubation the samples were illuminated from above with 395 nm at a radiant exposure of either 78.9 J/cm$^2$ or 157.8 J/cm$^2$. Survival of the insect was determined 8–9 h or 17 h (short dark incubation) post illumination
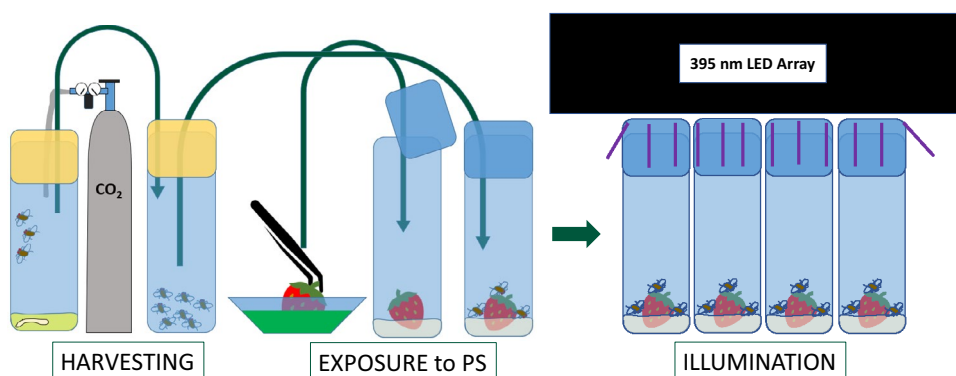


**Fig. 2** Treatment schematic of the strawberry feed assay setup to test photoinactivation of *Drosophila melanogaster*. Strawberries were dipped into the treatment solutions and transferred into breeding vials with agar. *Drosophila melanogaster* flies were added and allowed to feed on the strawberries for 8 h. Afterward, the samples were illuminated from above with 395 nm using a radiant exposure of 78.9 J/cm$^2$. Survival of the insect was determined 8–9 h post illumination

incubation period was 8 h and after that about one hour of illumination with the LED arrays resulted in a radiant exposure of 78.9 J/cm$^2$. Eight to nine hours after the end of the illumination, moribundity of the flies was assessed. Total experimental time was again 18 h (see Fig. 5). The treatment scheme of this setup is shown in Fig. 2.

## 2.7 Experimental procedure for the spray assay using LED illumination

At the bottom of a breeding vial an organic cotton pad (ebelin) was moistened with two milliliters of dH$_2$O. Flies were dazed with $CO_2$ and about 30 flies added into the breeding vial. The insects were then sprayed with two spray pumps (six spray pumps of Cuproxat due to higher recommended application rate/area) of a 20 ml glass spray bottle (Rixius AG, Mannheim,

Germany) resulting in about 0.28 g of deposited liquid. After spraying, a ~ 13 cm$^2$ piece of filter paper was fixed on the side of the vial with a piece of adhesive tape, which was moistened with 250 μL of 3% sucrose solution to prevent starvation of the flies. After one or eight hours of dark incubation at room temperature the flies were illuminated with a 395 nm LED array for 78.9 J/cm$^2$. Moribundity was assessed 18 h after start of the assay (see Fig. 5 for time schedule of setup). The experimental scheme is shown in Fig. 3.

## 2.8 Experimental procedure for spray assay using sunlight illumination

The sunlight spray assay was identical to the spray assay described above, but illumination was performed with natural sunlight for 5 h. The drug to light interval
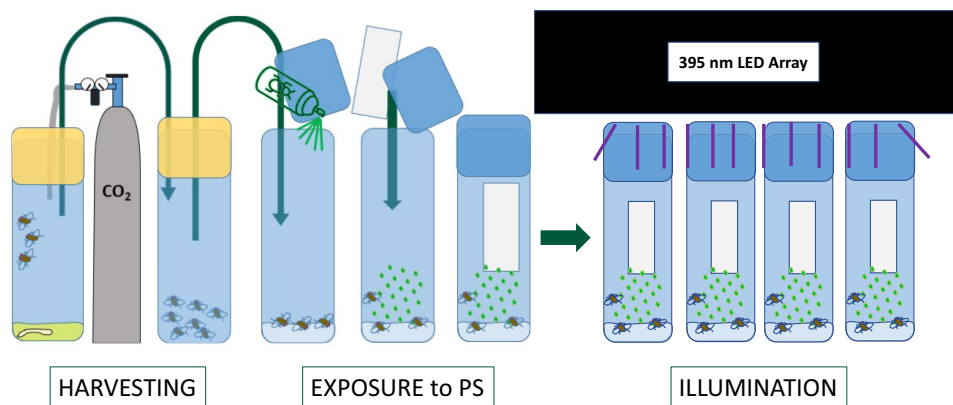
**Fig. 3** Treatment schematic of the spray assay setup to test photoinactivation of *Drosophila melanogaster*. Dazed flies were transferred into breeding vials and sprayed with the treatment solutions. Within the drug to light interval of either 1 or 8 h the insects were allowed to feed on 3% sucrose presented on filter paper. Afterward, the samples were illuminated from above with 395 nm at a radiant exposure of either 78.9 J/cm$^2$ or 157.8 J/cm$^2$. Survival of the insect was determined 8–9 h or 17 h (short dark incubation) post illumination



**Fig. 4** Treatment schematic of the sunlight spray assay setup to test *Drosophila melanogaster*. Dazed flies were transferred into breeding vials and sprayed with the treatment solutions. Within the drug to light interval of 14 h the insects were allowed to feed on 3% sucrose presented on filter paper. Afterward, the samples were illuminated with natural sunlight for 5 h
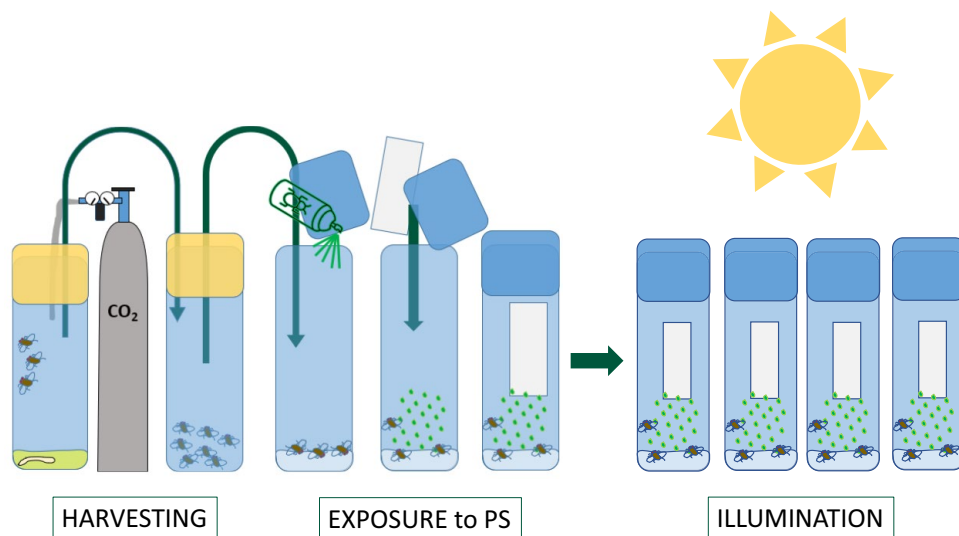
was 14 h (overnight) and the post illumination period to assess moribundity after illumination was 18 h. To obtain the roughly same intensity and duration of the illumination the four biologic replicates were performed simultaneously. Total experimental duration was about 37 h (see Fig. 5 for a schematic). The general treatment schematic is shown in Fig. 4.

## 2.9 Experimental schedule

A graphical representation of all the experimental schedules can be seen in Fig. 5

## 2.10 Statistical analysis

Data normal distribution was examined by applying the Shapiro-Wilks test, revealing significant deviations from normal distribution for most of the experimental groups. The Mann–Whitney $U$ test, performed as exact test, was used for pairwise comparisons of independent samples and the Wilcoxon-signed-ranks test (Exact test) was used for pairwise comparisons of related (i.e., non-glued vs. glued) samples. Pairwise comparison of groups showing normal distribution (Neem and Cuproxat controls of Sunlight spray assay) was performed using 2-tailed Student $T$ test for independent samples or the paired samples $T$ test for dependent samples. Statistical analyses were performed
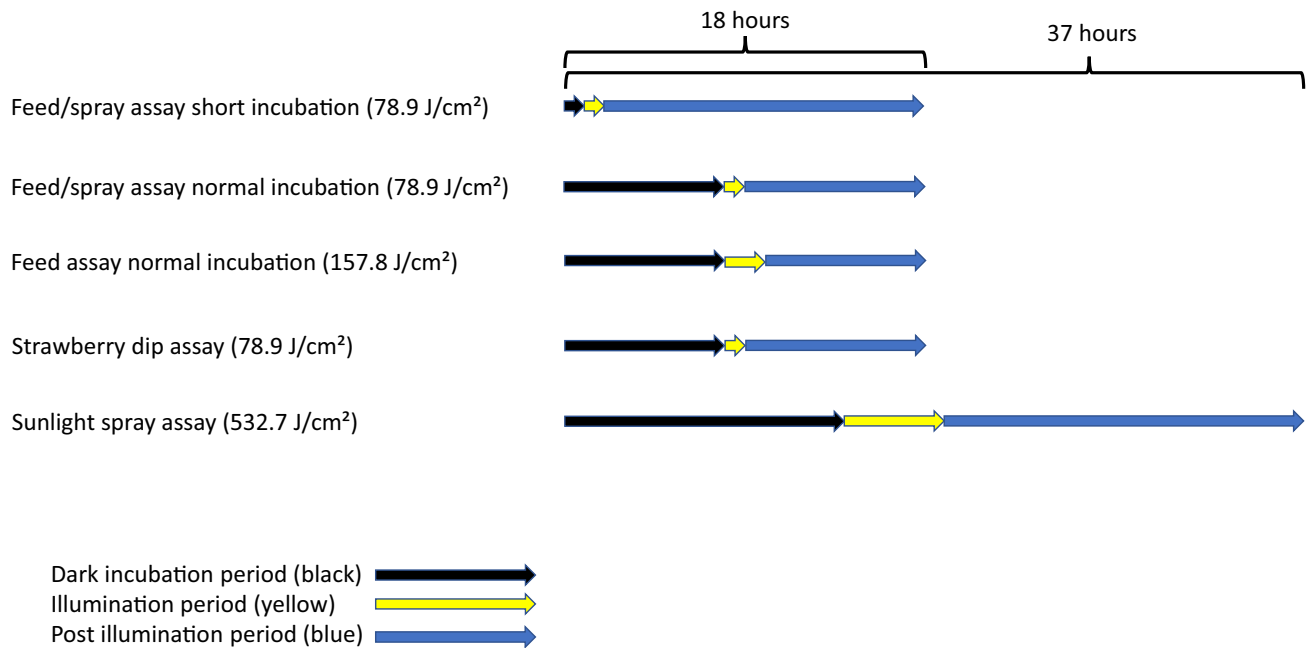
**Fig. 5** Duration schedule of the different experiments performed. The overall duration of each experiment was 18 h, except from illumination with sunlight, where each experiment took 37 h

using SPSS Version 27. Graphs were created using Origin 2021b (OriginLab Corporation, Northampton, MA, USA).

## 3 Results

### 3.1 Photosensitizers offered with sucrose: feed assays

In one methodological concept, the feed assay, Chl was offered to *D. melanogaster* as food containing 3% sucrose (Fig. 6 A-D). For a drug to light interval of 1 h there was no moribundity in the untreated controls, Neem-treated samples, the PDI 0.1 mM Chl treatment and the PDI 1 mM Chl treatment (Fig. 6A). For the treatment with Cuproxat the median moribundity was very low (1%). Photodynamic Inactivation based on 5 mM Chl increased the moribundity to 23.5% (Fig. 6A).

Increasing the dark incubation period from 1 to 8 h has a significant ($p < 0.05$) effect on the overall PDI based moribundity of 1 and 5 mM Chl treatments (Fig. 6B). Once more, there was no moribundity toward the untreated controls, the Neem control, and the PDI 0.1 mM Chl treatment while there was a weak toxic effect in the Cuproxat treatment (7%). For the photodynamic treatment with 1 mM Chl, the median moribundity is 68% and increases to a median total kill (100%) at 5 mM Chl (Fig. 6B).

Doubling the illumination period from 1 to 2 h (157.8 J/cm$^2$, drug to light interval 8 h) was not toxic to the insects

present in the control and the Neem samples (Fig. 6C). For PDI using 1 mM or 5 mM Chl the median moribundity was at 88% and 98%, respectively.

When feeding Drosophila with (photo-) insecticides present on strawberries (strawberry feed assay, 8 h drug to light interval) the moribundities are as follows: Cuproxat treatment 9.5%, dark control (5 mM Chl) 1%, PDI 1 mM Chl 23% and PDI 5 mM Chl 71%. The radiant exposure was 78.9 J/cm$^2$ for PDI-treated samples (Fig. 6D). Comprehensive tables with all values can be found in the supplementary file.

### 3.2 Photosensitizers sprayed onto insects: spray assays

As second insecticidal strategy the treatment solutions were sprayed onto *D. melanogaster*. The results are presented in Fig. 7 A-D.

For a drug to light interval of 1 h, no moribundity was determined for the untreated controls, the light only controls, the dark controls and for PDT treated samples with 0.1 and 1 mM Chl (Fig. 7A). The two positive controls Neem and Cuproxat showed a median moribundity of 10% and 34.5%, respectively. PDI treatment based on 5 mM Chl induced a moribundity of 35.5% (Fig. 7A).

Increasing dark incubation from 1 to 8 h has a significant ($p < 0.05$) effect on the moribundity (Fig. 7B). No notable moribundity was determined for the untreated controls, the dark controls, the light only controls and PDI
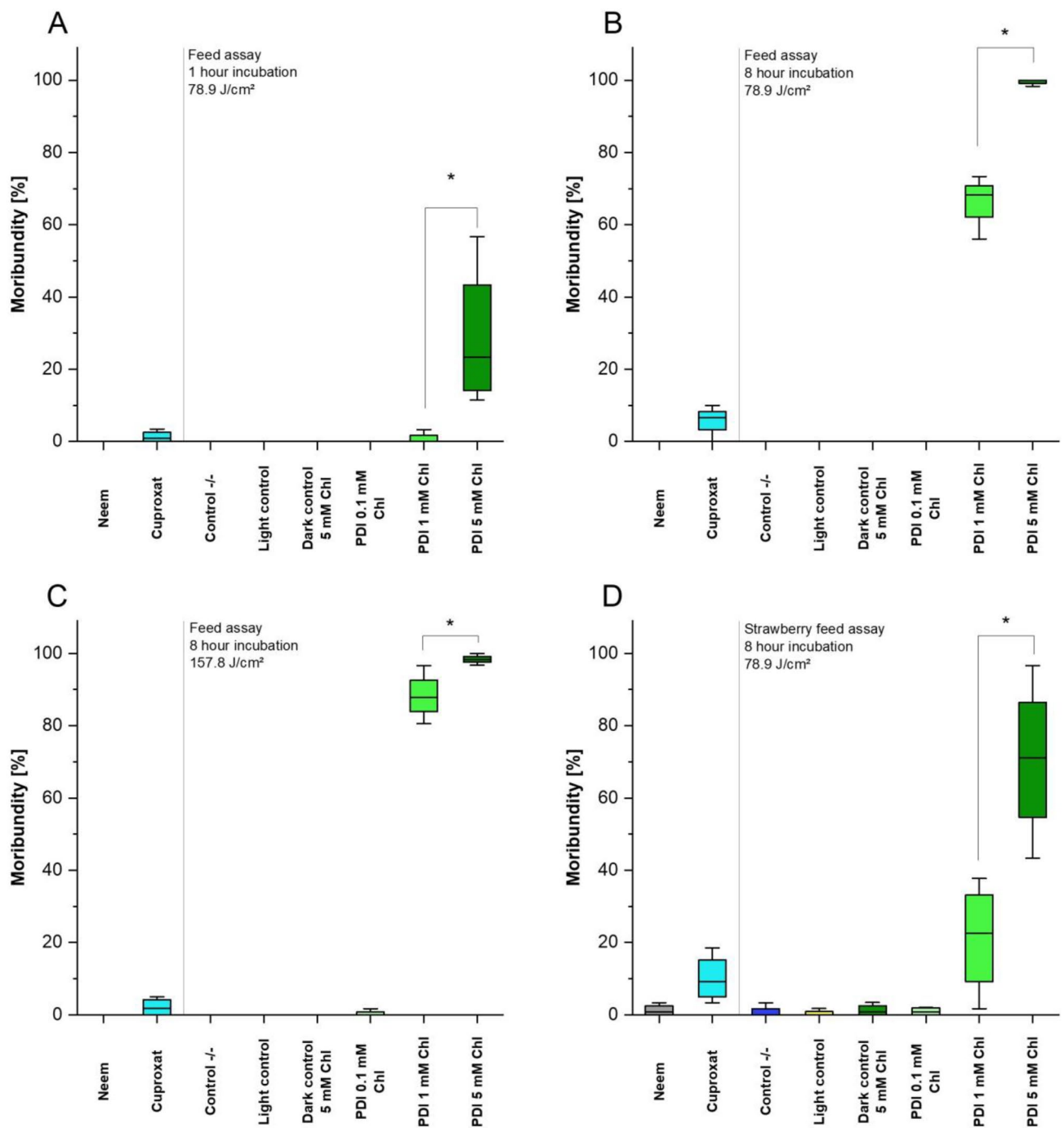
**Fig. 6** Moribundity analysis of *Drosophila melanogaster* after feeding on the (photo-) insecticides (feed assay). Data are presented as Box-Whisker-Plots of four biologic replicates. \*$p < 0.05$ as indicated (Mann Whitney *U* test [Exact test]). **A** One hour drug to light interval; radiant exposure 78.9 J/cm². **B** Eight hours drug to light interval, radiant exposure 78.9 J/cm². **C** Eight hours drug to light interval, radiant exposure 157.8 J/cm². **D** Insecticides offered on strawberries, 8 h drug to light interval, radiant exposure 78.9 J/cm²

with 0.1 mM and 1 mM Chl. The median moribundity for the positive control with its active copper sulfate ingredient in the Cuproxat treatment (Fig. 7B) was 30.5%, and for the insecticide positive control (Neem) 10%.

Photoactivation (78.9 J/cm²) of 5 mM Chl sprayed onto *Drosophila* is clearly toxic at 79.5%.

One effect of spraying insecticides onto *D. melanogaster* is that the flies get attached to the receptacle's surfaces by
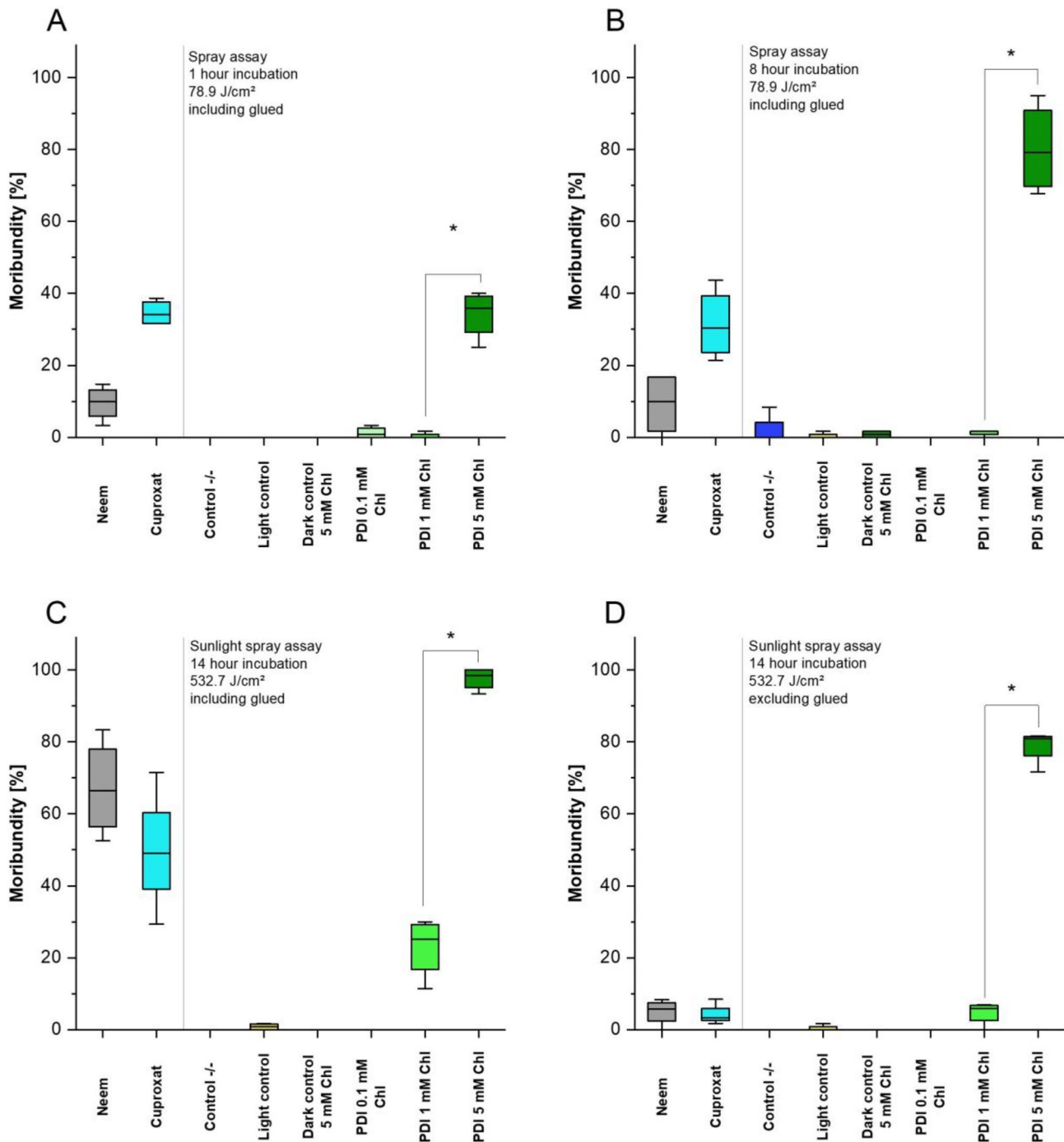
**Fig. 7** Moribundity analysis of the different spray assays. Data are presented as Box-Whisker-Plots of four biologic replicates. *$p < 0.05$ as indicated (Mann Whitney $U$ test [Exact test]). **A** Dark incubation: 1 h; radiant exposure: 78.9 J/cm$^2$. **B** dark incubation: 8 h; radiant exposure: 78.9 J/cm$^2$. **C** Sunlight spray assay including glued flies, dark incubation: 14 h; radiant exposure: 532.7 J/cm$^2$. **D** Same experiment as figure C but excluding glued flies

adhesive force and then die from starvation or drowning in the droplets. For this, the term *glued* is used in the following. To assess the contribution of this gluing effect to overall insect killing, the experiments using sunlight (532.7 J/cm$^2$, full spectrum) were evaluated in two ways: with (Fig. 7C)

and without counting glued insects (Fig. 7D). Since these data show a normal distribution, statistical examination of this dataset was performed using t-statistics. This revealed significant (Neem: $p < 0.005$; Cuproxat: $p < 0.016$) differences of moribundities comparing the evaluation where

glued insects were counted (Fig. 7C) versus an evaluation, in which glued insects were not considered (Fig. 7D). The median moribundity, including glued flies, of the sunlight spray assay in the light-control treatment is 1%, 49% for the Cuproxat treatment, 66.5% for the Neem treatment, 25% in the PDI 1 mM Chl treatment and 98.5% in the PDI 5 mM Chl treatment (Fig. 7C). Excluding glued flies, the moribundity rates drop to 3.4% for Cuproxat, 5.7% for Neem and 80.9% for PDI using 5 mM Chl (Fig. 7D).

## 3.3 Statistical comparison of the feed and spray assays results

For experiments applying the short-term, one hour incubation (Fig. 6A and 7A), no significant differences were found between the PDI 1 mM Chl treatments. The same applies to the PDI 5 mM Chl treatments. In contrast, for all three cases of the long-term, 8 h incubation experiments (regular feed assays with 78.9 and 157.8 J/cm$^2$ and spray assay with 78.9 J/cm$^2$) a substantial toxic effect was seen after the 18 h treatment (Fig. 6 A, B; Fig. 7B), while the dark controls with 5 mM Chl did not show any relevant moribundity.

## 4 Discussion

The aim of this study is to establish two experimental procedures, the feed assay and the spray assay, for standardizing tests on the photokilling efficiency of photodynamic insecticides. In addition, as proof of principle, photokilling of *D. melanogaster* using Chl as PS is demonstrated for both protocols.

For both strategies the drug to light interval is very critical for the photoinsecticidal effect: a relatively short dark incubation period of 1 hour resulted in lower moribundity when compared to 8 h in between spraying or feeding the PS and activation with light. If this is considered, a notable photokilling efficacy even for a relatively low concentration of the photoactive compound (1 mM, radiant exposure 78.9 J/cm$^2$) is achieved, allowing for economic treatment of an *D. melanogaster* infestation. The efficacy is further increased upon doubling the radiant exposure, resulting in almost 90% moribundity, indicating that the LD$_{50}$ is probably far below the 1 mM, after a mere 18 h. This observation is in line with other studies performed by Mamdouh Nassar et al. (2021) and Wohllebe et al. (2011) [28, 40], where several hours of dark incubation are required and that the LD$_{50}$ *D. melanogaster* is in between the more translucent *Chaoborus crystallinus* larva (LD$_{50}$ ~ 0,035 mM) and the less translucent honey bees (LC$_{50}$ ~ 3 mM).

Both experimental procedures in our study do not induce any toxicity in the control groups, proving the validity and applicability of the assays. However, for a significant photokilling of insects, higher concentrations of Chl are needed than for killing microorganisms with PDI: photoactivation of 0.1 mM did not affect *D. melanogaster*, but was demonstrated to be effective against plant pathogenic bacteria [18, 20] and fungi [19].

As general observation, feeding the insects with Chl is more effective than spraying the compounds onto them: for example, spraying 1 mM Chl onto insects does not cause acute toxicity after illumination (at 78.9 J/cm$^2$), but when *Drosophila* is allowed to feed on 1 mM Chl with sucrose a significant moribundity was induced. Increasing the radiant exposure to 157.8 J/cm$^2$ further enhances the photoinsecticidal effect. Therefore, for the field application, illumination with sunlight, using the full spectrum and further absorption bands of Chl, might improve the results.

The radiant exposures used in this study are considerably high and chlorophyllin is subject to rapid photodegradation under these light conditions [41]. However, the actual radiant exposure in the insect's body is most likely much lower than the one measured outside due to light scattering and absorbance by the cuticle and tissue. We therefore hypothesize, that due to the much lower radiant exposure within the semi-transparent *Drosophila* photodegradation of internalized Chl is much lower, too, by this justifying the need for such radiant exposures.

If the photoactive compound is presented on strawberries, the flies incorporate enough Chl for a rapid control of *Drosophila*. However, when comparing the results to Chl presented with sucrose, the photokilling effect is lower. We hypothesize that the natural content in fruits is somewhat more favorable to flies and therefore the flies did not consume as much Chl when Chl is offered with 3% sucrose.

Sodium magnesium chlorophyllin is per se not toxic in the dark at 5 mM concentration, which is in line with other studies [30]. The acute toxicity of Neem (active ingredient azadirachtin at 39.75 mg/l) containing Neem oil is negligible. In addition, Neem oil and its active ingredient azadirachtin are known mainly for non-lethal effects, like deformation in offspring or feeding aversion [42]. As these effects take several days to be detectable, our study might underestimate the effect of azadirachtin. However, we show that Neem oil cannot be deemed useful for combating an acute *D. melanogaster* infestation, which is in line with results from another study, where the main effect was reduction in progeny over longer time periods [37].

The effect of Cuproxat (active ingredient: copper sulfate) in the spray assays was mainly due to the higher recommended application rates, resulting in three times as much deposited liquid in comparison with the other treatments and thus many flies getting stuck on the side of the treatment vials. This is more evident in the sunlight spray assays. The acute toxicity within 18 h, when excluding

the stuck flies, in the other assays was negligible. However, longer observation or contact time might increase the lethality of this heavy metal [43].

Chlorophyllin undergoes rapid photodegradation [41]. This effect could probably make contamination of surface water with Chl less likely. The degradation products of chlorophyll itself are most likely not harmful [44] as Chl is approved as food additive E140. Therefore, Chl could theoretically also be used for treatments close to harvest, where conventional pesticides might not be applicable due to unwanted residues [45].

As for the conventional organic treatments, copper is highly toxic to aquatic invertebrates ($EC_{50} \sim 0.005$ mg/l for *Baetis tricaudatus* according to Mebane et al., 2020). Fungicides frequently exert toxic effects on soil organisms [6, 46]. Benthic invertebrates inhabiting streams near agricultural areas may encounter some runoff from the application of Chl PPP. However, besides significant dilution and photodegradation, many of these invertebrates reside in the riverbed to evade predation by fish. We therefore anticipate that Chl PPP will not exert significant effects on these communities [47, 48]. The little data available indicates that fishes are not affected by Chl [40]. Chlorophyllin does also not seem to affect healthy adult (> 8 mm) freshwater snail *Biomphalaria alexandrina*, as example of a aquatic invertebrate, not nessecarly hiding in the sediment [26].

Traditional pesticides typically exhibit a highly specific mechanism of action, making them susceptible to the rapid development of resistance. For example, a simple genetic mutation increased the resistance to Spinosad 1181 times [16]. Spinetoram will be banned in the EU in 2024 [49], leaving farmers with fewer options to combat fruit flies. While azadirachtin toxicity on *D. melanogaster* has been assessed [37] it has low acute toxicity, as shown in our studies, but it might reduce reproduction rates of *D. melanogaster*. However, it is highly toxic to aquatic organisms [13] and has potential to harm pollinators at field realistic doses [12].

As the options for pest-control diminish, farmers may find hope in the potential of PDI based on the food additive sodium magnesium chlorophyllin to combat smaller semi-translucent invertebrates, such as the various *Drosophila* species. Therefore, the approach presented here could serve as new avenue for farmers to manage invertebrate pests.

**Author contributions** Conceptualization, A.F. and K.P.; methodology, A.F., N.B. and K.P.; validation, A.F., N.B. and K.P.; investigation, A.F.; resources, J.L., M.F. and K.P.; data curation, A.F., statistics: A.F., N.B.; writing—original draft preparation, A.F.; writing—review and editing, A.F., J.L., M.F. and K.P.; visualization, A.F.; supervision, K.P.; project administration, K.P.; funding acquisition, K.P. All authors have read and agreed to the published version of the manuscript.

**Data Availability** Not applicable.

## Declarations

**Conflicts of Interest** The authors declare no competing interest.

**Informed consent** Not applicable.

**Institutional review board statement** Not applicable.

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