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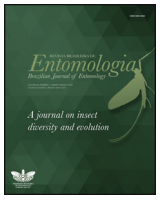

Exposure to Attractive Toxic Sugar Baits containing Piriproxyfen (ATSB-PPF) in surfaces reduces the number of eggs and larvae produced by *Aedes aegypti* (Diptera: Culicidae) females

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ABSTRACT

The significant role of *Aedes aegypti* as a vector of several arboviruses has intensified studies on vector control tools, including Attractive Toxic Sugar Baits containing Pyriproxyfen (ATSB-PPF). PPF is an insect growth regulator (IGR) that has been used to control immature mosquitoes, but it also has direct effects on female reproduction. This study evaluated the effect of physical exposure of *Ae. aegypti* females with ATSB-PPF on fecundity and fertility at different times before and after blood meal. Females were confined in cages impregnated with ATSB-PPF at different concentrations (5, 50, and 500 ppm). One group of females was exposed to the baits 24 and 48 hours Before the Blood Meal (BBM), and in the other group, exposure occurred 24 and 48 hours After the Blood Meal (ABM). The number of eggs laid, larvae, and pupation rate were analyzed. Exposure of *Ae. aegypti* females to ATSB-PPF bait 24 hours after the blood meal was able to reduce the number of eggs laid, especially at the higher concentrations used, but the number of larvae obtained was reduced regardless of the exposure time to ATSB-PPF, i.e., 24 hours before or after the blood meal, or the concentration of PPF used. No changes were observed in the number of pupae from larvae obtained from eggs laid by mosquitoes exposed to ATSB-PPF. Physical exposure of mosquitoes to sugar baits is sufficient to reduce the investigated reproductive parameters of *Ae. aegypti*.

Introduction

The Americas region recorded a total of 4,565,911 cases of dengue, including 7,653 (0.17%) severe cases and 2,340 deaths (case fatality rate of 0.051%) in 2023, the highest record within the historical series. Pan American Health Organization (PAHO) issued an alert regarding the dengue behavior recorded in the first weeks of 2024, highlighting a significant increase in the notification of this disease in several countries in the Americas region, urging Member States to intensify their *Aedes aegypti* Linnaeus, 1762 control actions (the main transmission vector). In some countries like Brazil, there were 455,525 cases of dengue in the first five epidemiological weeks of 2024, i.e., an increase of 218% compared to the last five years for the same period (PAHO, 2024).

According to PAHO (2019), the management of potential breeding sites, the use of larvicides and adulticides, among other means, are an important part of the integrated strategy for the prevention and control of arboviruses in the Americas, but the use of new technologies for vector control has already been considered and evaluated.

Although not yet evaluated for *Ae. aegypti* control by PAHO, Attractive Toxic Sugar Baits (ATSB), a strategy involving the use of sugar as a feeding stimulant for the ingestion of toxic substances, can cause a reduction of more than 90% in the mosquito population, e.g., *Culex pipiens* Linnaeus, 1758 (Müller et al., 2010) and *Anopheles sargentii* Theobald, 1907 (Beier et al., 2012), suggesting their use for mosquito control in the adult stage. Normally, ATSBs contain adulticidal substances, but recent studies suggest that the ingestion of ATSBs can be used to disperse Insect Growth Regulator (IGRs) like pyriproxyfen (PPF) through feces in *Ae. aegypti* (Silva et al., 2021) and *Aedes albopictus* Skuse, 1985 (Scott et al., 2017). Additionally, the exposure of adult females to PPF applied on surfaces may show a reduction or suppression in the number of eggs laid in mosquitoes such as *Ae. aegypti* (Itoh et al., 1994;

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Suttana et al., 2019) and *Anopheles arabiensis* Patton, 1905 (Harris et al., 2013), and in the larval eclosion of *Ae. aegypti* (Sihuincha et al., 2005). However, the effect of the exposure of *Ae. aegypti* females exposed to surfaces sprayed with ATSBs containing PPF was not studied. Therefore the present study investigated whether the exposure of females of this species to surfaces treated with ATSBs containing PPF, before and after blood feeding, during different exposure times (24 h and 48 h) and concentrations of PPF (5, 50, and 500 ppm) was able to alter the fecundity and fertility of adult females of *Ae. aegypti*.

Materials and methods

Ethical aspects

Human blood collection for blood feeding was authorized by the Research Ethics Committee of the Federal University of Rondônia, under protocol number 78543817.9.0000.5300.

Ae. aegypti rearing

A colony of *Ae. aegypti* at the Laboratory of Insect Bioecology (LaBEIn)/Federal University of Rondonia provided the mosquitoes used for experimentation. Female mosquitoes kept in plastic cages (17 cm x 15 cm) were starved for 48 hours and then fed with human blood through an artificial feeder with Teflon membrane (Siria et al., 2018). After 72 hours, a filter paper line plastic container (6 cm x 8 cm) with approximately 100 ml was placed inside the cage for oviposition. The eggs deposited on the filter paper were placed in plastic trays (8.5 x 36.0 x 43.5 cm) containing 1 liter of filtered water. The larvae were separated according to larval instar and fed daily. L1 larvae were fed ground fish food (Tetramin Tropical Flakes ad libitum), and from the L2 instar onwards, they were fed two grains daily of reptile food (Reptolife, Alcon Club). Tray cleaning was performed every 3 days. L4 larvae were transferred to screened plastic 2L pots (17 cm x 15 cm), containing 1 liter of water. Water change was performed every 3 days, and emerging adult mosquitoes were removed using a handheld aspirator and placed in screened cages. Adult mosquitoes were fed with 10% sucrose and kept in cages for 5 days to mate. The laboratory conditions were 27-28°C and 70-80% (RH).

Formulation of sugar baits and application in cages

The baits were prepared as described by Dias et al. (2023). The bait composition consisted of a mixture of concentrated guava juice (Maguary - Britvic Ebba S.A), filtered water, brown sugar (10%), and food coloring (Arcocolor, São Paulo, Brazil) (ASB-control). PPF was added to the ASB to obtain ATSB-PPF at concentrations of 5, 50, and 500 ppm. PPF was extracted from Sumilarv 0.5G® (Sumitomo Chemical Co. Ltd) using hexane.

For cage construction, 500 ml plastic pots (8 cm x 10 cm) were used and covered with tulle on the top. Two milliliters of the baits were spread on the inner wall of the cages and left to dry for 24 hours to prevent mosquito engorgement.

Evaluation of the effect of exposure time (24 h and 48 h) to ATSB-PPF Before Blood Meal (BBM) or After Blood Meal (ABM) on the number of eggs, larvae, and pupation rate of *Ae. aegypti* Females

Fifteen nulliparous females, 5-7 days old, were placed in cages previously brushed with ATSB-PPF or control (ASB). The females were kept in cages containing ASB and ATSB for 24 h. The experiments were performed in quadruplicate and repeated four times.

The exposure of females to the ATSB-PPF was set as follows: one group of females was kept inside cages with ATSB-PPF 24 h or 48 h before the blood meal (BBM), and another group was kept in cages with ATSB-PPF 24 h or 48 h after the blood meal (ABM). The blood meal was performed as described earlier, and from the initial 15 females per cage, 10 fully engorged females were selected and used throughout the experiment. After exposure to the baits and blood meal, the females were individually kept in clean cages, i.e., 200 ml plastic cups (8 cm x 7 cm), lined with filter paper, and 50 ml of water for oviposition, and fed with 10% sucrose solution. After 48 hours of oviposition, the filter paper with eggs was removed for drying, and the number of eggs was counted using a stereomicroscope. After egg counting, the filter paper with eggs was returned to the 200 ml cup, to which 100 ml of water was added, and larval hatching was monitored daily for 7 days, and the number of larvae was recorded. Hatched larvae were transferred to cages of the same size (200 ml plastic cups containing water) according to the treatment and hatching date, fed as described earlier until pupation. The pupation rate was calculates dividing the number of pupae/number of larvae obtained from experiments.

Statistical analysis

The effect of treatments on the variables was evaluated through three-factor ANOVA (concentration, type of exposure, i.e., BBM and ABM, and exposure period, i.e., 24 h and 48 h) and two-factor ANOVA, performed after combining the types of exposure to evaluate the effect of exposure period and concentration on the response variables. Comparisons between groups were evaluated by Tukey's test at a significance level of 5%. All tests were performed using PRISM 10 software (GraphPad Inc).

Results

In general, the mosquito exposure to ATSB-PPF 24 hs in BBM or ABM reduced the average number of eggs (F=40.3; P<0.001) compared to 48hs. Mosquitoes from 24 hs ABM group had a reduction in the average number of eggs, ranging from 45% (5 ppm) to 78% (500 ppm) compared to those exposed to baits for 48 hs ABM (Fig. 1). Overall, only exposure to baits containing 500 ppm of PPF in 24hs for BBM and ABM reduced the number of eggs compared to the control (P=0.011)

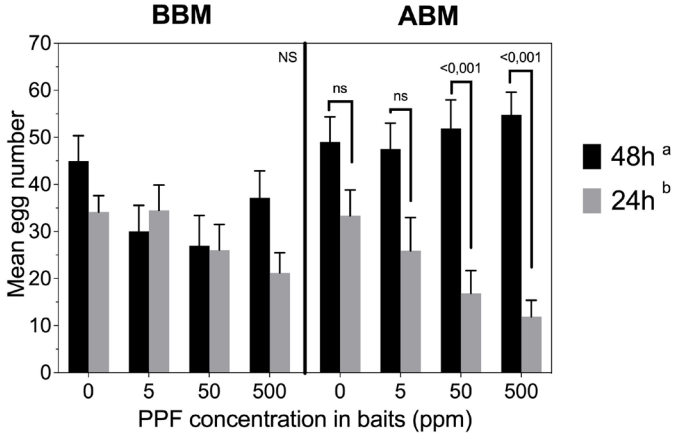

Figure 1 Average number of *Ae. aegypti* eggs laid from females exposed to ATSB-PPF at different concentrations, before (BBM) or after blood meal (ABM), and at different exposure time (24 and 48hs). NS: no statistically significant differences. Different letters indicate significant differences (P<0.001) between exposure times.

Mean larvae number was lower for BBM than ABM (F=5.74; P=0.017). Furthermore, female exposure to all tested concentrations of PPF reduced the number of larvae obtained by 60% compared to the control, except for ABM at 48 hours. Exposure to 24 hours in BBM and ABM reduced the number of larvae by 65% compared to the contact at 48 hours (F=57.1; P<0.0001) (Fig. 2).

The exposure of *Ae. aegypti* females to baits containing PPF at different concentrations, exposure time related to the blood meal did not affect the pupation rate (F=1.01; P=0.36; F=1.56, P=0.21 and F=1.21, P=0.27, respectively) and ranged from 0.74 to 1.0 (Fig. 3).

Discussion

The exposure of mosquitoes to surfaces containing PPF can alter some reproductive parameters of mosquitoes such as the number of eggs and larvae obtained (e.g., Ohba et al., 2013; Harris et al., 2013). However, the results depend on the concentration used, the timing of the exposure in relation to the blood meal, and the mosquito species studied.

The concentrations of PPF in the baits used in this study ranged from 5-500 ppm (0.00003 to 0.003 mg/cm^2), and overall, there was a reduction in the number of eggs laid when mosquitoes were exposed to baits containing 500 ppm (0.003 mg/cm^2). However, we observed that exposure to baits 24 hours after the blood meal (ABM), compared to exposure after 48 hours, was able to reduce the number of eggs at all concentrations.

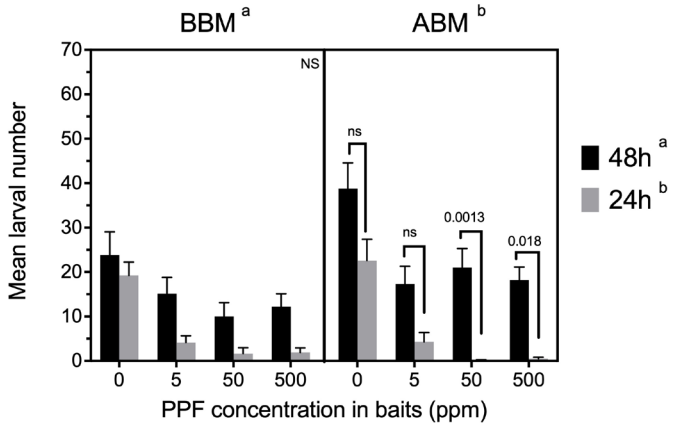

Figure 2 Average number of larvae obtained from *Ae. aegypti* females exposed to ATSB-PPF at different concentrations, before (BBM) or after blood meal (ABM), and at different exposure time (24 and 48hs). NS: no statistically significant differences. Different letters indicate significant differences (P<0.001) between exposure times.

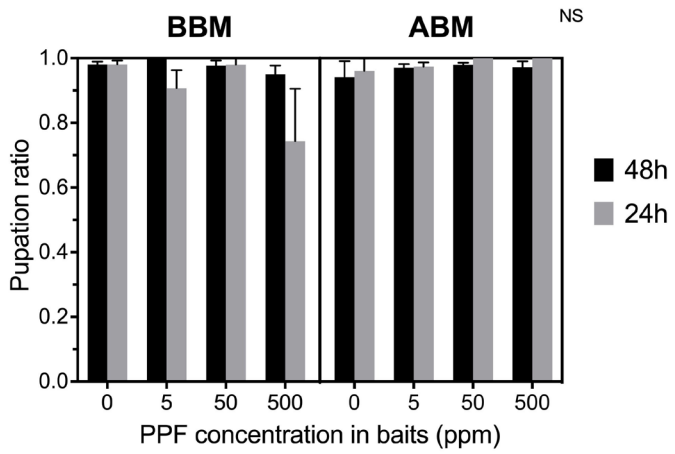

Figure 3 Pupation rate of *Ae. aegypti* pupae from females exposed to ATSB- PPF at different concentrations, before (BBM) or after blood meal (ABM), and at different exposure times. NS: no statistically significant differences.

Exposure with surfaces impregnated with PPF at high concentrations, e.g., 0.1 mg/cm^2, almost 40 times higher than that used in our study, was able to reduce the number of eggs laid by *Ae. aegypti* by over 80% at intervals ranging from 4 days before to 3 days after the blood meal (Itoh et al., 1994). Suttana et al. (2019) used 0.03 mg/cm^2 of PPF, 10 times higher than what we used, and reported a decrease of 60-80% in the number of eggs laid by the same mosquito species after exposure 24 hours before or after the blood meal, but there was no effect on this variable when contact was made 96 hours before or after the blood meal.

Interestingly, concentrations between 0.0003 mg/cm^2 (Sihuincha et al., 2005) or even 0.003 mg/cm^2 (Suttana et al., 2019), as used in this study, did not reduce the fecundity of *Ae. aegypti* as related by these authors, suggesting that the mosquito strain and its status of insecticide resistance affect susceptibility to PPF, e.g., Suttana et al. (2019) reported a lesser effect of PPF on the number of ovipositions and eggs laid by strains of *Ae. aegypti* resistant to insecticides (DDT, permethrin, and deltamethrin). In contrast, this range of PPF concentration was able to reduce the number of eggs laid by other mosquito species, e.g., *Ae. albopictus* (Ohba et al., 2013), *An. arabiensis* (Harris et al., 2013), *Anopheles gambiae* Giles, 1902 and *Culex quinquefasciatus* Say, 1823 (Mbare et al., 2014), after their exposure to impregnated surfaces.

Although the concentration of PPF may be determinant for the effect on mosquito reproduction, the timing of contact, i.e., before or after the blood meal, also affects the results. Some studies suggest that topical application of juvenile hormone analogs (JH) in *Ae. aegypti* causes a complete and permanent blockage in the follicular development of the ovaries 24 hours after the blood meal, but no effect was detected after 36 hours (Judson and Lumen, 1976). However, Patterson (1974) reported that the fecundity of this mosquito species after topical application of JH was reduced at later intervals after the blood meal, i.e., 32 to 40 hours.

In this study, the most robust effects of exposure to PPF present in baits were seen at 24 hours after the blood meal, causing a greater reduction in the number of eggs laid. While a reduction of about 60% in the number of *Ae. aegypti* larvae was obtained for virtually all concentrations tested at contact 24 hours before or after, and at contact 48 hours after. This corroborates other results for *Ae. aegypti* exposed to PPF directly impregnated on surfaces, e.g., contact 24 hours before or after the blood meal reduced the number of eggs laid by *Ae. aegypti* by about 80% and completely inhibited larval hatching (Suttana et al., 2019). Additionally, as mentioned earlier, although PPF did not affect the fecundity of this species, Sihuincha et al. (2005) observed a reduction of 70 to 90% in larval hatching from females exposed to PPF. Similarly, the number of larvae obtained from eggs of mosquitoes exposed to PPF seems to be species-dependent, as Mbare et al. (2014) observed a maximum reduction in larval hatching of only 37% in females of *C. quinquefasciatus*.

Exposure of mosquitoes to surfaces containing higher concentrations of PPF, i.e., 0.035 mg/cm^2, about 10 times higher than the highest concentration we used, resulted in sterilization of females of *An. gambiae* for three gonotrophic cycles (Koama et al., 2015), but concentrations above 500 ppm (0.003 mg/cm^2) were not viable for use in sugar baits for *Ae. aegypti* as they were not ingested (data not shown) when we evaluated the effect on PPF reproduction contained in ATSBs for *Ae. aegypti* (Silva et al., 2021).

It is important to mention that this study aimed to evaluate the effect of the physical contact and not ingestion of ATSB-PPF. However, the exposure period of 24 hours of females to baits in the BBM group, even after drying, resulted in low engorgement of some females, given

the presence of feces at the bottom of the cages. However, in the group of females exposed to baits in the ABM group, no baits were observed in the feces of these mosquitoes, which can be explained by the fact that the females were engorged with blood in this group.

Our results suggests that the mosquito tarsal or other body parts exposure to ATSB-PPF, besides ingestion (Silva et al., 2021), reduces the number of eggs laid and larvae, boosting the effect of the ATSB-PPF.

As related by Suttana et al. (2019), we did not observe any effect of the exposure to ATSB-PPF on the pupation rate of *Ae. aegypti*, nor on other variables such as the number of adults or sex ratio (data not shown). We did not find other studies investigating the pupation rate from larvae obtained from eggs laid by mosquitoes exposed to surfaces containing PPF, suggesting that the effect mainly occurs on the number of eggs laid and larvae obtained.

Hustedt et al. (2020) conducted a systematic review of PPF use for *Aedes* control and suggested that this substance may effectively control these mosquitoes in various environments and in various forms (granules, ULV sprays, fumingants, thermal fogging), but mentions that evidence on autodissemination and other products containing PPF (bednets, paints, candles, ovitraps) are not yet strong and consistent. In fact, the authors did not mention the potential of PPF to reduce the fecundity and fertility of *Aedes* after contact with surfaces or ATSBs sprayed with this substance as a complementary control tool. However, the reduction in fecundity and fertility with ingestion of ATSBs-PPF (Silva et al., 2021) or tarsal contact or other parts of the body with them (this study) suggest that the possibilities of using PPF for the control of this mosquito species can be expanded beyond its regular application. This study was conducted in a laboratory setting, and further research is needed to evaluate the effectiveness of ATSB-PPF baits in field conditions and as part of an integrated pest management (IPM) program that includes other control methods.

Conclusions

The exposure of female *Ae. aegypti* to ATSB-PPF 24 hours after the blood meal was able to reduce the number of eggs laid, especially at the highest concentrations used, but the number of larvae obtained was reduced regardless of the timing of contact with ATSB-PPF, i.e., 24 hours before or after the blood meal, or the concentration of PPF used in the exposure time. No changes were observed in the number of pupae from larvae obtained from eggs laid by mosquitoes exposed to ATSB-PPF.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contribution statement

CVCS Investigation and Original Draft. ACAD Supervision-Equal, Writing – review & editing - Equal. AAS Supervision – Equal, Conceptualization – Lead, Data Analysis – Lead, Writing – review & editing – Equal.

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