Artículos

Biological control of *Aedes aegypti* using *Metarhizium robertsii* (strain CEP 423): impregnated fabrics and sublethal effects on reproductive parameters

Control biológico de *Aedes aegypti* utilizando *Metarhizium robertsii* (cepa CEP 423): telas impregnadas y efectos subletales en los parámetros reproductivos

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Abstract: *Aedes aegypti* (L.) is an urban mosquito and the primary vector of dengue, Zika, yellow fever, and chikungunya in Latin America. Due to the emergence of chemical-insecticide-resistant populations, there is an urgent need for alternative control methods. Biocontrol with entomopathogenic fungi (EPF) is a viable strategy to reduce these mosquito populations. This study examines the use of the Argentine fungal strain *Metarhizium robertsii* CEP 423 for controlling *Ae. aegypti*. Adults were released in small cages with inoculated fabrics (10^6 conidia/cm^2), either white cotton-polyester (40/60 %) or red polyester (100 %) for 24 hours. Survival was significantly reduced compared to controls in both cases. Additionally, a sublethal concentration (5.7 x 10^5 conidia/mL) was tested on female reproductive parameters: number of bites, completed gonotrophic cycles, eggs laid, and egg viability. No statistical differences were found between treated and control adults. In conclusion, *M. robertsii* CEP 423 is effective for the biological control of adult *Ae. aegypti* that come into contact with conidia-treated surfaces. However, sublethal effects were not significant for vector control.
Keywords: Entomopathogenic fungus, Fecundity, Mosquito, Survival, Vector control.
Resumen: *Aedes aegypti* (L.) es un mosquito urbano y el principal vector de enfermedades como el dengue, Zika, fiebre amarilla y chikungunya en América Latina. Debido a la aparición de poblaciones resistentes a los insecticidas químicos, se deben implementar métodos de control alternativos. El control con hongos entomopatógenos (HEP) es una estrategia viable para reducir estas poblaciones de mosquitos. Este estudio examinó el uso de la cepa argentina *Metarhizium robertsii* CEP 423 para el control de *Ae. aegypti*. Se liberaron adultos en pequeñas jaulas con telas inoculadas (10^6 conidios/cm^2), ya sea de algodón-poliéster blanco (40/60 %) o poliéster rojo (100 %) durante 24 horas. La supervivencia se redujo significativamente en comparación con los controles en ambos casos. Además, se

probó una concentración subletal (5,7 x 10^5 conidios/mL) en los parámetros reproductivos de las hembras: número de picaduras, ciclos gonotróficos completados, huevos puestos y viabilidad de los huevos. No se encontraron diferencias estadísticas entre los adultos tratados y los controles. En conclusión, *M. robertsii* CEP 423 es eficaz para el control biológico de adultos de *Ae. aegypti* que entran en contacto con superficies tratadas con conidios. Sin embargo, los efectos subletales no fueron significativos para el control vectorial.
Palabras clave: Control de vectores, Fecundidad, Hongo entomopatógeno, Mosquito, Supervivencia.

INTRODUCTION

Aedes aegypti (L.) (Diptera: Culicidae) is an urban mosquito species highly anthropophilic which lays its eggs in artificial household breeding sites. It serves as the principal vector for arboviruses such as dengue, Zika, yellow fever, and chikungunya in Latin America (Segura et al., 2021). In Argentina, dengue outbreaks have been reported by the Health Ministry in 2009, 2016, 2020, 2023, and 2024. As the dengue vaccine remains largely unavailable, the most effective way to prevent arboviral diseases is through the control of mosquito populations (WHO, 2012; Wilson et al., 2020). However, the widespread use of chemical insecticides against mosquitoes has led to the emergence of resistant populations worldwide (Gray et al., 2018; Wang et al., 2023), including wild populations of *Ae. aegypti* in Argentina which were recently identified as pyrethroid-resistant (Barrera-Illanes et al., 2023, 2024; Gonzalez et al., 2024). Consequently, new strategies are needed to control vector populations and mitigate disease burden.

Biological control of insect pests utilizing entomopathogenic fungi (EPF) has gained increased interest as an environmentally friendly alternative. EPF have the potential for mosquito control by reducing adult and larval survival (Scholte et al., 2004; Evans et al., 2018; Cafarchia et al., 2022). In an urban-vector-control program for *Ae. aegypti*, optimizing the method of conidial application for adults is essential. Pieces of impregnated clothes or nets have been successful as a delivery system for conidia (Farenhorst et al., 2011; Paula et al., 2013, 2018; Carolino et al., 2014; Lee et al., 2023). Moreover, the sublethal effects of EPF on the reproductive parameters of mosquitoes further enhance control efforts by lowering the fitness of individual insects or populations. In females, observed effects include a reduction in host-finding, blood feeding, and fecundity (Scholte et al., 2006; George et al., 2011; Darbro et al., 2012; Blanford et al., 2012; Pelizza et al., 2013), while in males, EPF influence copulation activity and sperm production (Garza-Hernández et al., 2015).

Indigenous fungal strains are good candidates for augmentative or conservative biological control practices (Shah & Pell, 2003). In Falvo et al. (2020), fungal strains of *Metarhizium anisopliae* s.l. (Metsch.) Sorokin (Hypocreales: Clavicipitaceae) native from Argentina were tested against adult *Ae. aegypti* using a direct application method, i.e. spraying a conidial suspension. Among these, CEP 423, recently molecularly characterised (Schuster et al., 2023) as *M. robertsii* Bisch, Rehner & Humber, proved to be the most virulent against adult *Ae. aegypti* under laboratory conditions. This fungal strain is also pathogenic to *Ae. aegypti* larvae (Paixão et al., 2024). Therefore, further research on this fungal strain is crucial, as it holds the potential for developing a locally applicable mycoinsecticide against

this vector pest. The objectives of this study were to evaluate the efficacy of CEP 423-inoculated fabrics, provided as resting sites, for killing adult mosquitoes. In addition, we determined whether a sublethal concentration of *M. robertsii* CEP 423 has any impact on the blood feeding and fecundity of treated females.

MATERIALS AND METHODS

Mosquito origin and rearing

Mosquitoes were reared in the laboratory colony as in Gerberg et al. (1994), at 27±1 °C, 60±10 % relative humidity (RH), and 12/12 h photoperiod. Cardboard papers with eggs were placed in trays (25 x 42 x 7 cm^3) filled with dechlorinated water (3.5 L) for hatching. Larvae were fed with ground rabbit food (Toreanso, Argentina). Pupae were moved to a meshed cage (50 x 50 x 40 cm^3) for adult emergence. As a source of sugar, adults were provided with rehydrated raisins covered in a voile fabric from which they could feed. Females received a weekly supply of chicken blood. Black recipients for oviposition were placed in the cage. These consisted of the bottom of cut PET bottles that contained dechlorinated water (100 mL) and cardboard paper around its surface to collect new eggs.

For the bioassays, adults of 3-5 days old were used. Blood feeding was administered only for the specified assays; otherwise, adults were exclusively fed on a sugar diet. Adults were handled using handheld or mouth aspirators and immobilized with CO_2 when necessary.

Fungal origin and culture

The fungal strain *M. robertsii* CEP 423 was obtained from the Mycological Collection of Entomopathogenic Fungi of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE). It was originally isolated from a soil sample of La Plata, Buenos Aires, Argentina. It was cultured for 14 days on Sabouraud dextrose agar and yeast medium (quarter strength SDAY: 5 g dextrose, 2.5 g peptone, 2.5 g yeast extract, and 20 g agar/L of distilled water) in Petri dishes (100 mm diameter) at 25±1 °C, and 12/12 h photoperiod. Before performing the bioassays, conidial viability was assessed as reported by Lane et al. (1988) with SDAY (quarter strength) medium, and the percentage of germinated conidia was above 90 % for every trial.

Metarhizium robertsii CEP 423-inoculated fabrics

Two types of rectangular fabrics (7 x 5 cm^2) were used for the assays. Variations in colour, composition, and stitch density were as follows: i) white, 60 % cotton and 40 % polyester, 400 holes/cm^2, and

ii) red, 100 % polyester, 320 holes/cm^2. Each one was inoculated with 1 mL (0.5 mL over each side) of a suspension (3 x 10^8 conidia/mL) of *M. robertsii* s.l. CEP 423 in aqueous 0.01 % v/v Tween 80 (polyoxyethylene sorbitan monooleate, Merck, Germany). We used an airbrush sprayer model BD180 (FENGDA, China) to achieve a homogeneous dispersion of the inoculum (10^6 conidia/cm^2). The fabrics used as controls were sprayed with 1 mL (0.5 mL over each side) of aqueous Tween 80, 0.01 % v/v without conidia. Before the beginning of the bioassay, all the fabrics were left to dry for 16 h at 25±1 °C. The viability of the conidia attached to the fabrics was calculated 48 h after the inoculation. To this end, 1 cm^2 of each type of fabric was submerged in 1 mL of Tween 80, 0.01 % v/v, and agitated in a vortex at 2400 rpm for 3 minutes to prepare a conidial suspension of 10^6 conidia/mL. The percentage of germinated conidia was assessed as in Lane et al. (1988) with SDAY (quarter strength) medium amended with chloramphenicol 0.05 % w/v. Three repetitions were performed for each type of fabric.

Survival bioassays with *M. robertsii* CEP 423-inoculated fabrics

Each type of fabric (either treated with *M. robertsii* CEP 423 or untreated) was located inside plastic cages (7 cm height x 7 cm diameter). Per trial, 10 females *Ae. aegypti* were gently released inside each cage which was later closed at the top with a voile mesh and a rubber band. Mosquitoes were left inside the cage for 24 h. Afterwards, they were transferred to a clean plastic cage for 10 days. Raisins and water were offered during the whole process. Survival of individuals was checked daily, and the dead insects were removed and transferred to humid chambers (2 % w/v agar-water in a Petri dish of 60 mm diameter) for up to 7 days at 25±1 °C to observe mycosis over cadavers that confirmed the fungal infection. Each experiment was repeated four times using a total of 40 individuals per treatment.

Effects of a sublethal concentration of *M. robertsii* CEP 423 on *Ae. aegypti* blood feeding and fecundityEffects of a sublethal concentration of *M. robertsii* CEP 423 on *Ae. aegypti* blood feeding and fecundity

Preliminar considerations

1) According to previous research on the virulence of *M. robertsii* CEP 423 against adult *Ae. aegypti,* measured as the lethal concentration (LC) (LC_{90}: 2.2 x 10^8, LC_{70}: 2.3 x 10^7, LC_{50}: 2.4 x 10^6, LC_{30}: 5.7 x10^5, and LC_{10}: 4 x 10^4 conidia/mL) (Falvo et al., 2020), we define a sublethal dose as the higher concentration that produced a survival curve which was not significantly different to the

control using Kaplan-Meier analysis. In this case, we used the equivalent of LC_{30}.

2) It is well studied that larval nutrition has a direct relationship with adult body size which has been suggested to influence mosquito´s fitness (Yan et al., 2021). Thus, special care was taken to rear larval stages with equal amounts of food (0.05 g grounded rabbit food per tray (18 x 28 x 6 cm^3) with 200 eggs and 1.5 L dechlorinated water). Moreover, at the end of every trial, when females died, one of their wings was removed, and its length was measured as the distance from the axial incision to the apical margin excluding the fringe of the scales. The wing length was measured under a microscope (BX41, Olympus, Japan) using a scaled lens with 40X magnification. In this way, we checked the homogeneity among the individuals´ body sizes.

Bioassays

In order to evaluate the sublethal effects of *M. robertsii* CEP 423 in fecundity, mosquitoes were sexed at the pupal stage and separated individually until the adults emerged. Virgin males and females were used in the trials. The mosquitoes were inoculated according to Falvo et al. (2020). Briefly, adults were sprayed with the inoculum through a net using an airbrush, in groups of ten insects, either males or females. The treated adults were inoculated with a conidial suspension (0.5 mL, 5.7 x 10^5 conidia/mL) of *M. robertsii* CEP 423 in aqueous Tween 80, 0.01 % v/v, whereas the control individuals were sprayed with 0.5 mL aqueous Tween 80, 0.01 % v/v without conidia.

After inoculation, both male and female mosquitoes were released in the same cage (7 cm height x 7 cm diameter) to allow for copulation. One couple of a treated male and a treated female, or a control couple (a non-treated male and a non-treated female), was collocated per cage. Mating occurred in the next three days. Afterwards, females were provided with human blood twice a week, every three days. Sucrose solution 10% w/v was permanently offered to each couple. Thirty-six couples were used per treatment. Each cage contained a small ovitrap (4.5 cm height x 3 cm diameter) with 15 mL of water with a filter paper around where fully engorged females were able to lay their eggs. Upon completion of one gonotrophic cycle, starting at the blood meal and ending with egg-laying (Clements, 1992), the ovitraps were substituted by new clean ones. Papers that contained eggs were removed and allowed to dry for 8 h. After 2 days of storage under insectary conditions, these were immersed in trays with dechlorinated water for egg hatching. We recorded (per female along its whole life): the number of bites, the number of completed gonotrophic cycles, the number of eggs laid,

and the viability of the eggs. The assays continued until every adult died.

Data analysis

The median survival times (ST_{50}) were determined by the Kaplan–Meier method. Significant differences in adult longevity between treatments and controls were assessed by the Log-rank test. Comparisons between means (conidial viability over the fabrics, the number of bites per female, the number of completed gonotrophic cycles, the number of eggs laid, and wing length) were performed by a two-tailed t-test. Normality was checked by the Shapiro-Wilks test and homoscedasticity by the F-test for homogeneity of variances. If required, data were arcsine-square root transformed. The viability of the eggs was estimated from the hatching proportion and analysed by a proportion test with chi-square approximation. Calculations and figures were performed using the statistical software R version 4.3.1. (R Core Team, 2023).

RESULTS

Efficacy of *M. robertsii* CEP 423-inoculated fabrics in the survival of adult *Ae. aegypti*

Adults exposed to *M. robertsii* CEP 423-inoculated fabrics showed a significant reduction in survival compared to the controls (Fig. 1, white-cotton-polyester fabrics: $\chi 2= 16.3$, $p< 0.0001$; red polyester fabrics: $\chi 2= 19.3$, $p< 0.0001$). Survival of adults exposed to control fabrics remained above 77 %, thus the ST_{50} could not be calculated. No significant differences in survival were observed between adults exposed to the two types of treated fabrics ($\chi^2= 0.6$, $p= 0.4$). For adults exposed to the white cotton-polyester fabric, survival was 32.50±12.50 %, with a ST_{50} (95 % C.I.) of 6.5 (5-10) days. Among the dead mosquitoes, 37.5±10.31 % exhibited mycosis after incubation in humid chambers. Similarly, for the red-polyester fabric, survival was 37.50±14.93 %, with a ST_{50} (95 % C.I.) of 9 (6-10) days, and 45.00±17.09 % of the cadavers displayed signs of mycosis.

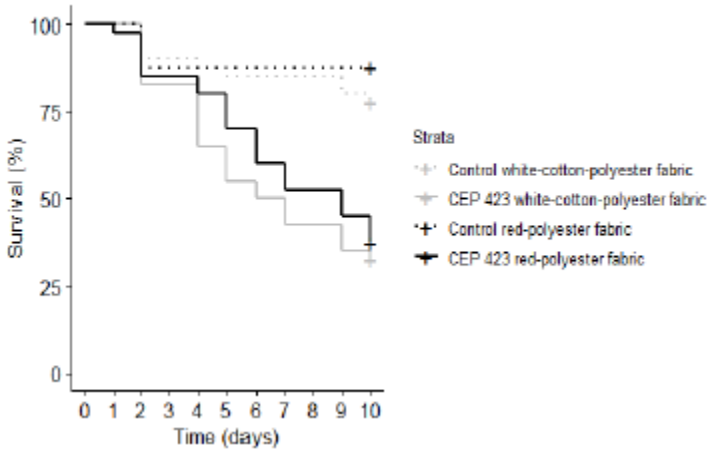

Figure 1.

Kaplan-Meier survival curves of adult *Aedes aegypti* exposed to either *Metarhizium robertsii* CEP 423-inoculated fabrics (10^6 conidia/cm) or untreated fabrics (control).

Crosses at the end of the curves represent censored data (surviving adults at the end of the experiment) (log-rank test, $p < 0.05$).

No significant differences were found in the conidial viability concerning the type of fabric used (t= -0.36, p= 0.74). The percentages of germinated conidia attached to each type of fabric were 80.00±9.56 % and 84.44±10.23 for the white-cotton-polyester fabric and the red-polyester fabric, respectively.

Effects of a sublethal concentration of *M. robertsii* CEP 423 on *Ae. aegypti* blood feeding and fecundity

As expected for a sublethal concentration, no significant differences were found between control and treated adults ($\chi 2$= 0.2, p= 0.7 for females; $\chi 2$= 2.7, p= 0.1 for males), but there was a significant difference in survival between sexes, with males living shorter than females ($\chi 2$= 42.5, p< 0.001 for control female vs control male; $\chi 2$= 56.7, p< 0.001 treated female vs treated males; Fig. 2). The ST_{50} ($C.I._{95}$) values were 41.5 (38-59), 19.0 (13-25), 40.5 (36-53), and 12.5 (8-18) days for control female, control male, treated female, and treated male, respectively.

In both the control and treated groups, 35 out of 36 females (n) completed at least one gonotrophic cycle (Table I). Two females, one from each group, mated and bit but did not lay eggs, resulting in an uncompleted gonotrophic cycle. The maximum number of completed gonotrophic cycles was eight for control females (n= 1) and six for treated females (n= 2). No significant differences were observed in the mean number of gonotrophic cycles completed: 2.97±0.33 for control females, and 2.40±0.21 for treated females (t= 0.33, p= 0.7). Each control female laid between 20-35 eggs per gonotrophic cycle, and, from those, only 20-35 % of the eggs hatched.

Similarly, each treated female laid between 25-41 eggs per gonotrophic cycle, and only 2-36 % were viable.

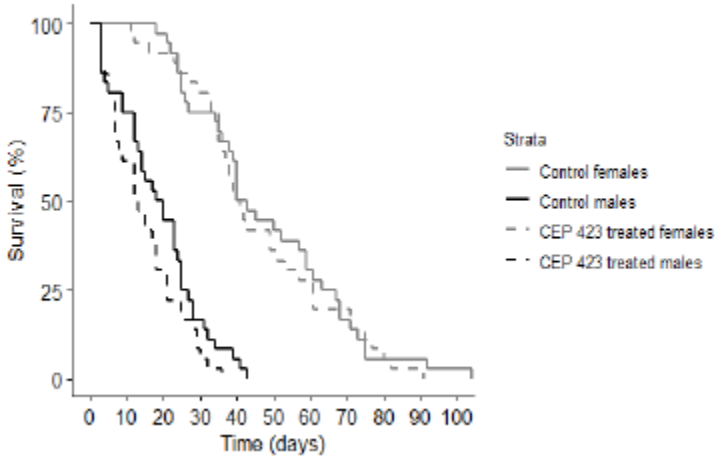

Figure 2.

Kaplan-Meier survival curves of adult male or female *Aedes aegypti* treated with a sublethal concentration (5.7 x 10^5 conidia/mL) of *Metarhizium robertsii* CEP 423 or untreated (control) (log-rank test, p< 0.05).

	Control Females			Treated Females		
GC	n	Eggs/posture	Viable eggs	n	Eggs/posture	Viable eggs
1	35	33.71±3.15	33.40±5.62	35	26.31±2.25	36.17±6.29
2	26	32.23±3.27	33.83±6.42	28	25.86±2.36	30.22±5.93
3	17	29.06±3.01	41.26±7.94	21	33.24±4.68	32.19±7.18
4	13	35.77±3.59	35.88±8.63	10	30.30±3.44	32.91±10.08
5	8	20.75±4.75	35.00±14.26	6	32.83±3.46	26.92±13.57
6	5	30.60±4.30	21.80±13.96	2	41.50±3.50	2.50±0.50
7	2	27.00±1.00	20.00±1.00		-	-
8	1	25.00	0.00		-	-

Table I.

Effect of a sublethal concentration (5.7 x 10^5 conidia/mL) of *M. robertsii* CEP 423 on the fecundity of *Ae. aegypti* females.

Eggs laid per female ($\bar{x}$±S.E.) and the percentage of hatched (viable) eggs per posture (%±S.E.) in each gonotrophic cycle (GC)

n: is the number of females that completed each GC

Furthermore, no significant differences were found in the wing length between control and treated females (t= 1.18, p= 0.2) (Fig. 3a). Along its life, the mean number of bites per female was 4.83±0.42 for controls, and 5.22±0.43 for treated (t= -0.65, p= 0.5) (Fig. 3b). Also, there were no significant differences in the total number of eggs laid per female, 93.75±10.85 for controls, and 81.28±10.97 for CEP 423 treated (t= 0.81, p= 0.4) (Fig. 3c). The percentage of hatched eggs was 33% in both cases (χ2= 0.14, p= 0.7) (Fig. 3d).

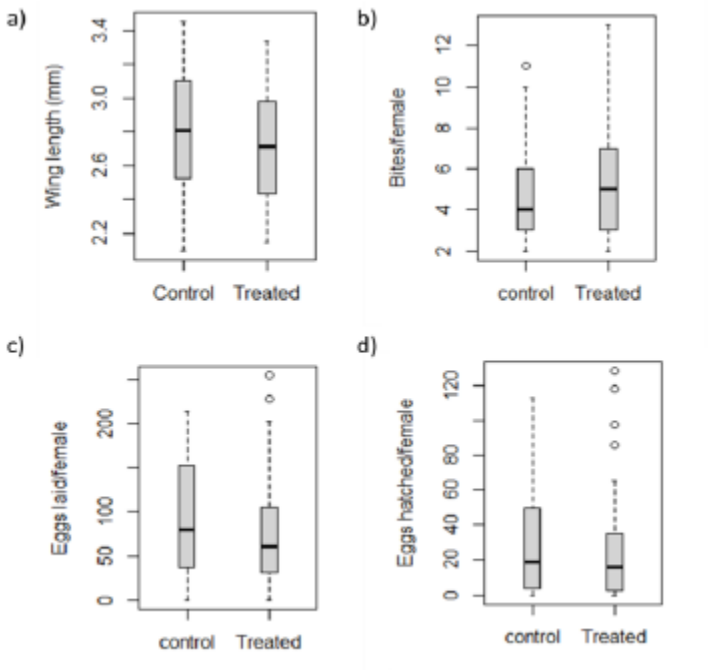

Figure 3.

a) wing length of the *Aedes aegypti* females used in the assays, b)-d) sublethal effects of *Metarhizium robertsii* CEP 423 in: the total number of bites, eggs laid, and eggs hatched per *Ae. aegypti* female along its life, respectively.

For all parameters, t-test p> 0.05.

DISCUSSION

Entomopathogenic fungi are a viable alternative to control mosquito populations (Scholte et al., 2004; Evans et al., 2018; Cafarchia et al., 2022). *Metarhizium robertsii* CEP 423 is an indigenous fungal strain from Argentina, an endemic area of Dengue fever, which is pathogenic to *Ae. aegypti* (Falvo et al., 2020; Paixão et al., 2024). We discuss here the implications of this fungal strain to control adult *Ae aegypti*. Due to mosquitoes´ flight activity and the need for the fungal propagules to contact the insects´ cuticle to kill them, the application methods of conidia may become a challenge and are key determinants in the efficacy of EPF as biocontrol agents. In previous reports, the strategy of inoculating a surface where adults could land, rest, and make contact with the inoculum successfully reduced the survival time of the treated adults compared to the controls (Mnyone et al., 2010a; Farenhorst et al., 2011; Paula et al., 2013; Lee et al., 2023). In agreement, our results demonstrate that fabrics of cotton and polyester, of everyday domestic use, could be used as a conidial delivery methodology to control adult *Ae. aegypti.* Our experimental design might seem contrived at first, but it is worth noting that the fabrics used here did not have any formulation

containing adjuvants or other compounds that may enhance conidial bioactivity or attraction of adults. Still, the results are promising. We expect that the addition of oil and lures (Paula et al., 2018; Lee et al., 2023) to *M. robertsii* CEP 423-inoculated fabrics could increase the efficacy of controlling adult *Ae. aegypti*. The treated fabrics could be used indoors, which is relevant for a domestic species such as *Ae. aegypti*.

Regarding sublethal effects, previous studies have shown that fungal infections reduced the responsiveness of *Anopheles stephensi* mosquitoes to host odour cues (George et al., 2011), and *Beauveria bassiana* (Balsamo) Vuillemin has been found to reduce blood feeding and egg production in *Ae. aegypti* (Darbro et al., 2012). However, in both studies, the concentrations used were high enough to also reduce the lifespan of treated adults compared to controls. In this study, we evaluated a sublethal concentration (LC_{30}) of *M. robertsii* CEP 423 that was useful for assessing reproductive parameters such as mating (not directly measured, but copulation was observed), blood feeding, fecundity, and progeny, as the lifespan of control and treated adults was similar. However, this concentration had no significant impact on the reproduction of *Ae. aegypti* suggesting it was too low to induce these side effects or it could have been quickly counteracted by the insects' immune system.

Notably, under the evaluated conditions, *M. robertsii* CEP 423 did not impair host detection or biting, which explains the lack of effect on egg production, a parameter that is influenced by the amount of protein in the diet (Scholte et al., 2006). The absence of an impact on blood feeding and the number of completed gonotrophic cycles is particularly relevant to dengue transmission, as it suggests that *M. robertsii* CEP 423 should only be used at lethal concentrations to prevent the spread of the virus. Furthermore, with respect to oviposition, further research is needed. Laboratory assays have limitations because *Ae. aegypti* exhibit skip oviposition behaviour, so future studies should consider adding multiple oviposition sites for females.

Additionally, it has been demonstrated that engorged females of *Ae. aegypti* (Paula et al., 2011) or *Anopheles gambiae* (Mnyone et al., 2010b) are less susceptible to lethal concentrations of EPF for a temporary period following a blood meal, suggesting that nutritional status may play a role in the immunological response. In our study, females lived longer than males, which could be attributed to nutrient transfer from male to female after mating (Villarreal et al., 2018). They were inoculated with *M. robertsii* CEP 423 while virgin and unfed, but afterward, they were allowed to mate and feed on blood *ad libitum* every three days. We hypothesize that this may have provided females with nutrients that enhanced their immunity, mitigating detrimental effects on fertility. The insect's immune system likely intervened early in the fungal infection process, potentially

preventing the expression of mycotoxins that would otherwise have negatively impacted the reproductive parameters tested.

Overall, this study provides evidence to integrate the fungal strain *M. robertsii* CEP 423 to sanitary programs and to arrange new strategies to control *Ae. aegypti*. Under laboratory conditions, this fungal strain had lethal activity on adults by indirect inoculation of conidia. However, as far as we could observe, it did not provide sublethal suppression of the vector population.

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Marianel L. FALVO, Eliana ORDOQUI, Juan J. GARCÍA, Claudia C. LÓPEZ LASTRA

Biological control of *Aedes aegypti* using *Metarhizium robertsii* (strain CEP 423): impregnated fabrics and sublethal effects on reproductive parameters

Control biológico de *Aedes aegypti* utilizando *Metarhizium robertsii* (cepa CEP 423): telas impregnadas y efectos subletales en los parámetros reproductivos

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