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Caffeine effect on mortality and oviposition in successive generations of *Aedes aegypti*

Efeito da cafeína sobre a mortalidade e oviposição em gerações sucessivas de *Aedes aegypti*

ABSTRACT

OBJECTIVE: Previous experiments showed that caffeine blocks the development of *Aedes aegypti* (*Diptera, Culicidae*) in the larval stage, consequently inhibiting the production of adults. This study aimed at obtaining data suggestive of caffeine resistance by these mosquitoes.

METHODS: Experiments were carried out in successive generations to assess adult production from eggs laid in previous generation and oviposition rate in every generation using 200 and 500 µg/mL caffeine. Tap water was used as control. Experiments were conducted in the city of São José do Rio Preto, Southeastern Brazil between 2002 and 2005. Statistical tests consisted of exploratory data analysis and smoothing algorithms.

RESULTS: Increasing reduction in productivity of adults occurred among generations at both caffeine concentrations but the differences were only significant at 200µg/mL caffeine. As for the oviposition rate, there was a decrease in the mean number of eggs per female over generations at both caffeine concentrations.

CONCLUSIONS: There was no evidence of caffeine resistance over generations. The study results corroborate caffeine as an alternative as an important *Ae. Aegypti* control agent to avoid resistance.

KEYWORDS: *Aedes*, growth & development. Larva, growth & development. Oviposition. Caffeine, toxicity. Insect control.

RESUMO

OBJETIVO: Experimentos anteriores mostraram que a cafeína bloqueia o desenvolvimento de *Aedes aegypti* (Diptera, Culicidae) na fase larval, inibindo conseqüentemente a produção de adultos. O objetivo do estudo foi obter dados que pudessem sugerir desenvolvimento de resistência dos mosquitos à cafeína.

MÉTODOS: Foi avaliada a produção de adultos em gerações sucessivas, a partir de ovos produzidos na geração anterior e a taxa de oviposição em cada geração, utilizando meios contendo cafeína a 200 e 500 µg/ml e água de torneira proveniente de poço artesiano como controle. Os experimentos foram conduzidos em São José do Rio Preto, entre 2002 e 2005. Nos testes estatísticos foram utilizados a análise exploratória de dados e algoritmos de alisamento.

RESULTADOS: Ocorreu redução crescente da produção de adultos, nas duas concentrações, ao longo das gerações, mas apenas no experimento a 200 µg/ml os dados foram estatisticamente significantes. Quanto à oviposição, a análise dos

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números mostra redução crescente e acentuada na média de ovos por fêmea, no experimento tratado.

CONCLUSÕES: Não houve evidência de resistência ao longo das gerações devido ao tratamento com cafeína. Os resultados encontrados podem reforçar a indicação da cafeína como uma alternativa aos principais agentes de controle do *Ae. aegypti* atualmente usados, contra os quais os mosquitos têm desenvolvido resistência.

DESCRITORES: *Aedes*, crescimento e desenvolvimento. Larva, crescimento e desenvolvimento. Oviposição. Cafeína, toxicidade. Controle de insetos.

INTRODUCTION

There are at least 447 species of insects and mites reported as having developed resistance to one or more chemical and/or organic groups in literature. Multiresistant species showing several mechanisms of resistance to different chemical groups are common. Resistance is defined by the World Health Organization (WHO) as "the development, in an organism, of the capacity to tolerate doses of toxics that are lethal for the majority of the individuals in a normal population (considered susceptible) of the same species" (WHO, 1957 *apud* Scott,[9] 1995). The development of alterations in the susceptibility of a population to toxics is due to specific selection of pre-adapted individuals over several generations of exposure (Scott,[9] 1995).

The level of resistance in populations of vector insects such as *Aedes aegypti* is dependent on the volume and frequency of insecticides applied. The rapid development of resistance in mosquitoes is favored by some characteristics including their short life cycle (11 to 18 days at 26ºC for *Ae. aegypti*,) and abundant progeny (about 70 to 150 eggs are laid per *Aedes* female) (Georghiou & Taylor,[2] 1977; Hemingway & Ranson,[3] 2000).

Aedes aegypti is among insects of medical interest as it is a vector of human disease viruses, including dengue, dengue hemorrhagic fever and yellow fever. Currently, the main *Ae. aegypti* control agents are pyrethroids and organophosphorous insecticides. However, the most used insecticides are mainly organophosphates, which are also highly toxic for vertebrates, even in relatively small doses. In addition, they are chemically unstable and require new applications at short intervals. Resistance to insecticides has already been detected in *Ae. aegypti* in several parts of the world, including Brazil. The city of São José do Rio Preto, Southeastern Brazil, has been subjected to intensive chemical control over the years but *Aedes aegypti* has shown reduced susceptibility (Macoris et al,[7] 1999; Macoris et al,[8] 2003).

Caffeine (CAF – 1,3,7-trimethylxanthine; $C_8H_{11}N_4O_2$) is a natural component of coffee, tea, guarana and chocolate. It has been used in successive research projects aimed at using it as an auxiliary agent in the control of *Ae. aegypti*. CAF showed the capacity to block larval development of these mosquitoes, causing their death at this stage (Laranja et al,[6] 2003).

The present study aimed at obtaining information suggestive of the development of *Ae. aegypti* resistance to CAF. Adult production from eggs and oviposition rate were studied over generations.

METHODS

Aedes Aegypti eggs, larvae and pupae (Diptera: Culicidae) were collected at breeding sites in the city of São José do Rio Preto, State of São Paulo, Southeastern Brazil, between 2002 and 2005. They were grown in the laboratory, producing the eggs for the study experiments.

The experiments involved treatment of *Ae. aegypti* at two different CAF concentrations: 200 and 500 mg/mL. These concentrations were chosen based on previous data showing weak and strong effect on larval mortality, respectively (Laranja et al,[6] 2003). At each concentration, eggs were allowed to develop in a 500 mL aqueous CAF solution. Adults of every generation were put into a cage and their eggs were used to produce the next generation in a medium with the same CAF concentration. In the experiment with 200 mg/mL CAF solution, 200 eggs laid in the previous generation gave rise to the next one, while in the treatment with 500 mg/mL CAF the number of eggs in the treated tests had to be increased because, as mentioned before, mosquitoes are strongly affected at this concentration (Laranja et al,[8] 2003). In both experiments, tap water was used as control.

When larvae reached L3 instar, 0.08 g of fish food was added to the medium. At pupal stage, they were

transferred to cages to develop into adults. Adults were fed with an 8% aqueous sugar solution. Females were blood fed (required for oocyte maturation) once a week using an immobilized mouse placed inside the cages for about 1.5 hour each time.

Eggs were laid on a filter paper strip placed inside a half-filled water glass at the level of water surface. Glasses (tumblers) containing a cone of filter paper and filled with water were also put inside the cages to increase relative air humidity. The filter paper containing eggs was removed two to three times a week (depending on the presence of eggs). Eggs were counted, dried for 24 hours and used (up to 30 days) to produce the next generation.

Statistical tests involved exploratory data analysis and smoothing algorithms for proportions of adults produced in each new generation (Resistant Smooth – Velleman,[10] 1980; Velleman & Hoaglin,[11] 1981). Z-test for two proportions comparison and polynomial regression statistics to adjust smooth data on adult production over generations in Experiment I (treatment with 200 mg/mL CAF solution) were also used (Zar,[12] 1999).

RESULTS

The number and proportion of male and female adults produced from eggs and the number of eggs laid in each generation up to the last female died or adult production ceased for both experiments are shown in Table 1. Environment characteristics for the development of *Ae. aegypti* such as room temperature and humidity of growth media during the experiments are shown in Table 2.

Experiment I was interrupted in the 10th generation due to the fact that nineth generation eggs were unable to develop into adults. In this generation, the control experiment produced only three females and four males, while the treated test, prepared with a total of 189 eggs obtained in F8, did not produce any adults.

The productivity of adults from 200 eggs, used to start each new generation in Experiment I, was variable for both the treated and control experiments in the different generations. In the control generations, the productivity ranged between 11% and 63.5% and in the treated generations, it ranged between 3.2% and 52%.

Table 1 - Productivity of adults and egg laying capacity over generations of *Aedes aegypti* under treatment with 200 µg/mL CAF (experiment I), 500 µg/mL CAF (experiment II) and controls. Percentages in the two last columns indicate the difference of oviposition in the treatment compared to controls. Southeastern Brazil, 2002-2005.

Exp	Gen	Media	N of eggs initials	F	M	Total	Productivity of adults (%)	Total of eggs produced	Productivity of eggs per female
I	F0	Water	200	23	10	23	11.5	1,012	44.0
		CAF 200	200	12	12	24	12.0	1,200 (+19%)	100.0 (+127%)
	F1	Water	200	40	7	47	23.5	5,654	141.3
		CAF 200	200	52	22	74	37.0	2,321 (-59%)	44.6 (-68%)
	F2	Water	200	65	62	127	63.5	8,366	128.7
		CAF 200	200	53	51	104	52.0	5,614 (-33%)	105.9 (-18%)
	F3	Water	200	27	11	38	19.0	1,362	50.4
		CAF 200	200	12	15	27	13.5	901 (-34%)	75.1 (+49%)
	F4	Water	200	30	39	69	34.5	4,546	151.5
		CAF 200	200	38	37	75	37.5	1,472 (-68%)	38.7 (-74%)
	F5	Water	200	22	30	52	26.0	2,909	132.2
		CAF 200	500	6	10	16	3.2	732 (-75%)	122.0 (-8%)
	F6	Water	200	49	37	86	43.0	4,073	83.2
		CAF 200	200	25	23	48	24.0	579 (-86%)	23.2 (-72%)
	F7	Water	200	12	10	22	11.0	2,041	170.1
		CAF 200	200	18	24	42	21.0	295 (-86%)	16.4 (-90%)
	F8	Water	200	21	18	39	19.5	6,066	288.9
		CAF 200	200	3	5	8	4.0	189 (-97%)	63.0 (-78%)
	F9	Water	200	3	4	7	3.5	-	-
		CAF 200	189	0	0	0	0	-	-
II	F0	Water	200	23	28	51	25.5	1,693	73.6
		CAF 500	330	15	18	33	10.0	692 (-59%)	46.1 (-37%)
	F1	Water	600	5	5	10	1.7	412	82.4
		CAF 500	619	5	2	7	1.1	221 (-46%)	44.2 (-46%)
	F2	Water	220	20	30	50	22.7	3,001	150.0
		CAF 500	217	9	7	16	7.4	556 (-81%)	61.8 (-59%)
	F3	Water	567	6	2	8	1.4	1,333	222.2
		CAF 500	556	4	1	5	0.9	537 (-60%)	134.2 (-40%)
	F4	Water	230	30	16	46	20.0	2,042	68.0
		CAF 500	224	8	4	12	5.4	79 (-96%)	9.9 (-85%)
	F5	Water	200	22	12	34	17.0	2,206	100.3
		CAF 500	79	3	1	4	5.1	159 (-93%)	53.0 (-47%)
	F6	Water	362	17	14	31	8.6	1784	104.9
		CAF 500	159	4	0	4	2.5	-	-

CAF: Caffeine

Table 2 - Room temperature and humidity of the *Aedes aegypti* growth media for the time each generation lasted in the treatment with 200 mg/mL CAF (Experiment I), 500 mg/mL CAF (Experiment II) and control (water). Southeastern Brazil, 2002-2005.

Exp.	Gen.	Time lasted	Temperature (°C)			Humidity (%)		
			Min.	Max.	Mean	Min.	Max.	Mean
I	F0	02/22/02 - 03/28/02	25.9	32.7	29.3	55.0	86.0	70.5
	F1	03/21/02 - 05/07/02	25.2	31.1	28.1	46.0	86.0	66.0
	F2	04/24/02 - 07/16/02	19.3	31.9	25.6	46.0	81.0	63.5
	F3	06/20/02 - 09/26/02	19.3	28.7	24.0	37.0	84.0	60.5
	F4	10/07/02 - 11/28/02	25.1	32.6	28.8	30.0	77.0	53.5
	F5	01/08/03 - 02/25/03	25.3	33.5	29.4	51.0	89.0	70.0
	F6	02/20/03 - 05/22/03	20.9	33.3	27.1	49.0	85.0	67.0
	F7	06/11/03 - 09/15/03	19.9	27.6	23.7	34.0	77.0	55.5
	F8	09/23/03 - 12/19/03	23.4	35.1	29.2	32.0	75.0	53.5
	F9	01/06/04 - 03/26/04	25.2	31.7	28.4	42.0	84.0	63.0
II	F0	09/24/03 - 01/05/04	23.4	35.1	29.2	32.0	75.0	53.5
	F1	01/06/04 - 04/07/04	25.2	31.7	28.4	42.0	84.0	63.0
	F2	04/02/04 - 09/01/04	17.5	30.0	23.7	33.0	82.0	57.5
	F3	09/22/04 - 12/15/04	22.8	32.6	27.7	27.0	76.0	51.5
	F4	11/12/04 - 02/10/05	25.0	33.0	29.0	38.0	81.0	59.5
	F5	12/21/04 - 04/05/05	25.0	32.9	28.9	32.0	82.0	57.0
	F6	03/01/05 - 07/06/05	21.0	31.0	26.0	46.0	82.0	64.0

In light of the numbers, the productivity of adults was higher in the control than in the treatment tests in five generations (F2, F3, F5, F6 and F8). In F0 and F4, the productivity of adults in the control and treated experiments was equivalent, and in F1 and F7, the productivity of adults in CAF 200 was higher than in the control. The highest proportion of adults was obtained in F2 for both control and treatment tests. However, in the treatment of F5 generation the initial number of eggs (produced in F4) had to be increased to 500 in order to obtain enough adults able to produce eggs to begin a new generation.

Experiment II (treatment with 500 mg/mL CAF) was carried out similarly to experiment I, only at a different CAF concentration. This experiment lasted up to the seventh generation because the eggs of the treated produced only few adult females (and males were not produced). The number of eggs starting each new generation was variable due to low productivity of adults in the treated (i.e., high larval mortality rate) and low egg laying capacity of females produced at this CAF concentration.

Charts including the proportion and smooth proportion of adults versus generation for Experiments I and II are shown in Figure 1. The different levels of the lines corresponding to control and treatment in the proportion charts (Figure1A and 1C) reflect larval mortality due to CAF effect. However, using the test of two independent proportions and normal approximation, the results showed no significant difference between treatment and control in every generation for both experiments.

As to the difference of productivity of adults between generations, the values for treatment and control were not significant in Experiment II. For example, the highest difference of smooth proportions was obtained between F0 and F1 in the treatment (0.03; Z=0.50, p=0.31) as well as in the control (0.03; Z=0.28, p=0.39), indicating that the productivity of adults remained the same along generations. However, in Experiment I, there was a decreasing trend of adult productivity over generations in the treatment and in the control . The model of polynomial regression grade 2 adjusted to data showed statistic significance for differences between generations. Considering Y= productivity of adults and X= generation, the resulting quadratic regression equation for control is:

$Y=0.19+0.05X-0.006X^2$; $R^2=82.1\%$, $F=16.03$, $p=0.002$;

and for the treatment is:

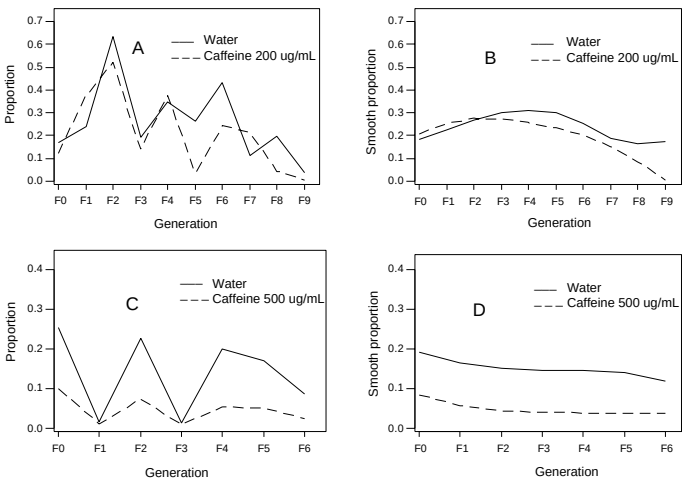

Figure 1 - Proportmon and smooth proportion charts for productivity of adults. A, B = Experiment I; C, D = Experiment II. Southeastern Brazil, 2002-2005.

$Y=0.21+0.04X-0.007X^2$; $R^2=99.7\%$, $F=1129.0$, $p=0.000$.

An additional observation in plots of Figure 1 A, C (Experiments I and II, respectively) is the alternated increase and decrease of adult production in the successive generations. Such variation occurred simultaneously in the treatment and control tests.

In Experiment I, the total productivity of eggs in F1 was higher in the treatment than in the control tests. From F1 onwards, the treated tests produced a total number of eggs much lower than controls. Such difference was greater from F4 to F8, and egg production in the treated of F8 was 97% lower than in the control. Mean egg productivity per female in F0 and F3 was higher in the treatment but, in the remaining generations, this mean was higher in the control. In the three last generations (F6, F7 and F8), oviposition rate per female in the control exceeded the treated by 72%, 90% and 78%, respectively.

In Experiment II, the total number of eggs and mean egg productivity per female were also lower in the treatment tests. In the control, the total number of eggs ranged from 412 to 3,001, and in the treatment, from 0 to 692, while the number of eggs per female varied from 68 to 222.2 in the control, and from 9.9 to 134.2 in the treatments.

Charts of productivity of eggs are shown in Figure 2 for both experiments and tests. Although statistical tests could not be applied in this case, the greater differences between control and treatment in each new generation (for Experiments I and II) and between generations (mainly for Experiment I) are adequate to evaluate the results.

DISCUSSION

Because the development of resistance to insecticides is one of the greatest problems affecting the success of vector control programs, it is also important to obtain information concerning resistance to possible alternative control agents. Bti, produced from toxins of *Bacillus thuringiensis ssp. israelensis*, which is currently the most widely used alternative control agent, has already showed resistance in some sites (Ffrench-Constant et al,[1] 2004). Caffeine (CAF), which was previously used in biological tests with *Ae. aegypti*, confirmed its efficacy as an auxiliary agent in the mosquito control. It blocks mosquito development at larval stage, inhibiting the production of adults at variable percentages, depending on CAF concentration used (Laranja et al,[6] 2003).

As to the productivity of adults in each new generation, statistical tests showed no significant difference between treatment and control in both Experiments but showed significantly reduced productivity in the tests of Experiment I as the generations progressed. Differences between generations seen in Experiment II were not significant. The fact that treatment and control tests remain constant or similarly decreased throughout generations shows that CAF is not promoting resistance detectable by production of adults. It is remarkable the occurrence of alternated decrease and increase of adult productivity in successive generations observed in the charts of Figure 1A, C, for treated and control tests. This observation needs further study.

While the production of adults in the treated and control tests have shown a statistically significant tendency to remain the same or to decrease as the generations progressed, the number of eggs laid in each new generation was differently affected in treatment tests when compared with the control tests. Although statistical tests could not be applied in the analysis of this characteristic, the differences are considerable, showing a reduction up to 90% in the mean number of eggs per female when compared with the controls, especially in the most recent generations. This suggests that, instead of promoting resistance, the impaired effect of CAF increases in succeeding generations.

Temperature and humidity do not seem to have affected the study results. Mosquito generations produced under established conditions showed different results (for example, in the experiment using CAF 200 temperature and humidity in the F4 generation were the same as those in F8 but adult productivity and oviposition rate were different). Besides, in several cases, control and treated tests in the same gen-

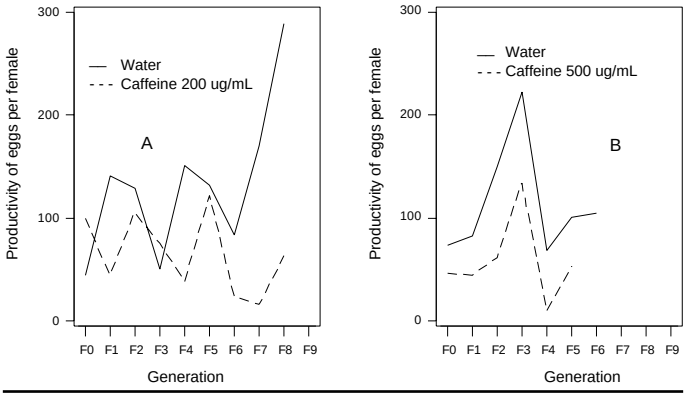

Figure 2 - Charts of mean productivity of eggs per female in every generation. A = Experiment I; B = Experiment II. Southeastern Brazil, 2002-2005.

eration (consequently in the same environment conditions) also yielded different results.

Although the possibility that CAF can promote an increase in insect resistance over time in nature could not be discarded, the present results obtained in laboratory did not corroborate this assumption. CAF increasingly affected the production of adult descendants and oviposition rate in successive generations, a finding corroborating those obtained by Itoyama et al[5] (1998) in *Drosophila prosaltans*. In this organism, progeny productivity studied over 10 generations decreased in a dosage-dependent manner in every generation. At lower doses, the normal productivity of progeny was recovered when treatment was stopped but not at the highest doses used. In addition, tests aimed at studying the egg-laying capacity of *D. prosaltans* grown in a control culture medium and media containing CAF showed that this characteristic also decreased according to the CAF concentration used, from 22% at 50 mg/mL to 87% at 1,500 mg/mL (Itoyama & Bicudo,[4] 1992).

Previous and present data on effect of caffeine treatment may recommend this agent as an alternative control of *Aedes aegypti*.

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