Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos

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In Brazil, decades of dengue vector control using organophosphates and pyrethroids have led to dissemination of resistance. Although these insecticides have been employed for decades against Aedes aegypti *in the country, knowledge of the impact of temephos resistance on vector viability is limited. We evaluated several fitness parameters in two Brazilian* Ae. aegypti *populations, both classified as deltamethrin resistant but with distinct resistant ratios (RR) for temephos. The insecticide-susceptible Rockefeller strain was used as an experimental control. The population presenting the higher temephos resistance level, Aparecida de Goiânia, state of Goiás (RR_{95} of 19.2), exhibited deficiency in the following four parameters: blood meal acceptance, amount of ingested blood, number of eggs and frequency of inseminated females. Mosquitoes from Boa Vista, state of Roraima, the population with lower temephos resistance level (RR_{95} of 7.4), presented impairment in only two parameters, blood meal acceptance and frequency of inseminated females. These results indicate that the overall fitness handicap was proportional to temephos resistance levels. However, it is unlikely that these disabilities can be attributed solely to temephos resistance, since both populations are also resistant to deltamethrin and harbour the* kdr *allele, which indicates resistance to pyrethroids. The effects of reduced fitness in resistant populations are discussed.*

Key words: *Aedes aegypti* - insecticide resistance - temephos - fitness

Aedes aegypti (L., 1762) is a mosquito with wide geographic distribution, predominantly in tropical and subtropical regions (Jansen & Beebe 2010). The females feed preferentially on humans and are strongly associated with the urban environment (Gubler 2002, Ponlawat & Harrington 2005, Siriyasatien 2010). This species is considered the main dengue vector, both its global distribution and the number of dengue cases having expanded since the 1950s (Mackenzie et al. 2004).

Insecticides still play a major role in the control of this mosquito, especially the organophosphate (OP) temephos which was the sole larvicide recommended for use in drinking water for a long period (Chavasse & Yap 1997). In Brazil, this pesticide has been employed since 1967 against *Ae. aegypti* larvae and its use was intensified after the 1986 dengue outbreak (Braga & Valle 2007a). Control of *Ae. aegypti* in the country was accomplished with OPs until 2000-2001, when pyrethroids were introduced (da-Cunha et al. 2005, Montella et al. 2007).

Vector control strategies based on intense and frequent insecticide applications result in increased frequency of resistance in a given population, a situation that culminates with the impairment or even complete failure of vector control. Resistance to temephos, as well as to other insecticide classes, such as pyrethroids, has already been detected in several Brazilian *Ae. aegypti* field populations (Macoris et al. 1999, Lima et al. 2003, Braga et al. 2004, da-Cunha et al. 2005, Montella et al. 2007). Both OP and pyrethroid insecticides act on the insect central nervous system. OPs bind to the enzyme acetylcholinesterase, while pyrethroids act on voltage-gated sodium channels.

Main resistance mechanisms include modifications in the insecticide target site, which can hamper or prevent insecticide binding. Pyrethroids, for example, keep the sodium channels across axons in the open conformation, resulting in repetitive nerve impulses leading to paralysis, an effect known as "knockdown". Pyrethroid-resistant insects exhibit modified sodium channels and are referred to as "knockdown resistant", or *kdr* (Martins & Valle 2012). Acetylcholinesterase hydrolyses the neurotransmitter acetylcholine, removing it from the synapse and interrupting impulse propagation. In susceptible insects, OP binding to this enzyme impedes its function, resulting in continuous transmission of nerve impulses (Hemingway & Ranson 2000, Ranson et al. 2004).

Another major resistance mechanism is activation of insect xenobiotic detoxification pathways, also known as metabolic resistance. Three groups of enzymes are involved in this process: esterases, glutathion-s-transferases and mixed function oxidases (Hemingway & Ranson 2000).

Resistant insects are supposed to exhibit a great adaptive advantage in an environment exposed to frequent or continuous insecticide pressure. Nevertheless, insecticide resistance is related to an energetic cost that can influence mosquito biology in the field. According to Roush and McKenzie (1987), fitness costs can be considered a

Financial support: IOC-FIOCRUZ, MS/SVS, PRONEX-Rede Dengue/MCT, CNPq, DECIT/SCTIE/MS, FAPERJ
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Received 14 November 2011
Accepted 12 April 2012

consequence of trade-offs between the allocation of energy underlying insecticide resistance mechanisms and insect fitness. As a result, when insecticide use is interrupted, resistant individuals tend to be less competitive compared to susceptible individuals, leading to decreasing frequencies of resistant insects over time. However, in certain situations, resistance can be unrelated to development or reproduction. Moreover, resistance can even display a positive effect, resulting in an adaptive advantage under field conditions (Rivero et al. 2010).

Several parameters of insect biology can be affected by pesticide resistance, such as development time, adult longevity, behaviour, reproduction and the immune system (Berticat et al. 2002, 2004, Rivero et al. 2010). Once altered, these aspects have the potential to influence both the dynamics of insecticide resistance dissemination and the relationship among vectors and the parasites they transmit. To address this correlation, the present study evaluated a series of life table parameters in two natural *Ae. aegypti* populations showing distinct levels of resistance to the OP temephos.

MATERIALS AND METHODS

Mosquitoes - Two populations of Brazilian *Ae. aegypti* (Fig. 1), Boa Vista, state of Roraima and Aparecida de Goiânia, state of Goiás, were chosen according to their levels of resistance to OP temephos, a major larvicide adopted for control of this mosquito. The Boa Vista (BVT) F2 and Aparecida de Goiânia (APG) F1 mosquitoes used in the assays were derived from eggs collected in 2007 and 2008, respectively. Mosquitoes from the Rockefeller (Rock) strain, which served as a reference lineage for both susceptibility and vigour, were also analysed as the control group (Kuno 2010).

Mosquito rearing - To synchronise development, eggs were allowed to hatch during 1 h. Groups of 1,000 first instar larvae were then transferred to plastic basins (33 x 24 x 8 cm) containing 1 L dechlorinated water and 1 g cat food (Friskies®, Purina, São Paulo, SP) and were maintained in a biological oxygen demand (BOD) incubator at 26ºC. When rearing proceeded until the adult stage, food was replaced every three days. Adult mosquitoes were maintained in an insectary at 26 ± 1ºC under 80 ± 10% relative humidity (rh).

Bioassays - Temephos resistance levels were evaluated in larvae from both populations through dose response bioassays (WHO 1981). In each assay 10 insecticide concentrations prepared with Temephos PESTANAL® (Sigma-Aldrich Brazil, São Paulo, SP) were tested. For each concentration, there were four replicates, each with 20 third instar larvae in 100 mL solution. Lethal concentrations (LCs) were calculated *via* probit analysis (software Polo-PC, LeOra Software, Berkeley, CA) (Raymond 1985). Resistance ratios (RR_{50} and RR_{95}) were obtained by dividing the LC of the field population (BVT or APG) by the equivalent LC from the Rock strain.

Deltamethrin resistance was also evaluated *via* the WHO (1998) protocol with slight modifications using impregnated papers. Adult females, one to three days old, non blood fed, were exposed to a deltamethrin diagnostic dose (3.65 mg/m^2), previously calibrated with Rock mosquitoes. It corresponded to twice the minimal dose resulting in total mortality of Rock strain after a 1 h exposure and 24 h recovery, as recommended for this assay. Each test consisted of three replicates using deltamethrin-impregnated paper plus one control, without the insecticide. For each replicate, 15-20 females were used.

Molecular assays - The allele-specific polymerase chain reaction (PCR)-based genotyping strategy was applied to investigate the frequency of the Val1016Ile *kdr* substitution in the *Ae. aegypti* voltage-gated sodium channel gene (*AaNa$_V$*) in BVT and APG populations. The protocol for genomic DNA extraction as well as the PCR conditions and subsequent analysis were according to Martins et al. (2009).

Adult longevity - Groups of 15 males and 15 females from each field population and the Rock strain were transferred to small carton cages (8.5 cm diameter and 8.5 cm high) and fed *ad libitum* with a 10% sugar solution. Mortality of each gender was recorded daily.

Blood meal acceptance and amount of ingested blood - Adult females three to five days old, collected in a cage containing males, were put in contact with a ketamine-anaesthetised guinea pig (Hawk & Leary 1995) for 30 min and the number of females that successfully fed on blood was recorded. To determine the amount of blood ingested, we calculated the difference between the average weights of three groups each of 10 non-fed and

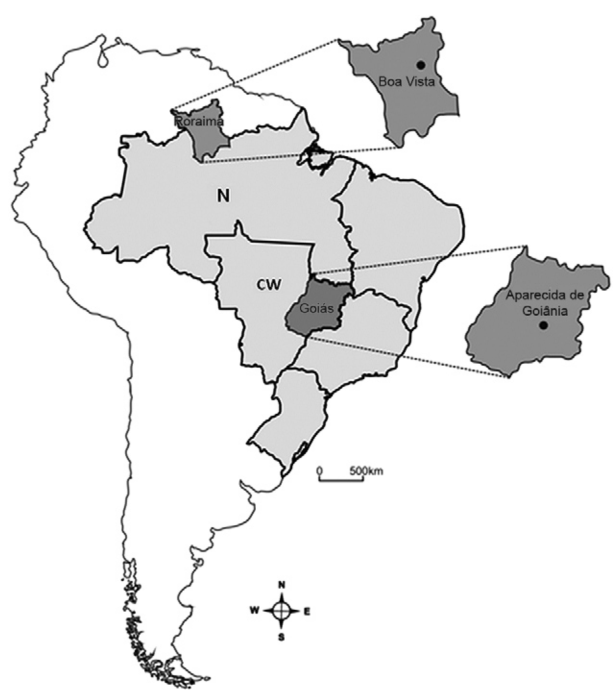

Fig. 1: Brazilian map indicating regions, states and the geographical location of *Aedes aegypti* populations under evaluation. The municipality of Boa Vista is located in the state of Roraima, in the North (N) Region, and Aparecida de Goiânia in the state of Goiás, in the Central West (CW) Region of Brazil.

10 blood-fed females, weighed on an analytical balance (APX-200, Denver instrument), as performed elsewhere (Belinato et al. 2009).

Egg laying - Three days after the blood meal, individual females were placed in inverted Petri dishes with the lid internally lined with a filter paper soaked with dechlorinated water (Valencia et al. 1996) and kept at 26ºC in a BOD incubator. After 24 h, females were removed and three parameters were recorded: the number of ovipositing females, the number and viability of eggs.

Frequency of inseminated females - For each population, 15 groups consisting of three females and one male each, all of which were two to three days old, were maintained together in transparent 50 mL Falcon tubes. All the specimens employed in this assay were reared individually since the pupa stage, in order to ensure the virginity of the adults. After three days, female spermathecae were dissected and the presence of spermatozoids was assessed with the aid of an optic microscope (Nikon Biophot, 200X).

Statistical analysis - All the experiments described herein were repeated at least three times. Data obtained for each parameter evaluated were compared using *t* tests or χ^2 analysis, as indicated in the results, except for longevity data that were subjected to Kruskal-Wallis followed by Dunn's Multiple Comparison Test. Graph-Pad Prism version 5.0 for Windows was adopted for all analyses (GraphPad Software, San Diego California USA) (graphpad.com).

RESULTS

Insecticide resistance status of field populations - Temephos produced a dose-response effect over the Rock strain and the two evaluated field populations. Table I shows LCs and resistance ratios obtained by the probit analysis. The APG population exhibited both higher temephos resistance ratio and greater heterogeneity. Nevertheless, both APG and BVT mosquitoes presented a high resistance status against this OP. In Brazil, *Ae. aegypti* populations with temephos RR_{95} above 3.0 are considered resistant and are subject to insecticide substitution (Ministry of Health, unpublished observations).

Resistance to the pyrethroid deltamethrin was also evaluated. Qualitative assays, performed with the deltamethrin diagnostic dose, detected 11.7% mortality in APG mosquitoes and 37.6% in BVT. According to WHO (Davidson & Zahar 1973), mortality levels below 80% in these assays are indicative of resistance. Additionally, the *kdr* mutation Val1016Ile was detected in both populations, as depicted in Table II.

Adult longevity - Statistical comparisons of female and male survivorship were performed arbitrarily on the 30th and 40th days following adult emergence. When each gender was compared, no differences in the longevity were noted among APG, BVT and the Rock strain on both days (Kruskal Wallis followed by Dunn's multiple comparison test, $p > 0.05$). In all cases, including in the Rock strain, females survived slightly longer, male attaining total mortality when 4.5% of females were still alive (Fig. 2A, B).

Blood meal - Blood meal acceptance was significantly different between field populations and the Rock strain (Fig. 3A). In the latter, 96% (86/90) of the females were able to feed. Significantly fewer insects from BVT (73/90) ($\chi^2_{0.05,1} = 9.11$; p = 0.0013) and APG (69/90) ($\chi^2_{0.05,1} = 13.42$; p = 0.0001) accepted the blood meal. However, no differences between field populations were noted ($\chi^2_{0.05,1}$ = 0,53; p = 0.2325).

When the amount of ingested blood was compared, that ingested by the APG population, which showed a higher level of temephos resistance, was significantly lower (by approximately 15%) compared to either the Rock strain ($t_{0.05(1),11}$; p = 0.008) or BVT mosquitoes ($t_{0.05(1),16}$; p = 0 .0087) (Fig. 3B). The amount of blood ingested by BVT females was 5% lower on average compared to Rock females, though this difference was not significant ($t_{0.05(1),11} = 1.253$; p = 0.1181).

Number and viability of oviposited eggs - A relationship was observed between the volume of blood ingested by each population and the number of eggs. APG females laid significantly fewer eggs (81 ± 30) than Rock (103 ± 19, $t_{0.05\,(1),79} = 4.096$; p < 0.0001) and BVT females (104 ± 17, $t_{0.05\,(1),79} = 4.393$; p < 0.0001) (Fig. 4). The average number of eggs per APG female was 21% lower than for Rock. By contrast, no significant differences were found between the quantity of eggs laid by Rock and BVT females ($t_{0.05\,(1),114} = 0.2226$; p = 0.4121). The

TABLE I

Susceptibility status of two Brazilian *Aedes aegypti* field populations, Boa Vista (BVT) and Aparecida de Goiânia (APG), to the organophosphate temephos, the sole larvicide employed in the country since 1967

Population	LC_{50} (ug/L)	RR_{50}	LC_{95} (ug/L)	RR_{95}	Slope
Rock	2.1	-	4.1	-	6.2
BVT	8.6	4.1	30.4	7.4	4.4
APG	32.9	15.7	78.7	19.2	3.1

the reference strain Rockefeller (Rock) was used as the susceptibility control. LC: lethal concentration; RR: resistance ratio.

TABLE II

Frequency of *AaNa$_v$* Val1016Ile *kdr* mutation in two Brazilian *Aedes aegypti* populations

Population	APG (F2)	BVT (F3)
1016Ile allelic frequency[a]	0.293	0.067
1016Ile/Ile genotypic frequency[a]	0.103	0.033

a: 29 and 30 individuals from respectively Aparecida de Goiânia (APG) and Boa Vista (BVT) were evaluated.

viability of eggs was comparable for the three strains evaluated, all of which hatched at rates greater than 90% ($t_{0.05\,(1)}$; $p > 0.05$).

Frequency of inseminated females - At the end of the three-day mating period, 98% of the Rock males had inseminated their three available females (Fig. 5). The remaining 2% inseminated only two females. In contrast, there was a great reduction in the number of females inseminated by the field males. Only 54% of the BVT males successfully inseminated their three females, whereas 7% did not inseminate any female. The plight was even more pronounced for the APG males, among which only 7% successfully inseminated their three females and the majority (68%) did not inseminate any female.

DISCUSSION

Presently the main *Ae. aegypti* control strategy relies upon chemical insecticides. In Brazil, OPs were widely adopted for the control of both larvae and adults of this mosquito, resulting in resistance dissemination throughout the country. In 2001, pyrethroids started to be employed against adults by the Brazilian dengue control program (Braga & Valle 2007b). However, the dissemination of pyrethroid resistance was soon verified (da-Cunha et al. 2005), initially as a consequence of metabolic resistance (Montella et al. 2007) and later as a mutation in the insecticide target site (Martins et al. 2009). It is well known that in many cases one of the effects of insecticide resistance is the impairment of a series of mosquito fitness parameters. We investigated, in two field *Ae. aegypti* Brazilian populations, several viability parameters in order to evaluate the effect of resistance on vector fitness.

In this study, a slight difference in longevity was noted between males and females within each strain, but not between strains. There is great diversity among the results of previous reports assessing insecticide resistance and longevity, particularly with regard to the insecticide class or culicid species addressed. This may be explained in part by differences in methodology. Deltamethrin selection of *Ae. aegypti* or *Culex pipiens pallens* under laboratory conditions for nine (Martins et al. 2012) or 12 (Li et al. 2002) generations, respectively, was found to result in decreased longevity. In both cases, survival assays were performed with a sugar solution provided *ad libitum*. In contrast, Hardstone et al. (2010) verified that *Culex quinquefasciatus* female adults resistant to the pyrethroid permethrin survived longer than susceptible ones when sustained with sugar. However, the authors did not find any differences in survival between males and females or between susceptible and resistant strains when adults were kept exclusively with water. In the latter case, the parameter evaluated was not longevity, but tolerance to starvation, with deprivation of energy resources derived from the immature stages. Agnew et al. (2004) reported greater vulnerability to starvation for three *Cx. quinquefasciatus* strains homozygous for the *ester[1]*, *ester[4]* or *Ace-1[R]* allele, all of which are related to

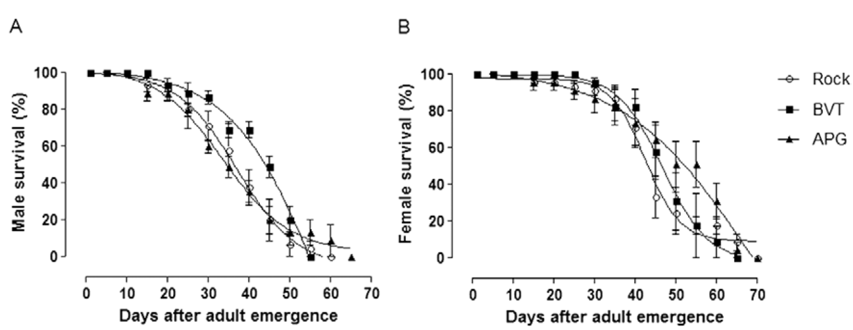

Fig. 2: non linear regression curves showing the longevity, registered every five days, of males (A) and females (B) from Rockefeller strain (Rock) and two field populations, Aparecida de Goiânia (APG) and Boa Vista (BVT).

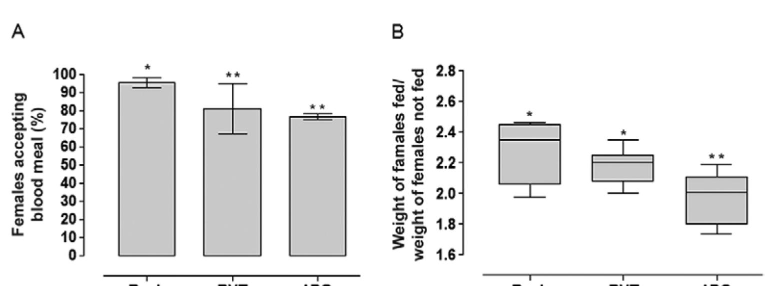

Fig 3: rate of blood meal acceptance (A) and amount of ingested blood (B) by Rockefeller (Rock), Aparecida de Goiânia (APG) and Boa Vista (BVT) *Aedes aegypti* females. Asterisks above columns mean significant differences ($p < 0.05$).

resistance to OPs. Adult starvation tolerance was also evaluated in an *Ae. aegypti* strain selected with *Bacillus thuringiensis* var. *israelensis* toxins. In this case, no differences were apparent between the selected group and the control group (Paris et al. 2011).

Under our experimental conditions, fewer field population females accepted the blood meal when compared to the Rock strain. Additionally, females from Aparecida de Goiânia, the population with a higher temephos resistance level, ingested 15% less blood than Rock females and, consequently, laid 21% fewer eggs. This is a consistent result because the amount of blood ingested by culicids is directly related to the number of eggs deposited (Clements 1992). A similar situation was observed by Martins et al. (2012) working with females after pyrethroid selection. To our knowledge, there are no previous reports for culicids dealing specifically with insecticide resistance influence over the acceptance of blood meal.

Kumar et al. (2009) studied the effect of deltamethrin selection on the fitness of an *Ae. aegypti* field population, with both larvae and adults. The number and viability of eggs laid per female were evaluated in the course of three gonotrophic cycles. A similar investigation was conducted by Kumar and Pillai (2011) with *Cx. quinquefasciatus*. In both cases, no significant differences were noted in the number of blood-fed females between control and insecticide-selected groups. However, in all cases, when compared to controls, a higher number of pyrethroid selected females accepted the blood meal upon the first offer. Nevertheless, pyrethroid-selected females from both species laid fewer eggs than control females. Although the amount of ingested blood was not evaluated, the number of eggs laid is an indirect measurement of this parameter (Clements 1992) and it is likely that pyrethroid-selected females consumed lower amounts of blood. In all cases, the viability of eggs laid by deltamethrin-selected females was also lower than that of susceptible females. Accordingly, deltamethrin-resistant *Cx. pipiens pallens* females laid fewer eggs than susceptible females, though in this case, viability differences were not noted (Li et al. 2002). Likewise, we did not find differences in egg viability for APG or BVT populations when compared to the Rock strain.

One of the most affected parameters was the mating efficiency of each population. After three days, almost all Rock males (98%) successfully inseminated their three available females. In contrast, the performance of males from field populations was much lower; only 54% of BVT and 7% of APG males inseminated their three females. Mating efficiency was inversely proportional to the temephos resistance ratio. Using a different approach, Berticat et al. (2002) demonstrated that OP resistance significantly affects the insemination rate of *Cx. pipiens,* resistant males being less competitive. Therefore, the majority of females were inseminated by susceptible males.

Although OPs and pyrethroids have been adopted in Brazil for the control of *Ae. aegypti* for more than four decades, there are few studies regarding the effects of these insecticides on vectorial capacity, particularly in field populations. Different aspects of insect biology, such as their development, reproduction or longevity, can be affected by insecticide resistance; the extent of these effects can depend not only upon the type and amount of insecticide used and the frequency of its application, but also on the resistance mechanism involved. Field populations can be exposed to different insecticides as a result of domestic or agricultural applications. Insect field populations can also be subjected to eventual non-integrated insecticide use when different disease vectors coexist in a given locality. Hence, perturbation of a given vector biology parameter can result from different selected mechanisms operating simultaneously. Therefore, the fitness cost related to resistance will ultimately depend upon the previous insecticide exposure of each vector population.

Although it is certain that insecticide resistance can alter vector biology, evaluation of individual parameters by different authors can reveal conflicting results. These differences may be attributed to peculiarities of the various vector species with respect to the effects of distinct insecticides or insecticide classes, differences in the genetic background of field populations leading to activation of a variety of potential resistance mechanisms or even to variations in the methodologies adopted in the evaluation of these effects. Moreover, it is important to take into account that insect fitness aspects are generally evaluated under optimal laboratory conditions. Therefore, it is expected that the extent of the effects analysed is underes-

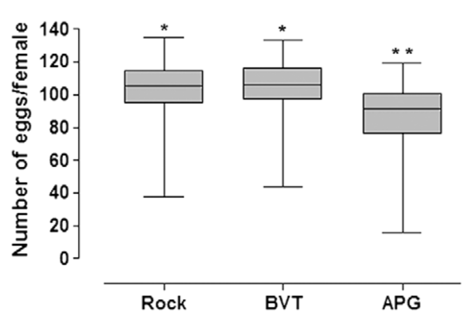

Fig. 4: median number of eggs laid by Rockefeller (Rock), Aparecida de Goiânia (APG) and Boa Vista (BVT) *Aedes aegypti* females. Asterisks mean significant differences (*: $p < 0.05$; **: $p < 0.01$).

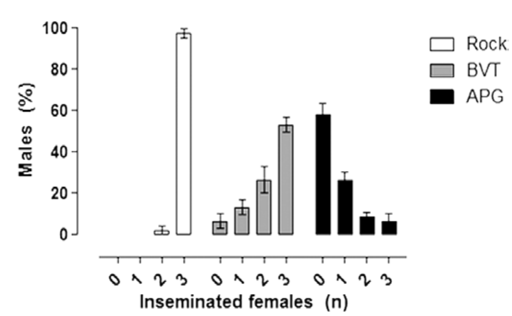

Fig. 5: mating effectiveness of Rockefeller (Rock), Aparecida de Goiânia (APG) and Boa Vista (BVT) *Aedes aegypti* specimens under confined conditions. In all cases, tubes containing one virgin male and three virgin females were kept for three days. Spermathecae of all females were then examined for the presence of sperm.

timated in the laboratory, where insects are not exposed to more stringent conditions, such as abrupt temperature changes, limited nutrition and predation. In contrast, in the field, insecticide resistance-fitness trade-offs can lead to more severe consequences (Kishony & Leibler 2003, Hardstone et al. 2009). Additionally, different alleles related to resistance can affect fitness positively or negatively, depending upon various interactions and the environment to which insects are subjected (Berticat et al. 2008).

It must be noted that both populations evaluated in this study were also resistant to the pyrethroid deltamethrin and that *kdr* mutations in the target site of this insecticide, i.e., the voltage-gated sodium channel, were detected. Previous reports suggest that these mutations spread rapidly among insect field populations (Garcia et al. 2009, Martins et al. 2009). In *Ae. aegypti*, this is likely related to the small impact of *kdr* on fitness, as determined through evaluations conducted in an insecticide-free environment under optimal laboratory conditions (LPBO Souza, unpublished obervations). According to our results, resistance to the OP temephos is directly related to the decreased viability and reproductive performance of the field populations compared to the Rock control strain. However, since the strains tested originated from the field, it is likely that other mechanisms involved in resistance to insecticides and *kdr* mutations may be contributing to the effects observed on fitness.

Because the application of chemical insecticides still plays an important role in the control of several insect vectors, a detailed analysis of the effect of resistance on the biology of these species can directly contribute to the development of novel control strategies as well as to the management of resistance in natural vector populations.

ACKNOWLEDGEMENTS

To Gilberto Couto Alcântara, for technical assistance, to Heloisa Maria Nogueira Diniz (Serviço de Produção e Tratamento de Imagem/IOC), for help with the figures, to Dr José Bento Pereira Lima, for critical evaluation of the bioassays, and to Mitchell Raymond Lishon (UCLA), for English revision.

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