Investigation of isoenzyme *a*-esterase in *Aedes aegypti* from two municipalities of Mato Grosso

Cristina Márcia de Menezes Butakka*
Leiliane dos Santos Novais Siqueira*
Fabiana Aparecida Caldart Rodrigues**
Rosina Djunko Miyazaki***
Sandra Mariotto ****
Lenicy Lucas de Miranda Cerqueira***
Walkiria Shimoya Bittencourt*

Abstract

Esterases are groups of enzymes that increase the mechanism of action of insect vectors in their metabolic activity while under environmental pressure. The aim of this study was to analyze the level of *a*-esterase expression in *Aedes aegypti* populations in the municipalities of Cuiabá and Várzea Grande, MT as a way to contribute to vector research. Samples were collected through ovitraps in each municipality and allele results were analyzed by factorial ANOVA between months, locations and seasonal phase. The alleles of 385 *Ae. aegypti* individuals demonstrated the highest expressions during the ebb period, significantly between months ($F_{7,377}=6.89$; $p<0.01$) and between sites ($F_{1,383}=11.01$). Expressed and superexpressed alleles decreased in Mar/2016, during the "peak water" period and resumed from May/2016 to Nov/2016. Regarding the frequency, expressed alleles increased in Várzea Grande, during the flood period with the highest precipitation. There was an increasing tendency of the expressed alleles with precipitation and the frequency values between Oct/2015 (30%±88) and Feb/2016 (89±55%); meanwhile, for the superexpressed alleles, this occurred during the ebb period (42±91%). Várzea Grande specimens reached a higher frequency of expressed alleles (75±41.36%), but there was a reduction of overexpressed alleles in both municipalities ($F_{2,13}=12.39$; $p<0.01$). The results obtained in this study indicate that the esterase isoenzyme method was sensitive enough to detect variations in allele frequency in natural *Ae. aegypti* populations, which implies an increased metabolic activity over the period examined.

Keywords: Mosquitoes, ovitraps, esterases.

INTRODUCTION

Aedes aegypti Linnaeus, 1762, the mosquito vector of the Zika-, chikungunya-, urban yellow fever- and dengue-causing viruses, is today one of the major public health problems in many regions[1]. There are difficulties in controlling it due to the movement of people in different places, which eventually contributes to the dispersal of arboviruses spreading across different countries[2,3,4,5].

There is an assumption that this species was introduced in Brazil in the colonial period, between the 16th and 19th centuries, during the slave trade. Although the species is not normally found in areas above 1,000 meters altitude, its presence has already been detected at heights of more than 2,000 meters in India

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*Universidade de Cuiabá – UNIC. Cuiabá/ MT, Brasil.
**Universidade do Estado do Mato Grosso - UNEMAT. CELBE – Cáceres/MT, Brasil.
***Universidade Federal de Mato Grosso - UFMT. Cuiabá, MT, Brasil.
****Instituto Federal de Educação, Ciência e Tecnologia de Mato Grosso. Cuiabá – MT, Brasil.
E-mail: cristinabutakka@yahoo.com.br

and Colombia[6].

Chikungunya epidemics have been reported since the 1960s[7] and the Zika virus was first isolated in 1947 in a monkey (*Macaca mulatta*) inhabiting the Ugandan forest, which was used as a "sentinel" in a yellow fever study[6]. The emergence of Zika occurred in 2014 in Brazil and has been the subject of epidemiological studies that seek to understand the dynamics of viruses in the human organism[8]. In Brazil, in recent years, this virus has already caused severe epidemics in a large number of Brazilian municipalities[3].

The scientific investigation of the populations of *Ae. aegypti* would increase the knowledge concerning the prevention of problems that directly affect the health of the human population. This fact shows the need to establish monitoring networks to control the vector through environmental or mechanical management strategies, which are the most used by the municipalities[4]. These networks seek to eliminate the mosquito by monitoring spaces and environments so that favorable points for its reproduction are not produced. Another practice is to use appropriate containers, often in the form of traps[5], whose presence, under field conditions, identifies the presence of the vector and reduces the eggs that would be deposited on the breeding grounds[9].

The knowledge of the expression of enzyme groups such as genetically distinct non-specific esterases[10], classified as carboxylesterases and cholinesterases[11,12,3], makes it possible to identify the mechanism of action of vectors by increasing their metabolic activity which make them resistant to chemical control[13,14]. In the organism of *Ae. aegypti*, esterases play a role in the central nervous system as they develop their ability to detoxify their body from molecules of chemical compounds (such as insecticides), which result in their deactivation. As a result, the followings generations of the insects become more resistant to these compounds[14]. Ae. aegypti performs the detoxification process using esterases (α- and ß-) or glutathione S-transferases (GSTs) present in its body[15].

In Brazil, the studies by Braga and Valle[6] on the monitoring and surveillance of *Ae. aegypti,* and from Guirado and Bicudo[16], have contributed to elucidate some of these biological mechanisms of how insects act during their proliferation and this type of resistance.

The objective of this work was to evaluate Aedes aegypti populations from biomonitors, as a way to contribute to vector research in Cuiabá and Várzea Grande, MT, and their present state. The enzymatic variability of adults and their spatial and seasonal distribution were analyzed to identify the esterase enzyme expressions and their frequency in different municipalities and sampling periods. It was hypothesized that: 1) esterase bands (light and dark) differ in their expression between sampling sites and seasonal periods; 2) there is a difference in the degree of expression and frequency of alleles in different populations and in the variability of precipitation (rainy season) in the environments.

MATERIALS AND METHODS

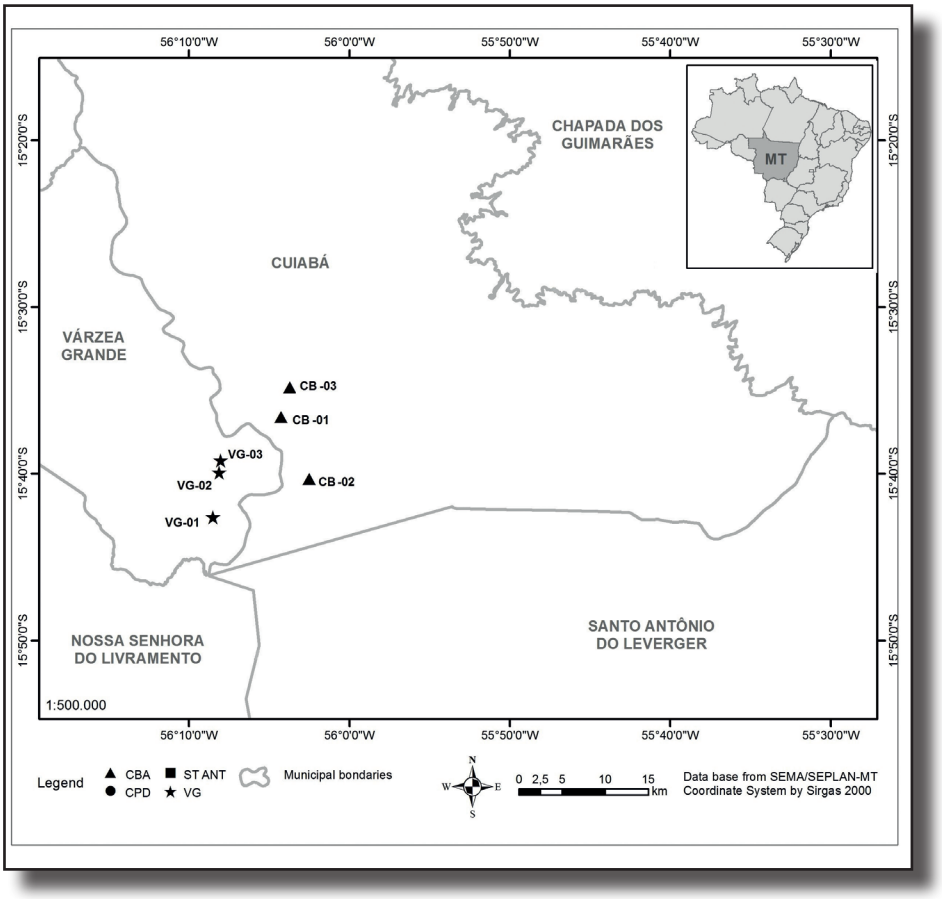
The municipality of Cuiabá is located on the left bank of the Cuiabá river and forms an urban network with the municipality of Várzea Grande, both located in the Midwest region of Brazil, in the Southcentral state of Mato Grosso. The information of the geographic coordinates of the sampling points, altitude, estimated number of inhabitants and demographic density agree with the IBGE[17,18] information and are listed in Table 1.

The collections were performed in both municipalities for a period of 08 months; from October 2015 to November 2016. Samples were established at 3 points in Cuiabá (CB-01, CB-02 and CB-03) and Várzea Grande (VG -01, VG-02 and VG-03), as shown in Figure 1. Precipitation climate data (mm), relative humidity (%), and maximum and minimum temperatures were obtained from the Meteorological Institute.

Table 1– Geographic coordinates, altitude (m), estimated number of inhabitants and demographic density (inhab/km2) of the municipalities of Cuiabá and Várzea Grande (IBGE, 2017), state of Mato Grosso.

Code	Coordinates	Altitude (m)	Estimated Number of Inhabitants	Demographic Density (inhab/km²)
CBA-01	15°36'36" S 56°03'76" W			
CBA-02	15°40'18" S 56°02'30" W	176	590.118	157.66
CBA-03	15°34'48" S 56°03'42" W			
VG-01	15°42'36" S 56°08'30" W			
VG-02	15°39'56" S 56°08'06" W	198	274.013	240.98
VG-03	15°39'12" S 56°08'01" W			

Subtitle: CBA-01; CBA-02; CBA-03 (Cuiabá); VG-01; VG-02; VG-03 (Várzea Grande).



Legend - CB-01, CB-02, CB-03 (Cuiabá), GV-01, GV-02, GV-03 (Várzea Grande).

Figure 1– Graphical representation and sites of the Monitored Study Area in the municipalities of Cuiabá and Várzea Grande of Mato Grosso State. Scale 1: 400,000.

For the collection and biomonitoring of *Ae. aegypti*, the entomological investigation method was the ovitrap (Figure 2), which is an oviposition trap devised by Fay and Perry[19] and has been shown to be a more sensitive, inexpensive and fast method for monitoring Aedes colonizers in breeding sites[3,20,21]. It consists of a 9x12 cm black 580 mL plastic container with a 13.5x2.5 cm Eucatex reed, whose rough part is facing outward for oviposition. 270 mL of an aqueous solution and 30 mL of straw infusion are added for female attraction and subsequent oviposition[22].

The ovitraps were installed every 15 days at a

1.5 m height with 3 traps at each sampling point (with a total of 9 traps for each municipality) in strategic locations with a greater circulation of people. The estimated insect population was determined by the number of eggs deposited on the straws.

Five days after installation, the traps were collected, and the material was taken to the laboratory for analysis. The eggs collected in these traps allowed for estimating the abundance of adults after its emergence and, later, the expression and frequency of alleles in infested areas.

Figure 2– Ovitrap model with an Eucatex reed and straw infusion for oviposition of Aedes aegypti specimens. Source: Photo by Rodrigues, 2019.

The work in the laboratory consisted primarily of counting the eggs performed on the straw through a stereoscopic microscope. To certify the presence of Ae. aegypti, the rearing was done in small aquariums (500 mL of water), with a quantity of 5 to 100 larvae in each one. The reeds were immersed in these aquariums, covered with a phyllo and were properly identified. The taxonomic identification of larvae was made to quantify their abundance in each ovitrap. 100 mg of fish feed was placed as food until the pupae developed. After passing through the four stages of development, pupation, and subsequent emergence of the adults, they were identified and packaged in polypropylene tubes, labeled and frozen in a freezer at a temperature of approximately -20°C.

The use of the electrophoresis technique aims to investigate the expression of esterase in biological samples by evaluating the total extract of macerated mosquitoes. This technique consists of exposing proteins to an electric current, causing the molecules to migrate through the gel towards the less anodic pole, separating according to their molecular weight and/or electric charge23. The polyacrylamide gel expresses the esterase through electrophoresis by means of isoenzyme separation, a method applicable to the model adapted by Paiva *et al.*[14].

The polyacrylamide gel preparation consisted of a 10% Gel composition associated with 29% acrylamide, 1% bis-acrylamide and tris gel buffer at 8.8 alkaline pH. TEMED (N, N, N, N, tetramethyl ethylenediamine) and 10% ammonium persulfate were added to polymerize to give a final volume of 25 mL of gel. After placing the polyacrylamide gel plates in the apparatus, samples of 8 to 12 loci were added to each electrophoresis plate[10].

To verify isoenzyme expression (light band) or superexpression (dark band), the following procedure was used: 1) after running, the gels were immersed in preincubated dye solution for 30 minutes in 100 mL of phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M Na2HPO4) at pH 6.0 and with the addition of 4 mL of h-naphthyl acetate and 15 mL of isopropyl alcohol; 2) then, 600 mg of dye (RR-Salt) dissolved in 30 ml isopropyl alcohol and 50 ml phosphate buffer were added; 3) 30 minutes of agitation was needed to reveal the polyacrylamide gel; 4) finally, it the result was observed after resting in an incubator at 37°C for one hour revealing isoenzyme expression or superexpression.

The 8-month sampling results were analyzed for formatting and statistics of the two sampling sites (Cuiabá and Várzea Grande). Data on the expression of esterase enzyme and frequency of alleles at locations and periods (and their relationship to precipitation) were analyzed using the Statistic 7.0 program using ANOVA - Factorial Analysis of Variance. From the number of copies of *Ae. aegypti* (n) identified during the months and sampling sites (municipalities), the "months" or "location" factor was considered as the independent predictor variable while the esterase factor was the dependent variable.

To use ANOVA, data were adjusted to logx. Subsequently, their normality was tested by the Shapiro-Wilk test with the significance factor set at $p<0.05$. Levene's test confirmed that the variances were homogeneous after adjustments.

The results of the "expressed alleles" and the climate precipitation variable were important descriptors for the dependent variables: 1. expressed alleles and 2. superexpression alleles; which are arbitrarily defined units to estimate allelic changes over the analyzed periods and environments.

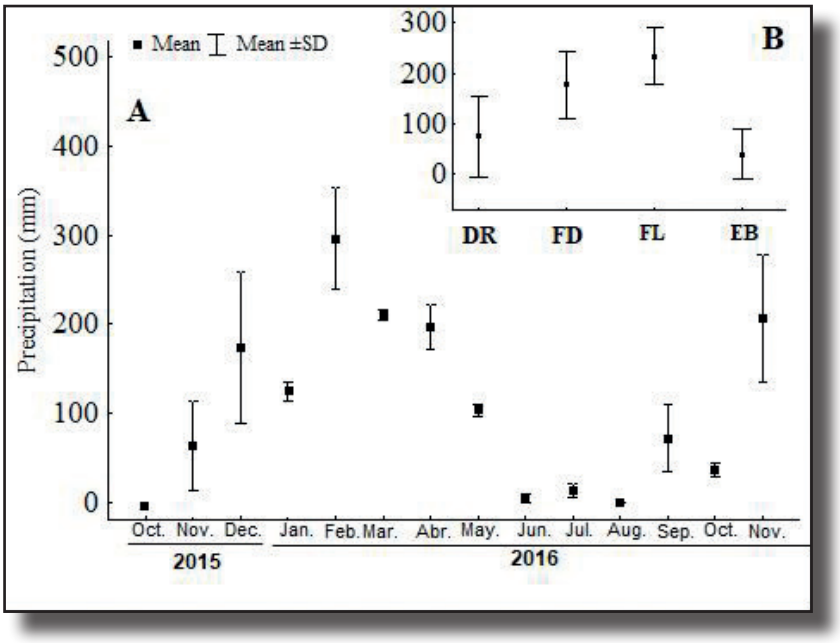
RESULTS

Average precipitation values are available on the box plots chart in Figure 3 from Oct/2015 to Nov/2016. Factorial ANOVA identified significant differences between the sampling periods (Figure 3a; F13,14=11.31; p<0.01) and the seasonal phases (Figure 3b; $F_{3,24}$=11.48; p<0.01) for this variable. The flood peak was recorded in Feb/2016, with the highest rainfall (297±56 mm) and relative humidity (83±4%). The periods with little or no rain were recorded between Jun/2016 (5±4 mm) and Aug/2016 (0±0 mm), due to the strong drought. The average values of the maximum temperature occurred in Oct/2015 (35.79±0.65°C) and the lowest values were in Jun/2016 (29.21±0.97°C); meanwhile the minimum temperature reached the lowest value in Jul/2016 (15.52±1.18°C).

A total of 385 specimens of *Ae. aegypti* was analyzed for differential expression (minimum=1, light-band – expressed alleles; maximum=2, dark-band – superexpression alleles) at both sampling sites (Cuiabá: n=191; Várzea Grande: n=194).

Figure 4 shows the distinction in the intensity of the expressed and superexpressed alleles with a characteristic expression of lighter bands and darker bands of the isoenzyme, respectively. The degree of coloration of the bands in the gels reflected the degree of synthesis or activity of esterases (α-esterases), having for each individual *Ae. aegypti* analyzed at each locus.

Factorial ANOVA identified highly significant differences in the frequency of expressed and superexpressed alleles between the considered periods (Figure 5A; $F_{7,\ 377}$=6.89; p<0.01) and between seasonal phases (Figure 5B, $F_{3,381}$=14.74; p<0.01). Both declined in Mar/2016 (Figure 5a) during the "peak water" period and resumed to increase from May to November/2016. An upward trend in the frequency of alleles expressed between Oct/2015 (30±88%) and Feb/2016 (89±55%) was recorded. Meanwhile, superexpressed alleles demonstrated populations



Legend: Number of samples analyzed: Drought (n=68); Flood (n=161); Full (n=161); Ebb (n = 38).

Figure 3– Average precipitation values (mm) between sampling periods (A) and between seasonal phases (B), from Oct/2015 to Nov/2016 of the municipalities of Cuiabá and Várzea Grande, state of Mato Grosso.

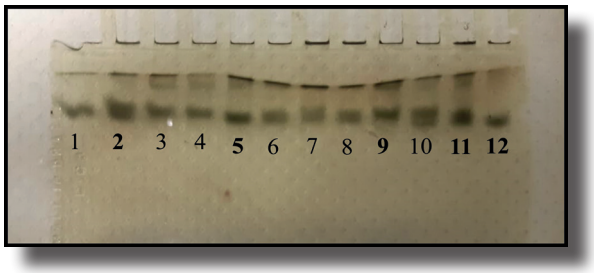
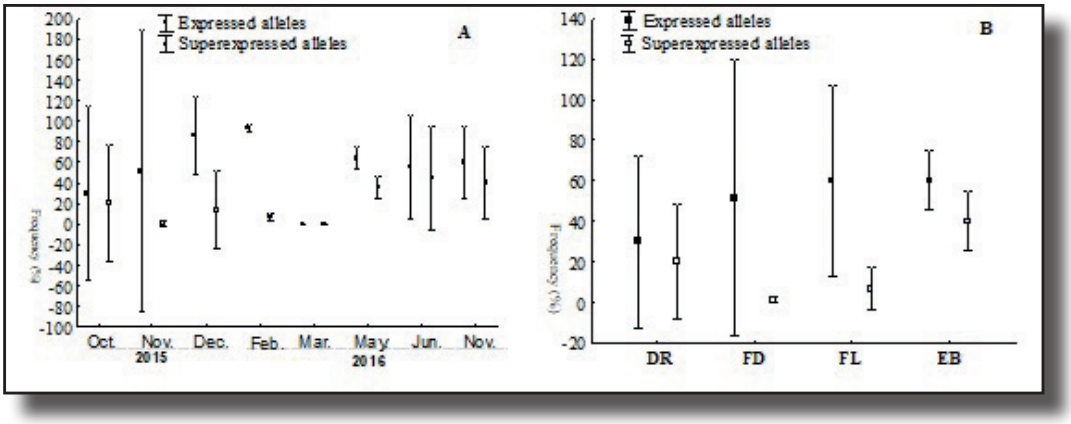


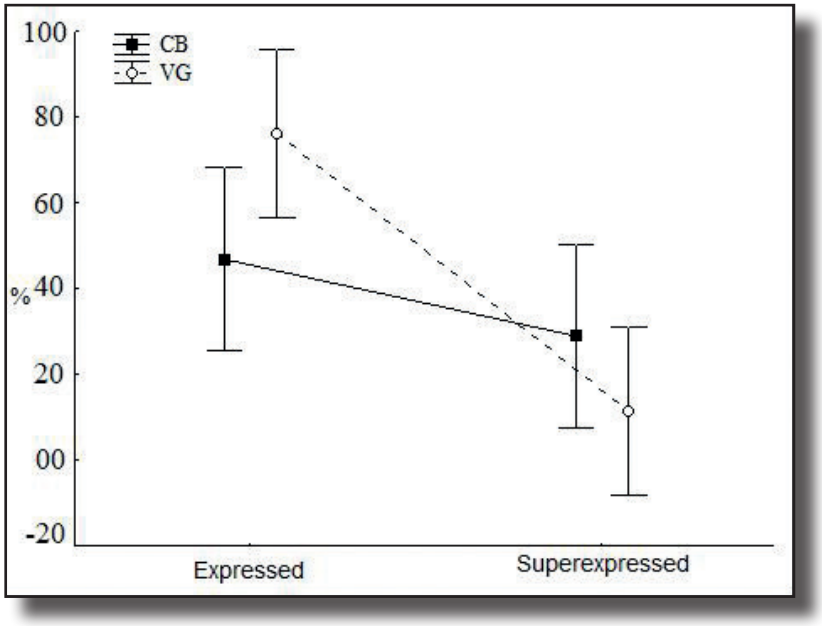
Figure 4– Polyacrylamide gel with *Ae. aegypti* showing the results of the expressed alleles (1, 4, 6, 7, 8, 10) and superexpressed alleles (2, 3, 5, 9, 11, 12) at each locus.

with higher metabolic activities in Jun/2016 (42±91%) during the ebb period (Figure 5b). The average frequency values of the expressed and superexpressed alleles also varied among the municipalities. Expressed alleles increased slightly in the Várzea Grande specimens (75±41.36%) and superexpressed alleles decreased in both municipalities (Figure 6). There was a slight upward trend in Cuiabá (46±24%), significant between sampling sites ($F_{2, 13}=12.39$; $p<0.01$).



Legend - Number of specimens analyzed: DR-Drought (n=68); FD-Flood (n=161); FL-Full (n=161); EB-Ebb (n=38).

Figure 5– Average values of alleles expressed between sampling periods (A) and seasonal phases (B), from Oct/2015 to Nov/2016 of the municipalities of Cuiabá and Várzea Grande, state of Mato Grosso.



Legenda - CB: Cuiabá; VG: Várzea Grande.

Figure 6– Average allele frequency values between sampling sites (municipalities of Cuiabá and Várzea Grande) - period from Oct/2015 to Nov/2016.

Figure 7 represents the variation of mean precipitation values (mm) and frequency of significantly expressed alleles between periods ($F_{14,14}=4.22$; $p<0.01$). With the absence of rainfall in Oct/2015, the average values of precipitation and frequency of alleles expressed were reduced (63±50 mm; 30±42%, respectively) and from Nov/2015 (174±86 mm; 52±68%; flood) to Feb/2016 (185±48 mm; full) there was an increase in alleles together with precipitation. Peculiarly, the opposite was recorded in Mar/2016 (197±26 mm), with a reduction in the allele frequency (0±0%).

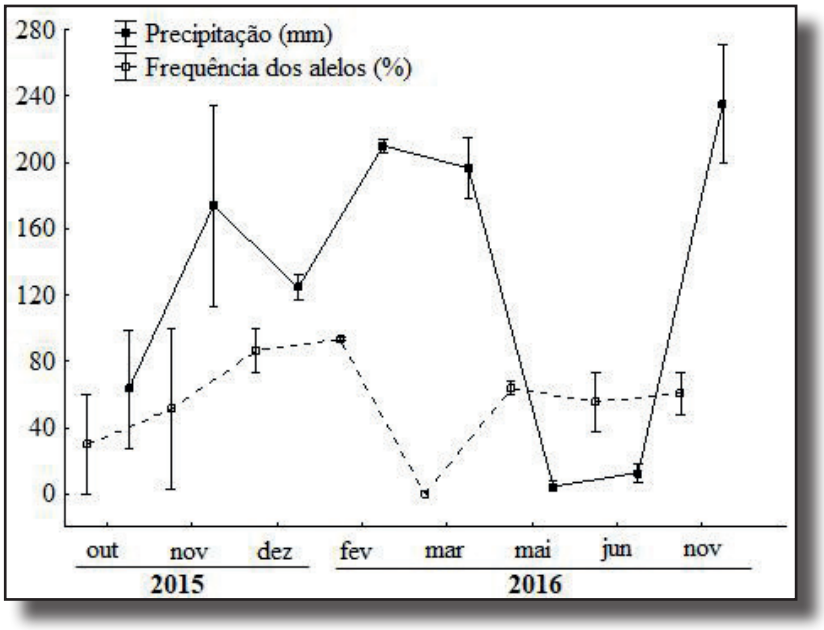


Figure 7– Mean values of precipitation (mm) and frequency of alleles expressed, from Oct/2015 to Nov/2016 of the municipalities of Cuiabá and Várzea Grande, state of Mato Grosso.

DISCUSSION

The highly significant differences in the frequency values of alleles expressed in the populations studied between the periods and seasonal phases showed the complexity involved in the peculiarities of the vector and revealed the difficulty of maintaining a single control model that is used throughout the year.

The reduction in allele expression during the Mar/2016 flood was probably related to the availability of a larger number of breeding sites adjacent to the sampling site, or the presence of other substrates in microhabitats that attract females to lay their eggs in places other than the traps, consequently reducing vector abundance in the ovitraps. However, it was observed that in the preceding periods, even during the flooding (Feb/2016), the ovitraps were sensitive for capturing adults and detecting the presence of mosquito eggs after their oviposition in the straws. Thus, the allele frequency increased by 89% for that period. Their registration in biomonitored areas in both municipalities showed that these traps were important tools[24,21] for surveillance and detection in risk areas with the presence of *Ae. Aegypti*; even though abundance has been sporadically reduced in some places and periods.

The variability in the expression of esterase bands between sampling sites and between periods was significantly supported the first hypothesis, especially in relation to seasonal precipitation. Therefore, there were significant differences in frequency of the esterase bands. These results showed that, in general, the variability of allele expression responded to the rainfall regime in a given period of increased rainfall (Feb/2016), and consequent increase of expressed alleles. The rainfall regime determines the population growth of *Ae. aegypti*[2], as was recorded for allele expression in the results

of this study. On the other hand, the influence of environmental factors, especially precipitation and temperature, was notable within the population dynamics of the species in question[25].

The analysis of esterase isoenzymes provides important data regarding the studied populations. The results of superexpressed allele gels showed an increase in metabolic mechanisms of some *Ae. aegypti*, probably pertinent to the reduction of the use of insecticides in drought/ebb periods or resulting from older populations that left few individuals with the highest allele expressions[23]. The increase of esterase in dry periods (Jun /2016) and the highest average values of enzyme expression in Várzea Grande (75±41.36%) may be related to some biological characteristics of mosquitoes, and even their reproductive fitness in face of the disease and environmental pressure with the use of insecticides by public agencies or particularly by people residing near the breeding sites. Many vector organisms favor the development of their resistance to this pressure through chemical compounds, incorporating higher concentration of esterases14 in their organisms in the next generations as a form of adaptation. Their descendants may support the greater number of alleles expressed[15] for this factor (resistance)[3]. Thus, chemical compounds will not produce the expected toxic effect on their body[26]. Box plot results showed that "superexpressed" alleles reached around 30% in the populations analyzed in their frequency in the city of Cuiabá, and the vectors left a greater allele expression to their offspring adapted to risk areas[23].

Genetic differentiation of populations was observed in the first genetic structure studies carried out in Ae. aegypti in Brazil[27]. The second hypothesis was relevant to the variability of the expression degree and frequency of alleles in the different vector populations. When rainfall decreased during the ebb period, there was a slight increase in superexpressed alleles either in response to processes that may generate environmental adaptability, or these variations are due to chance. Souza-Polezzi and Bicudo[12] suggested that the replacement of alleles due to environmental pressure or even genetic oscillation would be a factor in this uncertainty.

Zara *et al.*[13] stated that egg quiescence allows the maintenance of the cycle in the wild during seasonal climatic variations, since the viability of *Ae. aegypti* lasts up to about 492 days in drought, hatching after contact with water. The lack of sustainability in the fight against the mosquitoes ends up favoring the species, which goes through the most critical phase in the form of resistant eggs[25].

The results of the present study with the variability of α-esterase observed in adult populations through the average frequency values between municipalities and between seasonal phases. The frequency ratio of expressed and superexpressed alleles in the sites revealed the need for more and more punctual and temporal programs and interventions. The number of insects, periodic distribution and metabolic characteristics change much from one space to another, this may denote the most appropriate type of biomonitoring in the management of its resistance[16].

Muthusamy and Shivakumar[28] showed that a population of *Ae. Aegypti* from Namakkal (India) had a change in their susceptibility status. Meanwhile the other populations were moderately susceptible to insecticides and showed increased local α-ß-esterase activity as well as evidence of acetylcholinesterase insensitivity. However, Alvarez et al.29 found susceptible larval populations in western Venezuela with low resistance rates and no enzyme superexpression. Most works with Ae. aegypti focused on the analysis of the esterase profiles refers to the quantification of total esterase, evaluated in the extract of whole macerated mosquitoes[11,23], the information from which helps in obtaining resources for management focused on the use of insecticides. This study with the ovitraps helped in the capture and surveillance of vectors in the two biomonitored municipalities and the use of the collected samples identified the presence/absence of specific enzymes, investigating their local and temporal metabolic activity.

The record and the increase of the esterase variability in the analyzed samples proved that the populations were metabolically active using the primarily used mechanisms. As pointed out by Vasconcelos[30], it is a consensus among researchers that combating the vector requires a growing effort from the scientific community to verify

mutation and adaptation processes, as well as the rapid spread of new viruses; as had occurred in Brazil at the end of 2015, when a large number of cases of microcephaly caused by Zika virus were reported31. All necessary efforts must be made to combat this arbovirus, and the various public health sectors are responsible for carrying out surveillance actions in Brazil.

CONCLUSION

Investigation of the resistance mechanism of Ae. aegypti in the municipalities of Cuiabá and Várzea Grande, shown by the results of the expressed and overexpressed alleles, consisted in obtaining esterase levels. The results recorded in this study indicated that the method of using ovitraps and the markers of esterase isoenzymes was sensitive enough to detect variations in the frequency of expressed alleles in natural populations of this vector in both municipalities. In general, the frequency of the resistance allele was different between the two municipalities studied, which may indicate an effect of selecting different environments upon the alleles, such as the presence of breeding sites.

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