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The Influence of Fatty Acid Methyl Esters (FAMEs) in the Biochemistry and the Na^+/K^+-ATPase Activity of *Culex quinquefasciatus* Larvae

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Abstract *Culex quinquefasciatus* is the main vector of lymphatic filariasis and combating this insect is of great importance to public health. There are reports of insects that are resistant to the products currently used to control this vector, and therefore, the search for new products has increased. In the present study, we have evaluated the effects of fatty acid methyl esters (FAMEs) that showed larvicidal activity against *C. quinquefasciatus,* on glucose, total protein, and triacylglycerol contents and Na^+/K^+-ATPase activity in mosquito larvae. The exposure of the fourth instar larvae to the compounds caused a decrease in the total protein content and an increase in the activity of the Na^+/K^+-ATPase. Furthermore, the direct effect of FAMEs on cell membrane was assessed on purified pig kidney Na^+/K^+-ATPase membranes, erythrocyte ghost membranes, and larvae membrane preparation. No modifications on total phospholipids and cholesterol content were found after FAMEs 20 min treatment on larvae membrane preparation, but only 360 µg/mL FAME 2 was able to decrease total phospholipid of erythrocyte ghost membrane. Moreover, only 60 and 360 µg/mL FAME 3 caused an activation of purified Na^+/K^+-ATPase, that was an opposite effect of FAMEs treatment in larvae membrane preparation, and caused an inhibition of the pump activity. These data together suggest that maybe FAMEs can modulate the Na^+/K^+-ATPase on intact larvae for such mechanisms and not for a direct effect, one time that the direct effect of FAMEs in membrane preparation decreased the activity of Na^+/K^+-ATPase. The biochemical changes caused by the compounds were significant and may negatively influence the development and survival of C. *quinquefasciatus* larvae.

Keywords Na^+/K^+-ATPase · Insecticide · *Culex quinquefasciatus* · Fatty acid methyl esters · Membrane lipids

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Introduction

The mosquito *Culex quinquefasciatus* is the main vector of the bancroftian filariasis in the Americas, as well as transmitting several arboviruses. It has anthropophilic and endophytic habits, and its larvae are able to develop in almost all types of aquatic habitats modified by humans (Forattini 2002). The main limiting factor to control the population of *C. quinquefasciatus* and other insect vectors is their resistance to commonly used chemical insecticides. In Brazil, some studies indicate that *C. quinquefasciatus* populations are resistant to different insecticides (Alves et al. 2011; Bracco et al. 1997; Campos and Andrade 2003; González et al. 1999; Yébakima et al., 1995). Thus,

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multiple products are being applied to control unwanted insect populations, with an unknown effect on the environment (Barreto 2005). For this reason, the demand for plant extracts and natural substances has grown as a natural alternative for combating the insect vectors as these substances are less toxic to the environment.

Vegetable oils, produced in the secondary metabolism of plants, were considered as sources of materials with insecticidal, larvicidal, and repellent activities (Costa et al. 2005; Murugan et al. 2007). Studies have also shown that plant extracts have activity against *C. quinquefasciatus* revealed that the extract of *Adhatoda vasica* has a strong activity against *C. quinquefasciatus* and *Aedes aegypti* (Kovendan et al. 2012; Sharma et al. 2012; Thanigaivel et al. 2012). Another study showed that extracts of some Indian plants, mainly *Andrograp hispaniculata,* also have insecticidal and repellent properties against *C. quinquefasciatus* and *A. aegypti* (Govindarajan and Sivakumar 2012). Vegetable oils also have biological activities such as antibacterial, antifungal, and antioxidant activities, among others. The activities observed for vegetable oils are attributed mainly to the fatty acids present in the oils, being the main constituents of the oils (Chandrasekaran et al. 2011; Erdemoglu and Kusmenoglu 2003; Lima et al. 2011; Lima et al. 2012). Further, the active fraction of the bark of *Punica granatum* or from the root of *Canna indica* inhibits the activity of acetylcholinesterase and Na^+/K^+-ATPase in *Lymnaea acuminate* (Tripathi et al. 2004).

Vegetable oils contain fatty acids, which through a transesterification reaction, form methyl esters (FAMEs—*fatty acid methyl ester*). In the transesterification, a triacylglycerol reacts with an alcohol in the presence of a catalyst, which can be a base or a strong acid, to produce a mixture of fatty acid esters and glycerol (Barreto 2005; Geris et al. 2007; Pinto et al. 2005). Studies with fatty acid methyl esters are few, especially regarding their larvicidal activity against *C. quinquefasciatus* larvae. However, it was observed that FAMEs obtained from three species of *Vitex* showed larvicidal activity against *C. quinquefasciatus* (Kannathasan et al. 2008).

In addition, little is known about the biochemical changes that chemical insecticides and plant-derived substances cause to the mosquito larvae. However, changes in the synthesis of proteins and total lipids after the exposure of the larvae of *C. quinquefasciatus* to imidacloprid have been observed (Rao et al. 2008). Moreover, it was observed a significant change in the biochemical profile of the larvae of *Anopheles stephensi* and *C. quinquefasciatus* exposed to certain phytoextracts (Sharma et al. 2011).

Therefore, the objective of this study is to evaluate the effect of FAMEs on glucose, total protein and triacylglycerol contents and on the Na^+/K^+-ATPase activity in the larvae of the *C. quinquefasciatus* mosquito; and to evaluate their effects on the content of phospholipids and total cholesterol of the membrane and on the activity of purified Na^+/K^+-ATPase.

Materials and Methods

FAMEs Synthesis

The FAMEs used in this study were obtained from a transesterification reaction of vegetable oils (sunflower oil and soybean oil). Vegetable oils (1 g) were refluxed with 1.0 mol/L methanolic sodium hydroxide solution for 30 min and then extracted with ethyl ether. The aqueous phase was acidified with 1.0 mol/L chloridric acid solution and extracted with ethyl ether. The organic phase was dissolved in hexane and then refluxed with sulfuric acid methanolic solution 2 % v/v for 60 min. Fatty acid methyl esters were obtained after extraction and solvent elimination (Lima et al. 2011). The FAMEs, called 2 and 3, were produced in the Phytochemistry Laboratory of the Federal University of São João Del Rei (UFSJ) and kindly provided for use in this study.

GC/MS Analysis of FAMEs

Gas chromatography mass spectrometry (GC–MS) was performed with Shimadzu CG-MS QP5050A, using impact electron at 1.2 kV and helium as the carrier gas. A Supelco PTE-5 column (30 m × 0.25 mm) was used. The temperature was held at 120 °C and programmed to 300 °C with increase of 5 °C/min; injection and detector temperature were 230 °C. Split ratio was 1/10. Oven flow was 2 mL/min and mass range was 40–500 *m/z*. The fatty acid methyl esters were identified by comparison of their retention times with those of standards and by the NIST 2.0 Library database search.

Exposure of the Larvae to FAMEs

The fourth instar larvae of *C. quinquefasciatus* were separated into three groups with 50 larvae each with 100 mL of water. We treated one group with 1 h to FAME 2 and the other group to FAME 3, and the LC_{50} obtained was 90 µg/mL for FAME 2 and 20 µg/mL for FAME 3, previously determined in the Insect Vectors Laboratory of UFSJ. We compared both treated groups with the control group in all experiments. We homogenized the groups in 10 mL 20 mM Tris buffer pH 7.4 and centrifuged (Macro IV) for 10 min at 1500 *g*, and the supernatant was used for the determination of protein, glucose, and triglyceride contents.

Determination of Protein, Glucose, and Triglyceride Contents

The protein were determined according to Hartree (1972) using bovine serum albumin as standard and 30 µL aliquot of the total homogenate obtained from 50 larvae. The measurements were made with a spectrophotometer at a wavelength of 650 nm. Glucose were determined according to Trinder (1969) and triglycerides according to Megraw et al. (1979) using diagnostic kits according to the instructions of the manufacturer (Doles®).

Preparation of the *Culex quinquefasciatus* Larvae Membrane Preparations

1200 larvae with no treatment were obtained and were homogenized in 250 mM sucrose, 0.1 mM EGTA, and 25 mM imidazole HCl, pH 7.4, using a motor-driven Teflon Potter–Elvehjem homogenizer. The sample was then subjected to centrifugation at 10,000×*g* for 10 min. The resulting supernatant was centrifuged at 70,000×*g* for 1 h. The final pellet was resuspended in the homogenization solution. The membrane preparation was incubated for 20 min with different concentrations of FAME 2 and FAME 3 for the analysis of phospholipids, cholesterol, and Na^+/K^+-ATPase activity.

Preparation of the *Culex quinquefasciatus* Larvae Crude Membrane Homogenates

After exposure of the larvae to FAME 2 and FAME 3, they were homogenized in a motor-driven Teflon Potter–Elvehjem homogenizer for 20 times in buffer solution containing 6 mM Tris–HCl pH 6.8, 20 mM imidazole, 250 mM sucrose, 3 mM EDTA, 0.01 %, SDS, 2 mM PMSF, and 1 mM orthovanadate. Subsequently, the samples were sonicated in ice at 4 pulses of 15 s each, at 45 % power with 10 s intervals between each pulse and centrifuged at 4500×*g* for 10 min. Then the homogenate was centrifuged at 9000×*g* for 1 h at 4 °C in a refrigerated centrifuge. After the centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 mL of the same buffer solution and stored in eppendorf. The samples were storage in a freezer at −80 °C.

Determination of the Activity of Na^+/K^+-ATPase from Membrane Preparation of *C. quinquefasciatus* Larvae

ATPase activity was determined through the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis by the Na^+/K^+-ATPase, using the FISKE's colorimetric method (Fiske and Subbarow 1925). The assay was performed in reaction medium containing: 50 mM Hepes, 120 mM NaCl, 20 mM KCl, and 2 mM $MgCl_2$. This experiment was performed in 96-well ELISA plates. The same amount of protein from each sample (15 µg) and reaction medium were added to each well and the reaction was initiated by the addition of 3 mm ATP. The experiment was conducted with and without the specific inhibitor of the enzyme, 1 mM ouabain. The plate was incubated at 37 °C for 1 h, and the reaction was stopped with the addition of 100 µL of 1 % SDS. Subsequently, 100 µL of 5:1 ammonium molybdate solution/Fiske reagent was added. Fifteen minutes after the addition of the solution, the ELISA plate was read in a spectrophotometer (Biotek®—Power Wave XS2), at a wave length of 660 nm.

Determination of the Activity of Purified Pig Kidney Na^+/K^+-ATPase

The purified Na^+/K^+-ATPase was courtesy of the Laboratory of Structure and Regulation of P-ATPases of the Institute of Medical Biochemistry of the Federal University of Rio de Janeiro (UFRJ), and was obtained according Cortes et al. (2006). The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 20 min with FAMEs 2 and 3 at different concentrations. The activity of the purified Na^+/K^+-ATPase was determined by the FISKE method as described above.

Erythrocyte Ghost Preparation

The preparation of the plasmatic membrane of human erythrocytes was obtained by the method described by Maia et al. (2014). The erythrocytes were separated from the plasma and leukocytes by centrifugation at 2000×*g* for 10 min at 4 °C using a refrigerated centrifuge (ThermoScientific®). The pellet was subjected to freezing and thawing to lyse the cells and then resuspended in buffer containing 5 mM Hepes, pH 7.4 containing 1 mM of EDTA, and 0.6 mg/mL of PMSF and centrifuged at 9000×*g* for 17 min at 4 °C. The obtained pellet was resuspended in 10 mM Tris–HCl buffer, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$, and 50 µM $CaCl_2$; centrifuged at 9000×*g* for 17 min, at 4 °C; and stored. The membrane preparation was incubated for 20 min with different concentrations of FAME 2 and FAME 3 for the analysis of phospholipids and cholesterol.

Determination of Phospholipid and Cholesterol Content

The extraction of phospholipids and cholesterol was performed by the method of Higgins (1987) with modifications.

In brief, homogenized larvae were dissolved in the organic solvent mixture (chloroform–methanol, 2:1), keeping the solvent/tissue ratio 20:1. The samples were stirred in vortex and let stand until phase separation. The upper phase was discarded, and half of the volume of distilled water was added to the remaining phase. The samples were stirred and let stand until the phase separation. Again the upper phase was discarded, and the remaining phase was washed by adding an equal volume of 1/1 (v/v) methanol/water and letting stand until a new phase separation. Then, the upper phase was discarded and the lower phase concentrated by rotary evaporation, resuspended in a known volume of chloroform and stored at −20 °C under nitrogen atmosphere, and finally used for identification and analysis. The total phospholipids were measured by quantification of inorganic phosphate released from hydrolysis (Chen et al. 1956).

For cholesterol measurement a 40 µL aliquot of the lipid extract, obtained as described above, was dried in nitrogen air flow, and then 1.5 mL of glacial acetic acid and 1 mL of Reagent B were added. The sample was let stand for 10 min and quantified in spectrophotometer at a wavelength of 550 nm. Reagent B was prepared by diluting 4 mL of Reagent A in 46 mL of sulfuric acid. Reagent A was prepared by dissolving 2.5 g of $FeCl_3.6H_2O$ in 100 mL 85 % orthophosphoric acid. The determinations were performed in triplicate, and the content was calculated by comparing with a standard curve. The standard curve was obtained using 0 to 400 µl aliquots of a standard 1 mg/mL solution of cholesterol.

Statistical Analysis

The statistical analysis of the results was performed using the program GraphPadPrism 5. The analysis of variance (ANOVA) was used, followed by the multiple comparison test of Dunnett. The analyses were performed considering 95 % of significance and p value <0.05.

Results

In this study, the fatty acid methyl esters, obtained from vegetable oils, were investigated by gas chromatography/mass spectrometry (GC/MS). The analysis by CG/MS revealed high percentage of unsaturated methyl esters (83.95–88.33 %) as compared to saturated methyl esters (11.67–16.05 %). In samples, methyl oleate and methyl linoleate were detected as unsaturated methyl esters. Methyl palmitate was the most abundant among the saturated methyl esters, followed methyl stearate (Table 1).

The compounds, FAMEs 2 and 3, showed larvicidal activity against *C. quinquefasciatus* larvae, with LC_{50} values of 90 µg/mL for FAME 2 and 20 µg/mL for FAME 3 (data not shown). After 1 h treatment of the larvae with FAMEs, we evaluated the biochemical changes caused by the compounds. The exposure of the *C. quinquefasciatus* larvae to FAMEs caused a significant decrease in the content of total protein. Both compounds had the same effect on the content of total protein in the larvae, leading to a decrease of approximately 30 % in relation to the control group, and we did not observed changes in the content of glucose and triglycerides (Fig. 1).

Table 1 Composition and percentages of fatty acid methyl esters (FAMEs)

Peak no.	Compounds	FAME 2	FAME 3
1	Methyl palmitate	6.99	12.71
2	Methyl stearate	4.68	3.34
3	Methyl oleate	22.40	28.28
4	Methyl linoleate	65.93	55.67
Total		100.00	100.00

The exposure of the larvae to FAMEs 2 and 3 also caused changes in the activity of the Na^+/K^+-ATPase. Both compounds significantly increased the activity of the enzyme, but FAME 2 caused a greater increase than FAME 3. The first tested compound caused an increase of about 43 % and the second increased approximately 36 % the enzymatic activity (Fig. 2).

As the tested compounds changed the activity of the Na^+/K^+-ATPase of the *C. quinquefasciatus* larvae, we further evaluated if these compounds have a direct effect on the enzyme. Purfied pig kidney Na^+/K^+-ATPase was incubated with different concentrations of the compounds. Only FAME 3 was capable to provoke the activation of the Na^+/K^+-ATPase in 60 and 360 µg/mL (Fig. 3a). However, when we incubated the membrane preparation of *C. quinquefasciatus* larvae, the FAMEs (Fig. 3b) caused an inhibitory effect on all concentration tested (only 240 µg/mL of FAME 3 showed no effect). These data suggest a direct effect of FAMEs on Na^+/K^+-ATPase.

The activity of Na^+/K^+-ATPase may be modulated by changes in its lipid environment, and therefore, we assessed whether FAMEs 2 and 3 would cause changes in the content of total phospholipids and cholesterol of the plasmatic membrane. For these experiments, we incubated FAMEs for 20 min with erythrocytes ghost's membrane (Fig. 4) and *C. quinquefasciatus* larvae membrane preparation (Fig. 5). In erythrocytes membranes, only 360 µg/mL FAME 2 caused the decrease of total phospholipids (Fig. 4b) and no effect on cholesterol content. However, FAMEs treatments were not able to cause any modifications on total phospholipids and cholesterol of *C. quinquefasciatus* larvae membrane preparation.

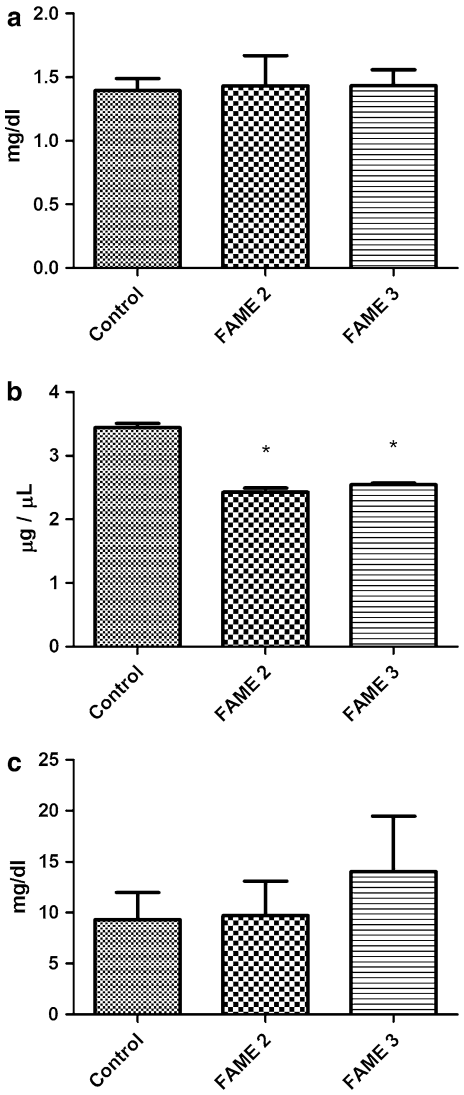

Fig. 1 Effect of FAMEs in the content of protein, glucose, and triglycerides in *C. quinquefasciatus* larvae. Fifty larvae were treated with 1 h FAMEs, and a total content of glucose (**a**), protein (**b**), and triglycerides (**c**) were measured. Data are mean ± SE ($n = 3$). *Asterisks* represent that result bars are significantly different from controls, as evaluated by ANOVA test ($p < 0.05$)

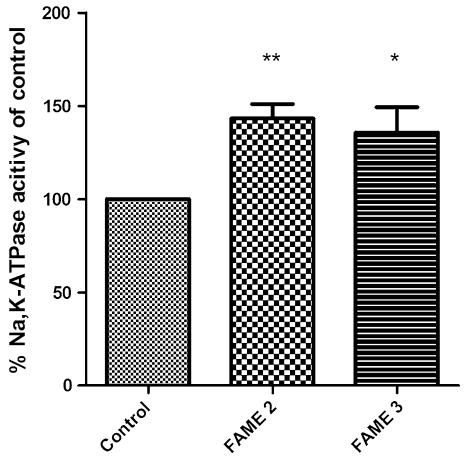

Fig. 2 Effect of FAMEs in the activity of the Na^+/K^+-ATPase of the *C. quinquefasciatus* larvae. The larvae were exposed for 1 h to 90 µg/mL for FAME 2 and 20 µg/mL for FAME 3. Data were presented as percent of control activity of the Na^+/K^+-ATPase. Data are mean ± SE ($n = 3$). *Asterisks* represent that result bars are significantly different from controls, as evaluated by ANOVA test ($p < 0.05$)

Discussion

Some larvicides have a negative impact on the growth and development of insect larvae and may cause changes in their metabolic and biochemical processes. The tested compounds showed some important biochemical changes in the larvae of *C. quinquefasciatus*. One hour of exposure of the larvae to FAMEs 2 and 3 may have not been enough to cause significant changes in the content of glucose and triglycerides, but we observed a significant decrease of the larvae protein content. This decrease in protein content may impair the survival and development of the larvae.

It has been shown that some plant extracts can decrease the level of protein of *C. quinquefasciatus* larvae. The *Artemisia annua* extract negatively affects the growth and the development of *C. quinquefasciatus* (Sharma et al., 2011). Plant extracts tested against larvae, and adults of *An. stephensi* reduced their content of protein. The extract may also interfere in the mechanism of protein synthesis, leading to a change in the content of total protein (Senthilkumar et al. 2009).

The protein content decrease may be due to a physiological adaptation to the stress caused by FAMEs. Under stress conditions and energy demand, protein catabolism may also occur, resulting in a decrease in the level of insect proteins. Insecticides may interfere in the energetic reserve by increasing the mobilization of these reserves in an attempt to a detoxification of these insecticides (Senthilkumar et al. 2009).

The membrane lipid environment may modulate the activity of the Na^+/K^+-ATPase. The complete hydrolytic activity of the enzyme depends on an association between the phospholipids and the cholesterol of the plasmatic membrane (Chapelle and Zwingelstein 1984; Habeck et al. 2015; Yeagle 1983). We evaluated the effects of FAMEs 2 and 3 on the content of total phospholipids and cholesterol in the membrane fractions in order to determine whether the activity of Na^+/K^+-ATPase would be modulated by modifications in the content of lipids of the plasmatic membrane. The FAMEs treatment in erythrocyte membranes and in *C. quinquefasciatus* larvae membrane preparation was not able to provoke modifications on total

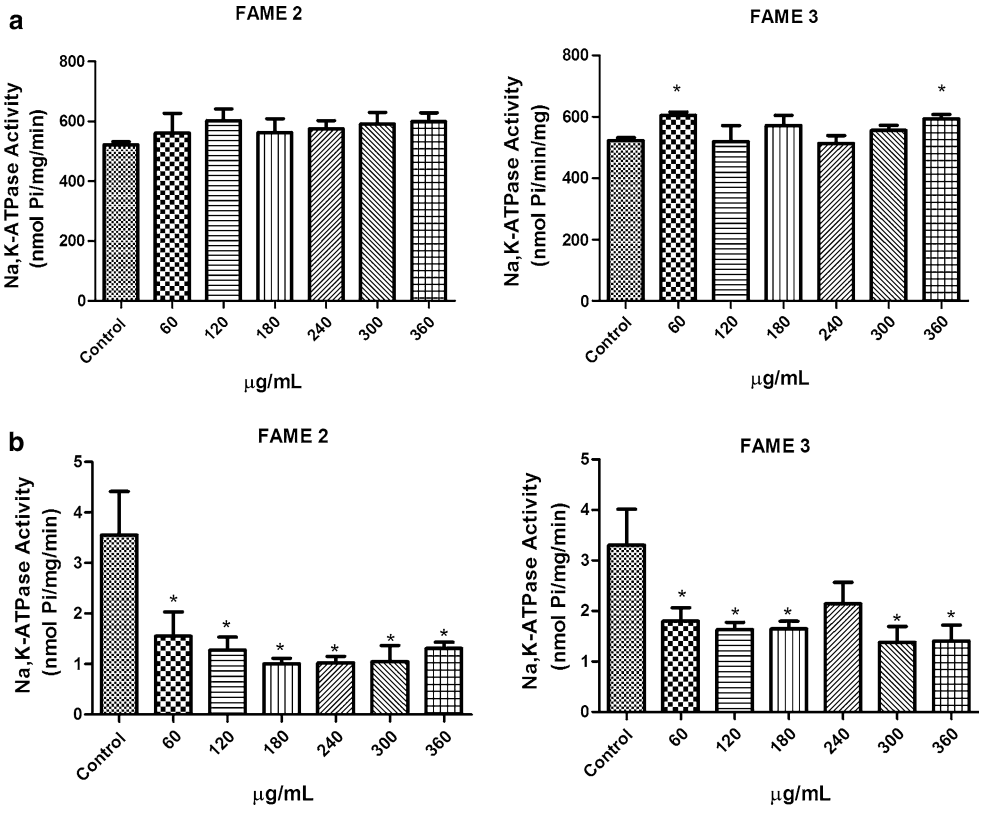

Fig. 3 Effects of FAMEs in the activity of the Na^+/K^+-ATPase. Purified pig kidney Na^+/K^+-ATPase (**a**) or larvae membrane preparation (**b**) was exposure to FAMEs for 20 min. Data are mean ± SE ($n = 3$). *Asterisks* represent that result bars are significantly different from controls, as evaluated by ANOVA test ($p < 0.05$)

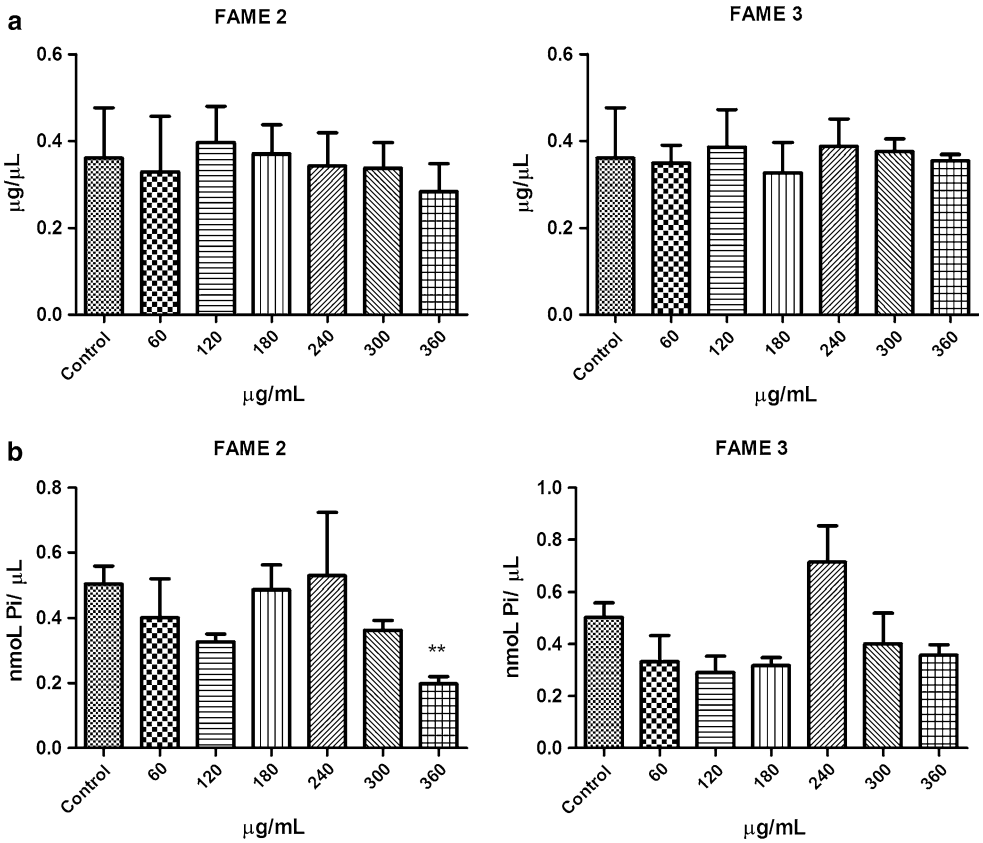

Fig. 4 Effect of FAMEs on the total content cholesterol (**a**) of and phospholipids (**b**) of the erythrocyte membrane. Erythrocyte ghost membranes were exposure to FAMEs for 20 min. Data are mean ± SE ($n = 3$). *Asterisks* represent that result bars are significantly different from controls, as evaluated by ANOVA test ($p < 0.05$)

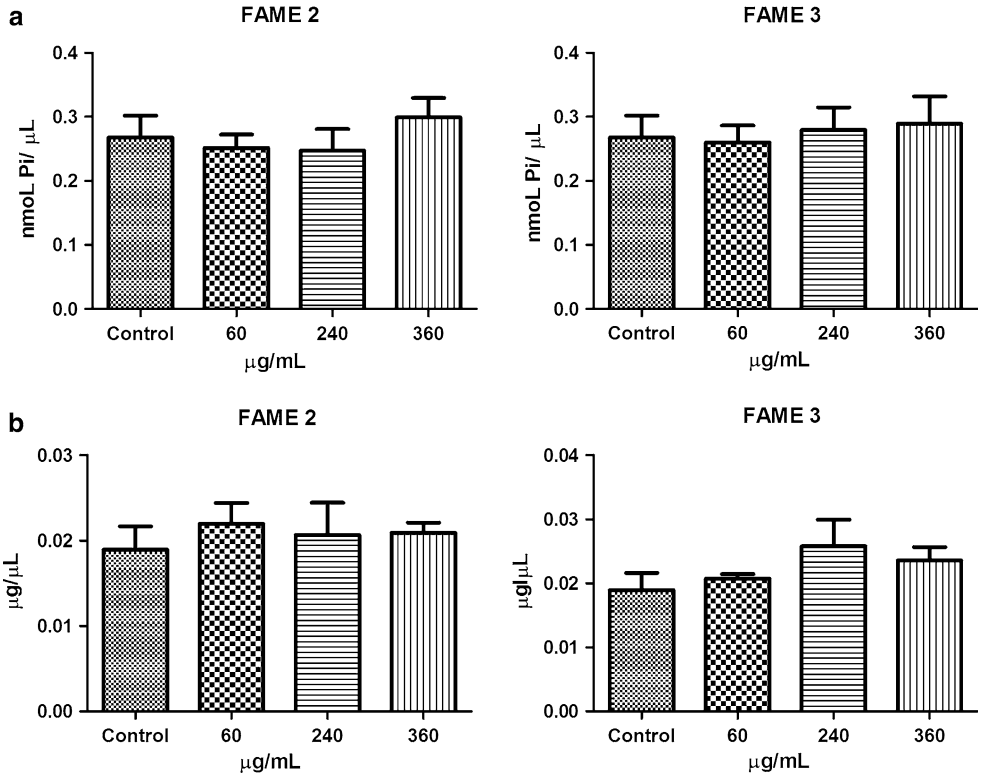

Fig. 5 Effect of FAMEs on the total content of phospholipids (**a**) and cholesterol (**b**) of the *C. quinquefasciatus* larvae membrane. The larvae membrane preparation were exposure to FAMEs for 20 min. Data are mean ± SE ($n = 3$). *Asterisks* represent that result bars are significantly different from controls, as evaluated by ANOVA test ($p < 0.05$)

cholesterol content, and only 360 µg/mL FAME 2 decreased the phospholipids of erythrocytes membranes.

The FAMEs contain a higher percentage of methyl esters of unsaturated fatty acids. These methyl esters may be, in some way, inserting themselves in the plasmatic membrane, thus leading to higher membrane fluidity and the subsequent increase in the activity of the Na^+/K^+-ATPase observed in our study (Ibarguren et al. 2014; Zavodnik et al. 1996, 1997).

Membrane fluidity is an important factor in cellular functions since the surrounding lipids can direct modulate the proper function of several proteins (Ibarguren et al., 2014). The lipids may influence the optimal conformation for the activity of proteins though modification of the membrane's biophysical properties. The effects of FAMEs can include non-specific interactions with the lipid content of plasma membrane, leading to a disturbance of optimum complementarity between proteins and surrounding lipids, inducing changes in the morphology of the membrane and its fluidity, finally leading to changes in the activity of the human erythrocyte membrane sodium pump (Zavodnik et al. 1996). It has also been shown that the surface tension of the membrane, which is in turn modulated by the lipid composition of the bilayer can be altered by insertions of fatty acids (Cordomi et al. 2010; Lopez et al. 2012; Prades et al. 2003). Thus, even that FAMEs have not caused modification on total phospholipids and cholesterol on plasma membrane, maybe specific point modifications on membrane environment might be enough to cause modification of the Na^+/K^+-ATPase activity.

The Na^+/K^+-ATPase may be involved in the transport of ions in the plasmatic membrane of the basal cells of the Malpighi tubules and middle intestine, and it is believed that it contributes to the regulation of the osmolarity of the larvae's hemolymph, which is essential for their survival (Patrick et al. 2006). Our results show that both compounds caused a significant increase in the activity of the Na^+/K^+-ATPase of *C. quinquefasciatus* larvae and purified pig kidney Na^+/K^+-ATPase. However, the compounds caused an inhibition of the Na^+/K^+-ATPase of *C. quinquefasciatus* larvae membrane preparations. It has been demonstrated antagonist effect on Na^+/K^+-ATPase activity of cardiotonic steroids on cell membrane preparations and intact cells (Oselkin et al. 2010; Rocha et al. 2014). In intact cells, we have complex modulators of the Na^+/K^+-ATPase that can cause modifications of their activity as: proteins kinases (Bertorello 1992; Cortes et al. 2006; Gonin et al. 2001; Zhang et al. 2008) and synthesis of new pumps to the membrane (Jiawei and Runming 2009; Tian et al. 2009). These data together suggest that maybe FAMEs can modulate the Na^+/K^+-ATPase of intact larvae for such mechanisms and not for a direct effect, one time that the direct effect of FAMEs in membrane preparation decreased the activity of Na^+/K^+-ATPase.

The alteration in the activity of the enzyme, caused by FAMEs 2 and 3, may have a negative impact on the mechanism of regulation of the osmolarity of the hemolymph hindering the survival of the larvae. We can also infer that the increase of Na^+/K^+-ATPase activity causes an increase in the energy expenditure and consumption of ATP by the enzyme, causing the impairing the activity of other carriers and some metabolic processes essential to the growth of the larvae. Thus, both compounds may affect the development and survival of the larvae of *C. quinquefasciatus.*

Conclusion

The compounds FAMEs 2 and 3 caused important biochemical changes in the larvae. We showed a decrease of the total protein, which might be caused by interference of FAMEs with the protein metabolism or an increase of their degradation in response to the physiological stress caused by the compounds, as the increase of the Na^+/K^+-ATPase activity. These biochemical changes observed may affect the development and survival of C. *quinquefasciatus* larvae.

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Compliance with Ethical Standards

Conflict of interest The authors declare that is no conflict of interest.

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