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# Age-Induced Carboxylesterase Expression in Acaricide-Resistant *Rhipicephalus microplus*

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**Abstract:** An acaricide-resistant and an acaricide-susceptible strain of *Rhipicephalus microplus* ticks, were evaluated during a 40 day larval development period for carboxylesterase expression on SDS-PAGE, with the objective of establishing the optimal larval age for carboxylesterase specific activity measurement. Experimental results demonstrated that carboxylesterase expression is located at 62 and 55 kDa polypeptides that exhibited enzymatic specific activity reaching a maximum between day 20 and 30 of larval development, larvae of this age showed carboxylesterase activity statistically higher ($p<0.001$ unpaired t-test) in acaricide resistant ticks when compared both strains, additionally, carboxylesterase activity in both strains was found to coincide with the vitellin degradation process. The assessment of the results suggested that carboxylesterase active enzymes are mature polypeptide products originating from higher mass protein precursors associated with the yolk nutrient storage reserves in preparation to pre-starving conditions. Experimental data obtained during this study demonstrated that in order to obtain reproducible results of carboxylesterase expression levels associated to acaricide resistance by zymograms, the enzymatic assays should be performed on 20 to 30 day old larvae.

**Key words:** *Rhipicephalus microplus*, *Boophilus microplus*, zymograms, acaricide-resistance, yolk, vitellin

## INTRODUCTION

One of the most insidious threats to the livestock industry in tropical and subtropical cattle grazing areas around the world, is by far the presence of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) (De Castro, 1997), this is because of the tick's role as vector of infective tick-borne diseases and blood-sucking debilitating effects on the tick infested cattle (Nuñez *et al*., 1985). Effective control of the cattle tick has been complicated because of the emergence of the acaricide-resistance phenomenon which is virtually against all commercial pesticide formulas available nowadays, reducing the control of the tick and increasing the costs of medical treatment on tick-born diseased cattle (Cossio-Bayugar *et al*., 2005). Currently, acaricide-resistance is better recognized on the field through failure to control the ticks rather than through early detection of acaricide-resistant

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ticks (Cossio-Bayugar *et al*., 2009), this is due to a grave technical void concerning reliable biochemical test for the assessment of acaricide-resistance in the laboratory (De Castro, 1997). Available acaricide-resistance diagnostic bioassays are based on tick larval packet test (Cossio-Bayugar *et al*., 2008), these bioassays are time-consuming and expensive, generating sometimes ambiguous results (Cossio-Bayugar *et al*., 2008). Because of the well-documented deficiencies of acaricide-resistance diagnostic methods, it is necessary to identify and characterize the biochemical mechanisms leading to acaricide-resistance in *R. microplus* in order to develop a rapid test oriented to identify acaricide-resistant ticks (Cossio-Bayugar *et al*., 2008). Carboxylesterases are ubiquitous enzymes among arthropods (Dauterman, 1982; Devonshire and Moores, 1982), these enzymes specialize in hydrolyzing ester bonds and share common affinity for some synthetic chromogenic substrates (Dauterman, 1982; Manchenko, 2003). Some of the *R. microplus* acaricide-resistance strains have been linked to detoxification of low-cost synthetic acaricide pyrethroids mediated by ester bond hydrolysis on the chemical structure by carboxylesterase (Riddles *et al*., 1983; De Jersey *et al*., 1985). Resistance to some other commonly used acaricide chemical formulations such as organophosphorous, has been related to an enzymatic detoxification process (Bull and Ahrens, 1988; Miranda *et al*., 1995; Cossio-Bayugar *et al*., 2009). Previous studies showed that carboxylesterase from *R. microplus*, isolated from larvae homogenates, exhibited 15 different carboxylesterase isozymes capable to hydrolyze non-specific synthetic substrates. Among these isozymes one 67 kilo-Dalton carboxylesterase was found to be capable to hydrolyze the acaricide cypermetrine (De Jersey *et al*., 1985). Other scientific works reported that the increases in esterase activity based on the enzymatic properties against synthetic substrates in *R. microplus* pyrethroid resistant strains (Jamroz *et al*., 2000; Baffi *et al*., 2008). Arthropods carboxylesterase function in nature, appears to be involved in the metabolism and the protection against the toxic effects of its xenobiotics compounds (Karunaratne and Hemingway, 2001), because of this protective role against natural toxins, previous reports attribute to this enzyme a first line of defense against pesticides in a large variety of pest arthropods including the sheep ectoparasite *Lucilia cuprina* (Newcomb *et al*., 1997; Campbell *et al*., 1997; Hartley *et al*., 2006), the human blood sucking mosquitoes *Culex* sp., *Aedes* sp. and *Anopheles* sp. (Hemingway and Karunaratne, 1998; Paton *et al*., 2000; Karunaratne and Hemingway, 2001) and the peach-potato aphid *Myzuz persicae* (Field and Devonshire, 1998; Field *et al*., 1999). However, the abundance of scientific information regarding the linkage between pesticide-resistance and carboxylesterase expression on a vast sample of pesticide-resistant arthropods including *R. microplus*, the carboxylesterase determination on the cattle tick has been unreliable due to fluctuation on enzymatic estimation during larval development. The unfed larvae are preferred for carboxylesterase measurement due to the abundance of bovine carboxylesterase in blood-engorged ticks that tend to mask the tick's enzymes.

The objective of this study was focused on carboxylesterase quantitative expression levels during larval development and established a possible age related fluctuation on acaricide resistant *R. microplus* carboxylesterase expression, compared to an acaricide sensitive strain of ticks.

## MATERIALS AND METHODS

This study was done from March 2008 to February 2009 at the facilities of: Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, INIFAP.

**Ticks**

Two *R. microplus* reference strains were used for this study. Both strains have been cultured and tested for acaricide resistance, as part of the cattle tick monitoring programs of the Agriculture Ministry (SAGARPA) of the Mexican Federal Government. Ticks were maintained at: Departamento de Ectoparásitos y Dípteros del Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA-SAGARPA). A multiple acaricide resistant ticks named Mora (Mo), was used as reference for high levels of esterase expression. The susceptible reference strain (Su) was used for comparison purposes. Each tick strain was cultured separately according to previous report (Cossio-Bayugar *et al*., 2008) by infesting a bovine with $2\times10^4$ 10-15 day old larvae, engorged females were collected 21 days after infestation, placed in Petri dishes for oviposition in groups of ten engorged ticks for each strain, then incubated at 28°C in 80% relative moisture until complete oviposition. The eggs were collected, weighted and aliquoted in vials with 200 mg each and kept at 28°C in 80% relative moisture until larvae hatching.

**Acaricide Resistance Bioassays**

Ticks assessment for their pyrethroids, organophosphorous and amidines toxicological profile demonstrated by acaricide discriminant doses bioassays (Cossio-Bayugar *et al*., 2008). Bioassays were run by using trichloro ethylene diluted acaricides at the following concentration: Coumaphos 0.2%, Chlorfenvinphos 0.2%, Cypermetrine 0.5%, Deltametrine 0.09%, Amitraz 0.0003%. One milliliter of each dilution was applied evenly to a 7 by 9 cm piece of filter paper. The trichloroethylene was allowed to evaporate from the filter paper for 2 h. Treated papers were then folded in half and sealed on the sides with paper clips, this formed a packet into which approximately 100 larvae were placed and then the top of the packet was sealed with another clip. The packets were kept at 28°C, 80% relative moisture for 24 h, after that the packets were removed from incubation and opened, live and death larva were counted and the data was processed as percentage of mortality for each tick group under every acaricide concentration.

**SDS-PAGE Carboxylesterase Zymogram Determination**

Two hundred milligram of larvae at 1, 10, 20, 30 and 40 day old were macerated separately in a ceramic mortar adding 1 mL of Phosphate Buffer Solution (PBS) 0.1 M pH 7.2 according to reported protocol (Miranda *et al*., 1995). The protein content of the tick extracts was determined by the Bradford methodology (Bradford, 1976). The 50 µg of protein from each tick extract were applied to separate wells. Sodium Duodecyl Sulphate Polyacrilamide Electrophorectic Gels (SDS-PAGE) was used with the electric current of 100 V/15 mA for 80 min (Laemmli, 1970). After electrophoretic separation, the SDS-PAGE gels were washed with gentle agitation for 15 min in 50 mL of PBS-Triton X100 0.2% for three times. Afterwards SDS-PAGE gels were submerged in a carboxylesterase substrate solution containing 100 mL of 0.05 M Phosphate buffer pH 7.2, 10 mg of α-naphtyl acetate and 50 mg of Fast Garner (Miranda *et al*., 1995; Manchenko, 2003). The SDS-PAGE gels were incubated at room temperature with gentle agitation until the appearance of red bands. The SDS-PAGE gels were washed in water after carboxylesterase enzymatic staining, afterwards, gels were protein-counterstained in 0.1% of coomassie brilliant blue (Sambrook and Russel, 2001). Processed gels were preserved in 3% acetic acid and zymograms were recorded as digital images using an Epi Chemi® (UVP life sciences, USA) image recorder, feeding the

software LabWorks 4® analysis software in densitometer mode. A Prestained Protein Molecular Mass Standard was commercially obtained (PageRuler™, Fermentas. USA); the densitometer was calibrated with digital images of this standard included in every SDS-PAGE zymogram in order to obtain automatically the Mass of each band within the zymogram according to the instructions of the LabWorks 4® software (UVP life sciences. USA). Densitograms of five different experiments for each strain, were converted to Optical Density (OD) measurements. Means and standard errors of each band registered for the five different experiments were graphically represented. Statistical comparisons between the carboxylesterase OD mean values from the acaricide-resistant ticks and corresponding carboxylesterase OD values from the susceptible reference tick strain, were made by an unpaired student's t-test using the GraphPad® Software (GraphPad Software Inc. USA).

## RESULTS AND DISCUSSION

In Table 1, the reference susceptible (Su) ticks showed 100% mortality when exposed to all acaricides used. The results of this table were considered a comparison reference for both acaricide toxicological test and carboxylesterase activity levels. Mo acaricide resistant strain showed high levels of Pyrethroid (Ps) and Organophosphorus (Op) resistance with a 0% mortality when exposed to: chlorfenvinphos, coumaphos, diazinon, chlorpiriphos, deltametrine, cypermetrine and flumetrine (Table 1), while it exhibited acaricide susceptibility with 100% mortality rate to Amitraz, under acaricide dosages capable of killing a 100% of the reference Su strain.

Carboxylesterase isozymes showed a strong specific reaction in two polypeptidic bands of 55 and 62 kDa, which were detected on 20 and 30 day old larvae, respectively (Fig. 1a). Specific carboxylesterase enzymatic reaction measured by densitometry showed an statistically significant increased on carboxylesterase activity with respect to basal carboxylesterase activity detected on day 1 after larvae hatching ($p<0.001$ unpaired t-test) on bands located at 55 and 62 kDa (Fig. 1, 2), enzymatic activity also showed a statistically significant difference ($p<0.01$ unpaired t-test) when compared both strains of ticks carboxylesterase activity at 55 and 62 kDa bands (Fig. 2a). Protein counterstaining showed vitellin polypeptides of 125, 77 and 68 kDa (Fig. 2b) which were consumed as time progressed reaching vitellin depletion and tick starvation conditions at day 40, same time at which carboxylesterase activity ceased.

Pesticide resistance in arthropods exhibited complex biochemical and genetic multifactorial origins (Hemingway and Karunarte, 1998; Cossio-Bayugar *et al*., 2008), these factors may include an altered pesticide target site (Bull and Ahrens, 1988) and/or increased enzymatic detoxification (Feyereisen, 1995), detoxifying enzymes are common responsible of arthropod pesticide resistance mechanism and among some other type of enzymes, carboxylesterases participate naturally in enhanced metabolism of drugs and pesticides in several arthropod models (Karunaratne and Hemingway, 2001). The availability of an extensive scientific background on this subject, makes a logical choice to search for an

Table 1: Acaricide bioassays results on two different strains of ticks

| | Organophosphorous | | | Pyrethroids | | | Amidines | |
|---|---|---|---|---|---|---|---|---|
| Tick strain | Chlorfenvinphos | Coumaphos | Diazinon | Chlorpiriphos | Cypermetrine | Deltametrine | Flumetrine | Amitraz |
| Su (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Mo (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |

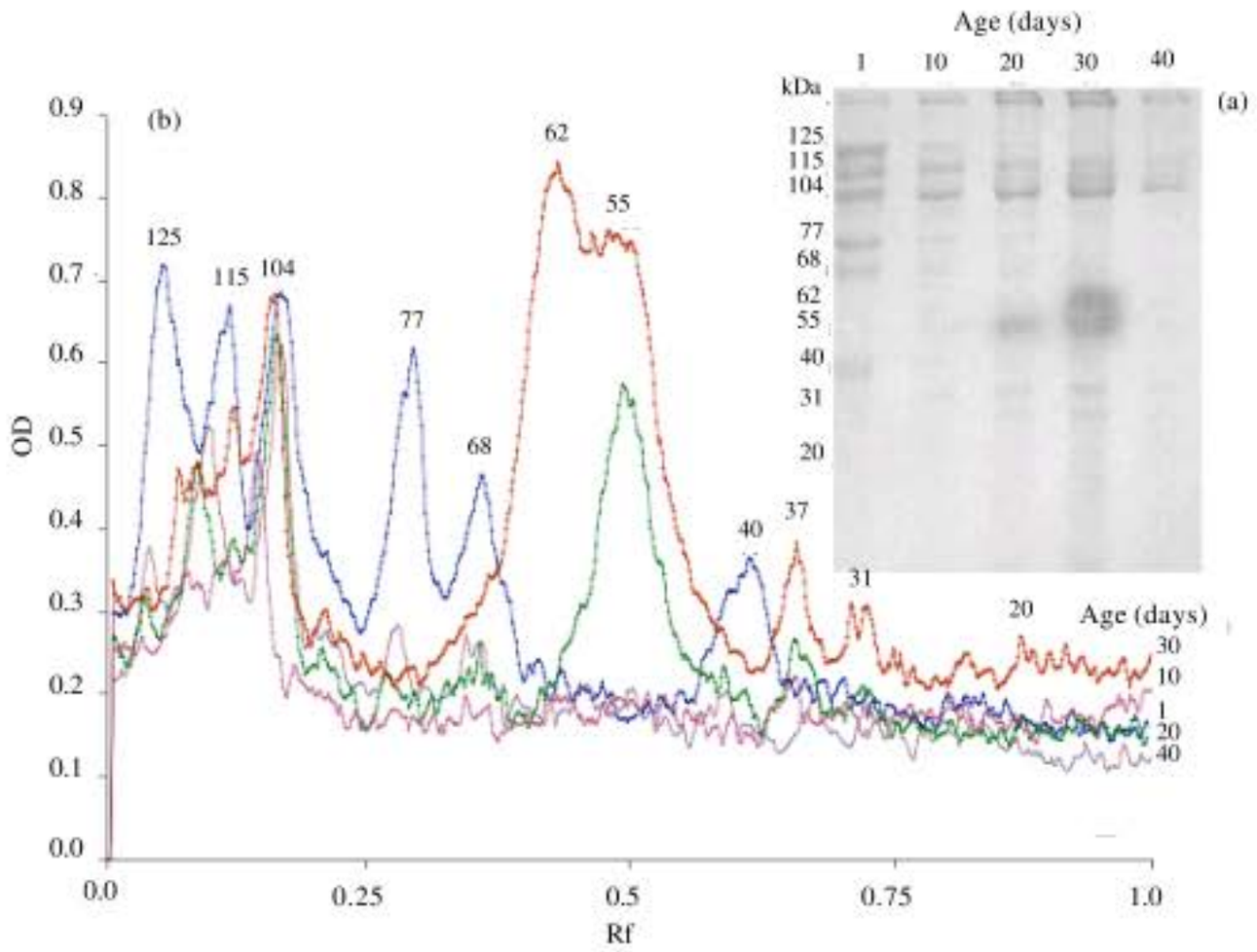


Fig. 1: Example of SDS-PAGE carboxylesterase zymogram and densitogram. Specific carboxylesterase activity was identified by zymograms in SDS-PAGE on larvae of different ages. (a) SDS-PAGE image of Mo strain during 1, 10, 20, 30 and 40 days of age. SDS-PAGE results were transformed into OD values by densitometry analysis. (b) Numbers at the left side of the SDS-PAGE image and on the main peaks on the densitogram represent the molecular protein mass of the main polypeptidic bands in kDa. Five different experiments for each strain were evaluated in order to compute the statistical comparisons between acaricide-susceptible and acaricide-resistant strains

increase in carboxylesterase activity when a pesticide resistant arthropod is detected. In accordance to the above mentioned literature, during our analysis we did find a significant increase in carboxylesterase activity levels ($p<0.001$ unpaired t test) in the resistant tick strain Mo (Table 1), (Fig. 1a). The results of Mo tick strain had high levels of carboxylesterase activity support a direct linkage relationship to the high levels of resistance found on this strain of ticks to organophosphorous and pyrethroid formulations used on the toxicological bioassays (Table 1). These results were in agreement with a carboxylesterase enhanced metabolic detoxification scenario conferring acaricide resistance in *R. microplus* as demonstrated in previous reports (Dauterman, 1982; Miranda *et al*., 1995). Carboxylesterase specific enzymatic activity Mo strain was located on two 55 and 62 kDa polypeptidic bands detected on 20 and 30 days old larvae (Fig. 1, 2), this specific carboxylesterase activity showed a statistically significant difference ($p<0.001$ Unpaired t-test) when compared to acaricide susceptible strain, carboxylesterase levels fluctuated during the larvae aging reaching a peak between 20 to 30 days of larvae development. Protein counterstaining of SDS-PAGE zymograms allowed detection of the vitellin degradation progress on polypeptides 125, 77 and 68 kDa (Fig. 1a, b) this polypeptide degradation is in accordance to previous reports associated with proteolytic activity of a aspartic proteinase precursor on high molecular weight vitellin polypeptides (Chinzei *et al*., 1983; Rosell-Davies and Cooms,

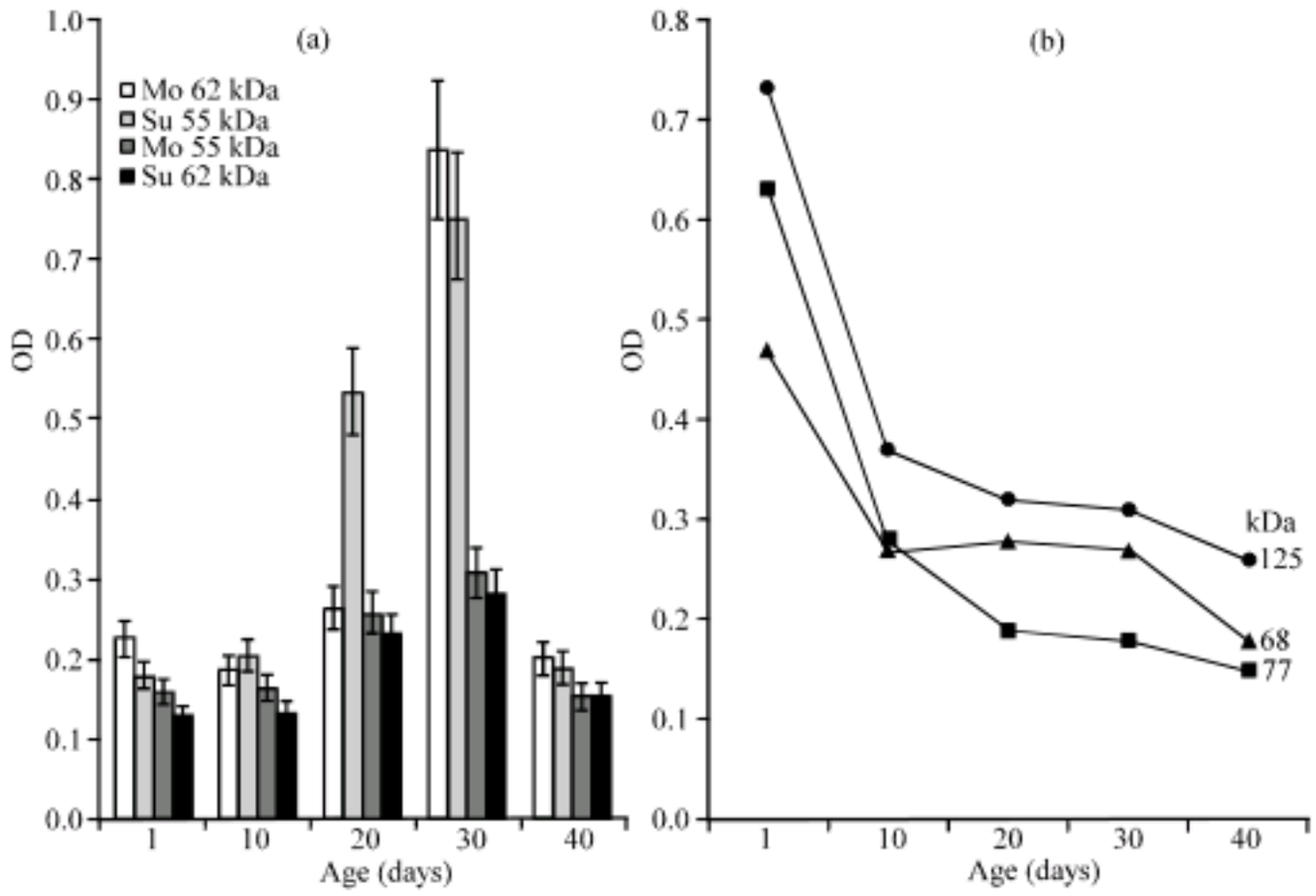


Fig. 2: Compared carboxylesterase values and vitellin degradation. (a) Carboxylesterase specific activity is located in two polypeptidic bands located at 55 and 62 kDa which are distinctively expressed on acaricide-resistant ticks (Mo) when compared against acaricide-susceptible ticks (Su) . (b) Vitellin polypeptides on both strains, were located at 125, 77 and 68 kDa bands and total vitellin consumption was achieved at 40 day old larvae B

1989; Logullo *et al*., 1998), supplying this way nutrients and metabolites during larval free living stage, SDS-PAGE polypeptidic pattern also exhibited vitellin polypeptides at 125, 77 and 68 kDa disappearing from detection both by visual and densitometry (Fig. 1a, b) at 40 days old larvae. Vitellin degradation showed coincide with carboxylesterase activity peak at day 20 and 30 which suggested a relationship between both phenomena. Vitellin proteolytic degradation carboxylesterase relationship may be explained by the fact that carboxylesterase activation has been related with the modulation of juvenoids, which is a powerful hormone-like molecules that promotes the ecdisys and ovarian development in soft and hard ticks (Chinzei *et al*., 1983; Rosell-Davies and Cooms, 1989). Belozerov (2006) illustrated that such hormone was like chemical compounds modulated by degrading its ester bound rendering an inactive metabolite, so that the carboxylesterase enzymatic activity was required during a very precise time line along the larvae's development and maturation. The experimental data presented on this study showed an evident chronological appearance of carboxylesterase enzymatic activity which coincided with vitellin degradation, the time-line of larval development occurred with vitellin polypeptides depletion and the pre-starvation condition settle for the larvae, under natural field conditions larvae of the same age were able to find a suitable bovine host within a month and start the blood feeding process, otherwise face death by starvation (Nuñez *et al*., 1985). This could explain why several critical metabolic processes appear to happen at 20 to 30 days of larval development identifiable during this study at the total polypeptidic pattern and carboxylesterase activity (Fig. 1, 2). Present results were consistent with distinctive carboxylesterase phenotypes in tested strains *R. microplus* which included the phenotypes with normal levels of carboxylesterase

activity for the acaricide sensitive ticks and phenotypes increased with carboxylesterase activity levels at 55 and 62 kDa on acaricide resistant ticks, therefore the phenotypes for carboxylesterase expression in tested strains of *R. microplus* should be tested in coming field surveys and acaricide resistance field monitoring.

## CONCLUSION

In order to establish a definitive trends and biochemical linkage relationship between acaricide resistance and carboxylesterase levels in the cattle tick, further studies should be done on 20 to 30 days old larvae in order to establish a diagnostic predictive parameter based on zymograms of carboxylesterases and other detoxifying enzymes, this scientific research may useful for the management of problems on acaricide resistance in *R. microplus.*

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