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## Research Article

## Comparative Activity of Metabolic Enzymes of Acaricides in F1 Larvae of *Rhipicephalus (Boophilus)* *Microplus* (Acar. Ixodidae) and Susceptible Strains IVRI-I: Correlation with Massive Selection of Resistant Ticks on Farms in Benin, West Africa -

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## Abstract

The role of enzymes in the metabolic processes of acaricides is well known for their crucial involvement in the resistance mechanisms of several arthropods, including ticks and other mites. The present study therefore aimed to evaluate the level of expression of esterases, monooxygenases and glutathione-S-transferases in F1 populations of *Rhipicephalus (Boophilus) microplus* not exposed to an acaricide molecule. The results obtained indicate a significant difference ( $p < 0.0001$ ) between the level of expression of these enzymes in *R. (B.) microplus* populations and the reference susceptible strain (IVRI-I). This may be positively correlated with resistance of *R. (B.) microplus* to common acaricides. These results thus lift a corner of veil on a massive selection of individuals resistant to conventional molecules used in the fight against ticks in Benin. However, this population is not yet multiresistant because the level of expression of these enzymes compared to that of the multiresistant IVRI-V strain, remains significantly ( $p < 0.0001$ ) lower. Therefore, it is urgent to adopt strategies for the use of acaricides in the control of these ticks.

**Keywords:** Enzymes; Metabolic resistance; Acaricides; *Rhipicephalus (Boophilus) microplus*

## INTRODUCTION

Tick resistance to acaricides is a global phenomenon and almost all tropical and sub-tropical countries face the problem of resistance to one or more acaricide formulations [1]. It renders available products and vector control strategies ineffective, resulting in increased prevalence of pathogens and the diseases they transmit [2-4]. Moreover it requires an increase in the quantities of products used and the development of new molecules or formulations thus increasing the cost.

Over the last two decades, tick research has shown numerous cases of acaricide resistance in *R. (B.) microplus* [5-11] and the role of overexpression of metabolic enzymes such as Esterases (ESTs), Uthathione-S-Transferases (GSTs) and cytochrome P450 is reported to be involved in the metabolic detoxification of acaricides [12, 13].

However, most of his work in the laboratory quantifies the enzymatic activity of field strains after bioassays such as AIT (Adult Immersion Test), LIT (Larval Immersion Test) and LPT (Larval Packet Test) with conventional acaricides. But, we all know that any organism that suffers an aggression of any kind is called up on to defend itself by reacting. Therefore does not account for the real level of expression of its enzymes in unexposed populations after years of massive and abusive use of synthetic molecules in the field. The present study therefore proposes to quantify the enzyme profile in an F1 population of *R. (B.) microplus* not exposed to synthetic acaricides. The objective is to evaluate the level of expression of detoxification enzymes in populations of *R. (B.) microplus* of Kpinnou.

## MATERIALS AND METHODS

### Obtaining the F1 larvae

A population of ticks engorged females (~200 ticks) was collected from Girolando cattle from the Kpinnou Breeding Farm (FEK) in the commune of Athieme (Mono County, 6°35'58.78"North and 1°44'26. 84"East). The animals have not been treated recently with a conventional acaricide formulation, the last treatment being more than 30 days ago.

The collected ticks were kept alive in an isothermal box, which guaranteed good aeration and relative humidity until the National Laboratory of Veterinary Parasitology (LNPV).

### Morphological identification and egg-laying of female *Rhipicephalus (Boophilus) microplus*

The morphological identification of the species *Rhipicephalus (Boophilus) microplus* was carried out at the National Laboratories of

Veterinary Parasitology using OPTIKA binocular magnifiers at 10X magnification, according to the dichotomous identification key of Walker, et al. [14]. The *Boophilus* subgenus was identified on the basis of the hexagonal-based capitulum, presence of distinct eyes, Coxa I with small paired spurs, Coxa II to IV without spurs, presence of small palm segments.

The ticks were then laid at laboratory room temperature ( $28^{\circ}\text{C} \pm 2$ ) until oviposition. After oviposition, which usually started 72 h after, and lasted about 16 days, the eggs were carefully separated from the females and weighed at a rate of 0.1g/package in standard FAO [15] larval packages using the precision balance (*Explorer® Pro*; max = 210 g; d = 0.1) and then incubated still at laboratory room temperature ( $28^{\circ}\text{C} \pm 2$ ) until hatching, which took place after 21- 23 days.

At hatching, F1 larvae take about 15 days to complete their chitinization. Once chitinization is complete, the larvae with negative geotropism move up to the upper edge of the packs where they gather in clusters (swarms). This behaviour is similar to that observed in nature where they climb to the end of the grass stems to wait for the passage of a possible host to take the blood meal. It is therefore warming larvae, i.e. larvae capable of taking the blood meal that have been manipulated for this study.

### Reference tick strains

The acaricide-sensitive reference strain IVRI-I (Reg. No. NBAII/IVRI/BM/1/1998) and the multiresistant strain IVRI-V (registration pending) of *R. (B.) microplus* maintained in the

Entomology Laboratory, Parasitology Division, Indian Veterinary Research Institute, and used for standardization and validation of biochemical assays were used as reference for resistance level characterization.

### Biochemical assays of detoxification enzymatic activities

We have worked on 3 of the most frequent enzyme families known for their considerable role in acaricide resistance, namely cytochrome P450 monooxygenases (Cyt P450), Glutathion-S-Transferases (GST) and esterases  $\alpha$  and  $\beta$  [ 16- 20].

The activities of Cyt P450, Esterase and GST are expressed as the amount of product formed per mg of protein from enzyme extracts. Protein quantities are determined with bovine serum albumin as reference.

### Preservation and preparation of ticks

The presence of enzyme activities appropriate for acaricide resistance is often performed using microplate assays .As larvae must



not be destroyed before use, the F1 obtained were stored at  $-80^{\circ}\text{C}$ . The standardized protocol for characterizing acaricide resistance to field ticks by Fular, et al. [21] (in press) was used to assess enzyme activity.

Pools of 40 *R. (B.) microplus* larvae with an average of 15 days were formed in Eppendorf tubes. A total of 40 samples were collected.

Since enzymes degrade quickly at room temperature, all the work was done on ice. The ticks were crushed in 200 ml of distilled water using sterilized plungers and an Argos brand pellet. The crushed material was centrifuged at 12,000 rpm for 2 min in a centrifuge of the type MIKRO<sup>®</sup> 200R V2.01, and the supernatant was recovered in sequence from wells of a microplate with a micropipette. A volume of  $2 \times 20 \mu\text{l}$  was distributed in 2 wells of a microplate for oxidases. The other 4 microplates were filled with 10 ml in 2 replicates for the amount of proteins and the evaluation of GST activities and then esterases.

### Measurement of Glutathione S-Transferase (GST) activity

The enzymatic activity of GST was determined by spectrophotometer monitoring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) with glutathione (microfluorometric method).  $10 \mu\text{l}$  of tick meal in two replicas was placed in each Nunc plate well and  $200 \mu\text{l}$  of a solution of 0.06 g glutathione in reduced form (GSH) and 0.013 g

CDNB (1-chloro-2,4-dinitrobenzene) previously and completely dissolved in 1 ml methanol was added.

The change in optical density resulting from the binding between the thiol group of glutathione and the CDNB (substrate) is measured at 340 nm for 5 minutes at contact ( $t_0$ ) and after 1 minute of reaction ( $t_1$ ), with a microplate spectrophotometer. For the control measurements, the same protocol was followed by replacing the extraction solutions containing the enzymes with the extraction solution alone (experimental blank). The results were expressed in nanomoles of products (conjugated form of glutathione) per milligram of protein per minute.

### Measurement of oxydase activity

After having put  $20 \mu\text{l}$  of broth in two replicas in each well, we added  $80 \mu\text{l}$  of a solution of 3,3',5,5' Tetramethyl Benzidine (TMBZ) 0.25M pH 5.0 dissolved in 6 ml methanol. Then 18 ml of 0.25M Sodium Acetate Buffer ( $\text{NaC}_2\text{H}_3\text{O}_2$ ) pH 5.0 and  $25 \mu\text{l}$  of a 3% hydrogen peroxide solution were added to each well. The plate was then incubated for 30 min at room temperature with a lid and read at 630 nm every 10 min.

The oxidase activity of each tick in nmol P450equivalent units/mg protein was calculated as follows nmol P450 equivalent units per  $20 \mu\text{l}$  of feed / ( $2 \times$  (mg protein in  $10 \mu\text{l}$  feed))

### Measurement of non-specific esterase activity ( $\alpha$ - and $\beta$ -naphthol)

The esterase activities were measured with the microfluorometric method by measuring the production of  $\alpha$ - and  $\beta$ -naphthol from a substrate of  $\alpha$ - and  $\beta$ -Naphthyl Acetate (NA). The reaction is performed in multi-well plates. Thus,  $10 \mu\text{l}$  of the broth in two replicas were put in each plate well to which we added  $90 \mu\text{l}$  of 1% Triton Phosphate Saline Buffer (PBS)  $\text{pH}^{\text{H}}$

After mixing, the plate is left for 10 minutes at room temperature. Then  $100 \mu\text{l}$  of a solution consisting of  $600 \mu\text{l}$  0.3 M alpha-Naphthyl acetate (or beta-Naphthyl acetate), 2.5 ml 1% Triton PBS buffer  $\text{pH}^{\text{H}}$

6.5 and 7 ml water is added. The plate will be incubated again for 30 min at room temperature. The reaction is stopped by adding  $100 \mu\text{l}$  of a solution consisting of 0.010 g Fast Garnett Salt (FGBC) dissolved in 10ml distilled water. After mixing, the plate is incubated for 10 minutes at room temperature with a lid. It will then be read at 550 nm as an endpoint. Absorbances of the reaction medium were recorded at 527 nm for  $\alpha$ -naphthyl acetate and at 505 nm for  $\beta$ -naphthyl acetate using a microplate reader. The tick esterase activity in  $\mu\text{mol}$  of alpha-Naphthol produced/min/mg protein was determined as follows (alpha-Naphthol in  $\mu\text{mol}$  produced by  $10 \mu\text{l}$  of grinding / amount of protein (in mg) in  $10 \mu\text{l}$  of grinding) / 30

### Statistical analysis

Comparative measurements of mean enzyme activities were performed by a one-factor analysis of variance (ANOVA) using Graph Pad Prism 5 software, between susceptible a IVRI-I strains and *R. (B.) microplus* from the Kpinnou farm on the one hand, and between *R. (B.) microplus* ticks and multi-resistant IVRI-V strains other hand. The nonparametric Mann-Whitney U test was used to indicate a significant increase in the different means.

## RESULTS

### Esterase activity

The average specific activity of  $\alpha$  and  $\beta$  esterases of *R. (B.) microplus* populations of the Kpinnou farm not exposed to acaricides is estimated to be  $0.160 \pm 0.045 \mu\text{mol}$   $\alpha$ - naphthol formed/min/mg protein and  $0.166 \pm 0.048 \mu\text{mol}$   $\beta$ -naphthol formed/min/mg protein, respectively, versus  $0.099 \pm 0.031 \mu\text{mol}$   $\alpha$ -naphthol formed/min/mg protein ( $\alpha$ -esterase) and  $0.110 \pm 0.034$  ( $\beta$  esterase)  $\mu\text{mol}$   $\beta$ -naphthol formed/min/mg protein for the sensitive reference strain (IVRI-I) (Table 1).

**Table 1:** Average enzyme activities of the *R. (B.) microplus* populations of the Kpinnou farm compared to IVRI-I and IVRI-V strains.

Ticks populations considered	$\alpha$ -esterase ( $\mu\text{mol}$ $\alpha$ -naph, /min/mg)	$\beta$ -esterase ( $\mu\text{mol}$ $\beta$ -naph, /min/mg)	Monoxygenase (nmol P450/mg)	GST (nmol GSH conj./ min/mg)
IVRI-I	$0.99^{\text{a}} \pm 0.031$	$0.109^{\text{a}} \pm 0.034$	$0.052^{\text{a}} \pm 0.018$	$0.13^{\text{a}} \pm 0.023$
Kpinnou	$0.159^{\text{b}} \pm 0.044$	$0.165^{\text{b}} \pm 0.047$	$0.094^{\text{b}} \pm 0.031$	$0.411^{\text{b}} \pm 0.159$
IVRI-V	$0.235^{\text{c}} \pm 0.042$	$0.243^{\text{c}} \pm 0.04$	$0.121^{\text{c}} \pm 0.039$	$0.521^{\text{c}} \pm 0.129$

Mean  $\pm$  SE; significant difference ( $p < 0.0001$ )

This observed difference is very significant ( $p < 0.0001$ ) and the  $\alpha$  and  $\beta$  esterases of *R. (B.) microplus* populations of Kpinnou are 1.61 and 1.50 times higher respectively than those of the susceptible IVRI-I strain (Figure 1). On the other hand, these esterase activities recorded for the field population are significantly lower ( $p < 0.0001$ ) compared to those of the multiresistant

IVRI-V strain ( $0.236 \pm 0.043 \mu\text{mol}$   $\alpha$ -naphthol formed/min/mg protein for  $\alpha$ -esterase and  $0.244 \pm 0.040 \mu\text{mol}$   $\beta$ -naphthol formed/min/mg protein for  $\beta$ -esterase).

### Cyt P450 monooxygenase activity

Biochemical tests showed that the oxidase activity of the sensitive IVRI-I strain ( $0.052 \pm 0.018$  nmol P450/min/mg protein) was significantly different ( $p < 0.0001$ ) from that recorded in *R. (B.) microplus* Kpinnou populations ( $0.095 \pm 0.031$  nmol P450/min/mg

protein). However, this activity remains lower than that of the multi-resistant IVRI-V strain ( $0.121 \pm 0.039$  P450/min/mg protein) (Figure 2).

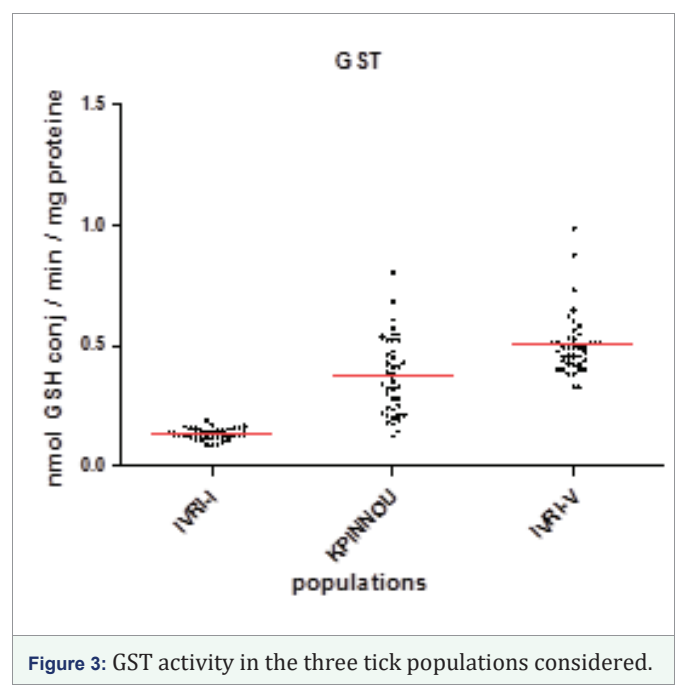
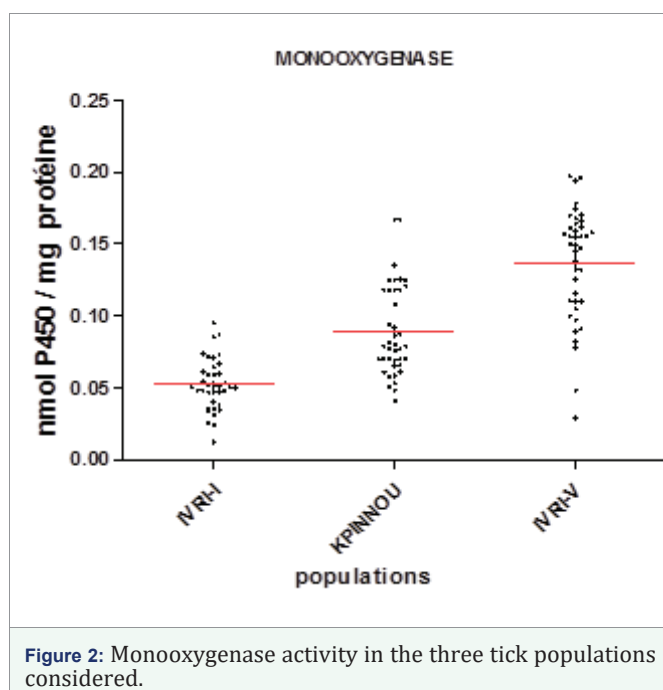
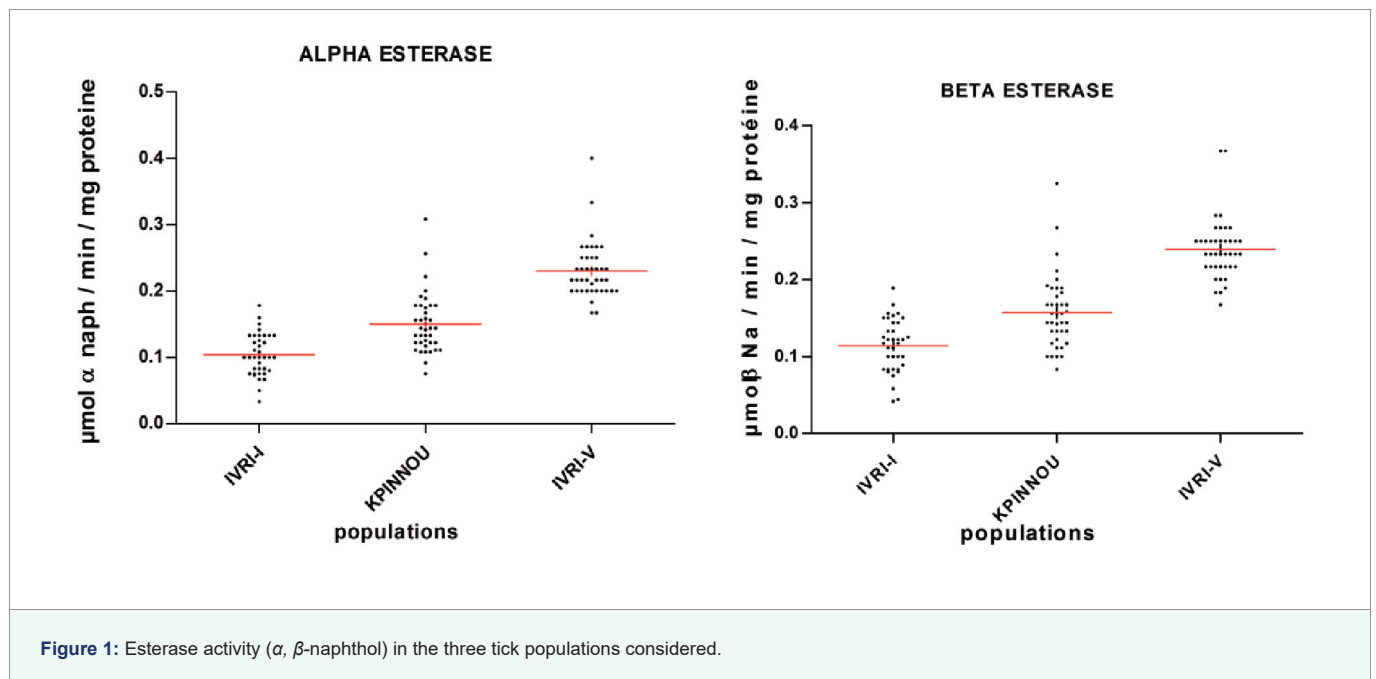
### Glutathione-S-transferase (GST) activity

The results in figure 3 show that the mean GST activity of *R. (B.) microplus* populations of Kpinnou was 3.14 times ( $0.411 \pm 0.160$  nmol GSH conj./min/mg protein) higher ( $p < 0.0001$ ) than that of the sensitive reference strain IVRI-I ( $0.131 \pm 0.023$  nmol GSH conj./min/mg protein). This overproduction of GST recorded in *R. (B.) microplus* populations of Kpinnou are, on the other hand, significantly lower ( $p < 0.0003$ ) than those of the resistant IVRI-V strain ( $0.522 \pm 0.129$  nmol GSHconj./min/mg protein) as well as in esterases and oxydases.

## DISCUSSION

Among the two major mechanisms involved in the development of resistance, the role of enzyme over expression on acaricide metabolism has been established in a number of arthropod species [22-26].

In this study, a significant difference ( $p < 0.001$ ) in enzyme activity (esterases, GST and cytochrome P450 monooxygenase) was observed between the reference susceptible tick strain (IVRI-I) and *microplus R. (B.)* populations not exposed to acaricide. This highlights the whole issue of massive selection of individuals resistant to acaricides commonly used in breeding. Indeed, esterase elevation is a well-known mechanism for detoxification of organophosphates







and, to a lesser extent, for carbamate resistance in insects and mites [27]. Its involvement in the detoxification of pyrethroids has also been demonstrated in *Tetranychus urticae* mites [28]. These enzymes hydrolyze ester bonds, cleaving xenobiotics and generating acid and alcohol as metabolites.

Although in arthropods, this class of enzymes is involved in various important physiological activities such as reproductive behaviour [29] and nervous system function [30], the esterase activity obtained from *R. (B.) microplus* populations of Kpinnou was higher than that of the reference susceptible strain and cannot be positively correlated with the development of resistance to commercially available acaricide molecules.

Similarly, the high level of GST expression recorded in Kpinnou's *R. (B.) microplus* ticks indicates a strong aptitude for the development of resistance. Li, et al. [5] reported that the increase in GST activity may be related to the metabolic detoxification of OPs that conferred resistance to San Roman *R. (B.) microplus* populations. He, et al. [31] and Li, et al. [5,32] showed a significant correlation between GST overproduction and resistance of *R. (B.)*

*microplus* to coumaphos, diazinon and amitraz. A 4 to 6-fold increase in GST activity among populations of *R. bursa* resistant to synthetic pyrethroids compared to a susceptible population was demonstrated by Enayati, et al. [33]. Their conclusion appears to be consistent with the present results where they found a 3.14-fold increase in GST activity of IVRI-I strain. Indeed,

GSTs represent a group of multigenic isozymes classified into five families of genes, namely alpha ( $\alpha$ ), mu ( $\mu$ ), pi ( $\Pi$ ), theta ( $\theta$ ), and sigma ( $\sigma$ ), based on their sequence similarity and cross immunoreactivity [34]. They are generally involved in the detoxification of endo- and xenobiotics. GSTs are present in all aerobic eukaryotes as catalysts for the conjugation of the reduced form of Glutathione (GSH) with molecules with an electrophilic center, a thioether bond is formed between the sulphur of the GST and the substrate, resulting in the resulting conjugates containing more soluble water and facilitating their excretion from the cells [35].

Also, studies have shown that cytochrome P450 is involved in the resistance of ticks to various acaricides. Metabolic resistance to synthetic pyrethroids due to cytochrome P450 activity has already been observed in the Mexican population of *R. (B.) microplus* [9]. Enayati, et al. [33] reported an increase in monooxygenase content up to 4.9- fold in the pyrethroid-resistant *R. bursa* population of Iran compared to the susceptible strain. In this study, the significant level of monooxygenase expression ( $p < 0.0001$ ) recorded in *R. (B.) microplus* ticks from the Kpinnou farm was 1.83 times higher than that of the reference susceptible strain (IVRI-I). This is a strong indication of the possible role of monooxygenases in detoxifying active acaricides or preventing them from reaching their target site.

Cytochrome P450 is a superfamily of enzymes, also called CYP, present in all organisms. More than 200 CYP enzymes have been described and code for proteins whose structural characteristics are retained even when sequence similarity is relatively low [36]. Cytochrome P450 enzymes play different roles in eucaryotes, including carbon uptake, biosynthesis of hormones and cellular components, and degradation of xenobiotics via an oxidation reaction that takes place through conformational changes.

Moreover, it should be noted that, despite this overproduction of detoxification enzymes in the *R. (B.) microplus* populations of the

Kpinnou farm, the level of expression of these enzymes is significantly different ( $p < 0.0001$ ) from that recorded in the multiresistant strain (IVRI-V).

## CONCLUSION

Resistance to acaricides is a widespread phenomenon in bovine tick populations. The role of enzymes such as cytochromes P450. Esterases and GST in the metabolic processes of acaricides has been well documented. This metabolic process actually results from the degradation and increased excretion of xenobiotic molecules, a mechanism that probably evolved from an ancestral ability to degrade food toxins. Therefore, these enzymes are expected to be present at a slightly moderate level in tick populations in livestock. In the present

study, significantly different values ( $p < 0.0001$ ) of these enzymes were recorded in *R. (B.) microplus* populations from the Kpinnou farm not exposed to an acaricide molecule compared to the reference susceptible strain (IVRI-I). These results therefore shed some light on the massive selection of individuals resistant to the conventional molecules used to control these ectoparasites. However, further studies are necessary to identify the classes of acaricide molecules to which these ticks are resistant in Benin in order to adopt strategies for the use of acaricides in the fight against ticks.

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