Esterase profile in the postembryonic development of *Rhipicephalus microplus*

Milla Alves Baffi[1], Cícero Donizete Pereira[2], Guilherme Rocha Lino de Souza[2], Carlos Roberto Ceron[3] and Ana Maria Bonetti[2]

[1]Universidade Estadual de Londrina, Dep. de Biologia Geral, Lab. de Genética Molecular, Rod. Celso Garcia Cid (PR445), Km 380, Caixa Postal 6001, CEP 86051-990 Londrina, PR, Brazil. E-mail: millabaffi@yahoo.com.br [2]Universidade Federal de Uberlândia, Instituto de Genética e Bioquímica, Lab. de Genética, Campus Umuarama, Av. Acre, s/nº, Bloco 2E, Sala 33, CEP 38405-902 Uberlândia, MG, Brazil. E-mail: cdpereira@ufu.br, grlino@hotmail.com, ambonetti@hotmail.com [3]Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas, Dep. de Química e Ciências Ambientais, Rua Cristóvão Colombo 2265, CEP 15054-000 São José do Rio Preto, SP, Brazil. E-mail: diretoria@ibilce.unesp.br

Abstract – The objective of this work was to analyze the pattern of esterase activity in the development stages of *Rhipicephalus microplus* by nondenaturing polyacrylamide gel electrophoresis using specific staining for esterase. The electrophoretical results revealed the presence of nine regions displaying esterase activity, stained with both alpha-naphthyl acetate and beta-naphthyl acetate, and classified as alpha-beta-esterase. Stage-specific esterases were found, with the first nymphal and larval stages showing the greatest esterase activity throughout the development. An esterase called EST-4 was detected only in males and was considered sex-specific. There are differences in the esterase profile among the different postembryonic development stages of *R. microplus*.

Index terms: cattle tick, resistance to acaricides, tick control.

Perfil esterásico durante o desenvolvimento pós-embrionário de *Rhipicephalus microplus*

Resumo – O objetivo deste trabalho foi analisar o padrão de atividade da esterase nos estágios de desenvolvimento de *Rhipicephalus microplus* por meio de eletroforese em gel de poliacrilamida não desnaturante, com uso de coloração específica para esterase. Observou-se a presença de nove regiões com atividade esterásica, coradas tanto pelo alfa-naftil acetato como pelo beta-naftil acetato, e definidas como alfa-beta-esterases. Foram detectadas esterases estágio-específicas, e os estágios de ninfa de primeiro estádio e de larva foram os que mostraram maior atividade esterásica durante todo o desenvolvimento. A esterase EST-4 foi detectada apenas em machos e considerada sexo-específica. Existem diferenças quanto ao perfil esterásico nos diferentes estágios de desenvolvimento pós-embrionário de *R. microplus*.

Termos para indexação: carrapato bovino, resistência a acaricidas, controle de carrapatos.

Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is one of the most important ectoparasites in Brazilian cattle ranching and, according to the Ministry of Agriculture, it is responsible for annual losses of two billion dollars (Grisi et al., 2002). This parasite increases mortality, causes huge losses in milk production, natality, massive use of pesticides, and losses to the leather industry. Moreover, *R. microplus* is the main vector of *Babesia* and *Anaplasma*, which cause the babesiosis and anaplasmosis diseases in cattle (De Castro, 1997).

The traditional control method is based mostly on the use of acaricides: arsenic derivatives, organophosphates, amidines, carbamates and pyrethroids. The indiscriminate use of these compounds has led to the selection of resistant strains to the lethal effects of these substances, the appearance of chemical residues in animal products (meat and milk) and environmental pollution (Foil et al., 2004).

The life cycle of *B. microplus* presents: a parasitic phase, in which the tick passes through the larvae, nymph and adult instar periods, all in a single host; and a free life phase that begins when the female drops to the ground and culminates when the larvae finds a host. At each laying, a female produces 2,000 to 3,000 eggs (Furlong, 1993). The newly hatched larvae migrate to the tips of leaves to locate a host. After attaching itself

to the host, the larvae initially feed on plasma and lymph, initiating the metamorphosis. The larvae undergo ecdysis, partially metamorphosing into nymphs in about seven days. In this phase, its food consists of lymph, plasma and blood until after a seven-day period, followed by another ecdysis, when the sexual differentiation occurs. The nymph can transform into a male that keratinizes, originating the adult males, or into a female (semiengorged females). In the adult stage, the semiengorged female begins feeding on blood, copulates, and her blood volume increases until she is completely engorged, whereupon she drops to the ground. The average duration of the parasite's life cycle is 21 days. The male tick remains attached to its host, surviving for up to twice as long as the female (Cordovés, 1997).

Esterases belong to a group of highly variable and multifunctional hydrolytic enzymes. In arthropods, these enzymes are related to physiological activities such as juvenile hormone regulation (Hidayat & Goodman, 1994), ontogenetic development (Bitondi & Mestriner, 1983), digestive processes (Argentine & James, 1995), reproductive behavior (Labate et al., 1990), functioning of the nervous system (Villate & Bachmann, 2002), and resistance to pesticides (Hemingway et al., 2002; Li et al., 2005).

Carboxylesterases (CaE) and acetylcholinesterases (AChE) are associated with pesticide resistance in arthropods (Guerrero et al., 1999). In *R. microplus*, Baxter & Barker (1998) demonstrated the relation between resistance to organophosphate compounds and altered AChE activity in an Australian strain; and Jamroz et al. (2000) found increased CaE activity in a Mexican strain of pyrethroid-resistant ticks. Point mutations have also been detected in the genes encoding these enzymes (Hernandez et al., 2000, 2002). Two acetylcholinesterases (EST-1 and EST-2) were identified in Brazil and associated to resistance in a strain of *R. microplus* resistant to cypermethrin (Baffi et al., 2005).

Rhipicephalus microplus poses a serious problem in cattle ranching, because it is a debilitating agent and a vector of highly virulent hemoparasites. Management programs have led to increased interest in the development of researches on the biology of this parasite, aiming to underpin control programs. The literature lacks data on the esterase pattern during the development cycle of *R. microplus*.

The objective of this work was the analysis of the esterase profile, during the development stages of *R. microplus*, aiming to contribute with biological knowledge for future research on the control of this tick in its immature phases.

Material and Methods

The Mozzo strain, which originated from Uruguay and was kept away from acaricide pressure for multiple generations, was used for the analysis of the esterase pattern, during the postembryonic development of *R. microplus* (larvae, nymphs, male and female adults). This strain was donated by the Experimental Parasitological Laboratory of Vallée S.A. (Uberlândia, MG, Brazil), where it had been kept under suitable conditions of temperature (27°C) and humidity (80%).

A pool of specimens from each phase (50 mg) was macerated in liquid nitrogen; then it was suspended in sodium phosphate buffer (0.01 M, pH 6.5, containing 10% glycerol, 0.001% bromophenol blue, 20% sucrose, 0.001 M EDTA and 0.5% Triton X-100), and centrifuged at 15,000 *g* for 15 min at 4°C. Adult engorged females weighing 50 mg were prewashed in 0.15 M PBS buffer (phosphate-buffered saline) and macerated individually. The pellet was discarded and the supernatant, containing soluble proteins, was collected. The protein dosage was carried out according to Bradford (1976) method. The absorbance was read at 595 nm in a Hitachi U-2000 spectrophotometer, and the mean protein concentration in the extracts was determined from the standard curve, using bovine serum albumin (BSA). The quantity of total protein was expressed in milligrams of proteins per gram of sample.

The enzymatic assays were carried out by non-denaturing polyacrylamide gel electrophoresis (Laemmli, 1970), using 12% separation gel and 5% stacking gel prepared in 0.375 M Tris-HCl buffer, pH 8.8. In each sample, 100 µg of total protein were applied. Electrophoresis was conducted for 3 hours, in a cold chamber, at constant amperage of 40 mA, in Tris (0.087 M) – Glycine (0.013 M) pH 8.3 running buffer.

After the electrophoretical separation, the esterases were identified by the method described by Lapenta et al. (1998). The gels were preincubated in 0.1 M sodium phosphate buffer, pH 6.5, for about 45 min at 37°C, after which they were stained with alpha- and/or beta-naphthyl acetate as synthetic substrates. The alpha and beta esterase activities were visible in the gels as black and red bands, indicating preferential hydrolysis of the alpha- or beta-naphthyl acetate, respectively. The staining

reaction was carried out in the dark, at 37°C for 1 hour, in a solution containing 30 mg of alpha- or beta-naphthyl acetate, prediluted in 1 mL of acetone, and combined with a freshly prepared and filtered solution of 85 mg of Fast Blue R/R Salt, in 100 mL of 0.1 M sodium phosphate buffer, pH 6.5. The bands showing esterase activity were numbered starting from the anodic end of the gel.

The esterases were biochemically characterized by means of assays with the following inhibitors: 1.0 mM copper sulfate, 1.0 mM p-chloromercurybenzoate (pCMB), 0.4 mM malathion, 1.0 mM eserine sulfate, and 1.0 mM phenylmethylsulphonyl fluoride (PMSF). The inhibitors were tested separately during the preincubation and in the staining solutions. Gels were preincubated for 30 min, in the dark, in sodium phosphate buffer (0.1 M, pH 6.5) containing the inhibitor, and then stained for detection of esterase activity in the presence of the inhibitor. A commercial malathion solution volume of 0.4 mM was added directly in the preincubation and in the staining solution. To prepare it for use, the PMSF was dissolved in 1 mL of methanol, and the pCMB in 1 mL of 0.1 M NaOH, pH 8. Eserine sulfate and copper sulfate were added directly to the preincubation and staining solutions. The esterases inhibited by malathion (an organophosphate) and by eserine sulfate (a carbamate) were classified as AChE, according to Oakeshott et al. (1993). The esterases inhibited only by malathion were considered CaE. Copper sulfate and pCMB were used as arylesterase inhibitors, and PMSF was used as a serine esterase inhibitor. Control gels without inhibitors were also analyzed to esterase activity.

Results and Discussion

Nine regions of esterase activity were detected during the ontogeny of *R. microplus*, using alpha-naphthyl acetate as substrate (Figure 1). These regions were numbered EST-1 through EST-9, starting from the anodic end of the gel. The bands identified when stained simultaneously with alpha- and beta-naphthyl acetates turned black, indicating preferential hydrolysis by alpha-naphthyl acetate. Gels stained separately with each substrate displayed the same pattern, without specific bands, and the enzymes were classified as alpha-beta esterases.

The results of the assays with inhibitors, for the biochemical characterization of these esterases, are presented in Table 1 with the degrees of inhibition classified in a decreasing order of band intensity. None of the esterases detected was affected by copper sulfate or pCMB. Eserine sulfate completely inhibited the activity of EST-1, EST-2, EST-3 and EST-4, which were also strongly inhibited by malathion and PMSF, and were classified as AChE. The enzymes EST-7, EST-8 and EST-9 were inhibited by treatment with malathion and PMSF, and were classified as CaE.

The electrophoretical analysis revealed differences in the pattern of esterase activity, during the immature phases of the cattle tick. Because pools of each phase were used, it is possible that allele overlapping occurred, preventing the identification of individual variations.

Two alleles corresponding to the esterases EST-8 and EST-9 were expressed throughout the ontogenetic development. These esterases were present in all the stages of development and exhibited highly intense staining. This homogeneous distribution in all the samples reflects the functional importance of these enzymes, which demonstrate constant and high gene activity throughout the tick's life cycle, and were considered products of *housekeeping* genes. Lima-Catelani et al. (2004) described these characteristics for the esterase bands of *Aedes aegypti*, detected in all the phases of development by electrophoresis in starch and polyacrylamide gel.

In our assays with inhibitors, the enzymes EST-8 and EST-9 were classified as CaE (Table 1). Carboxylesterases are associated with physiological processes of the

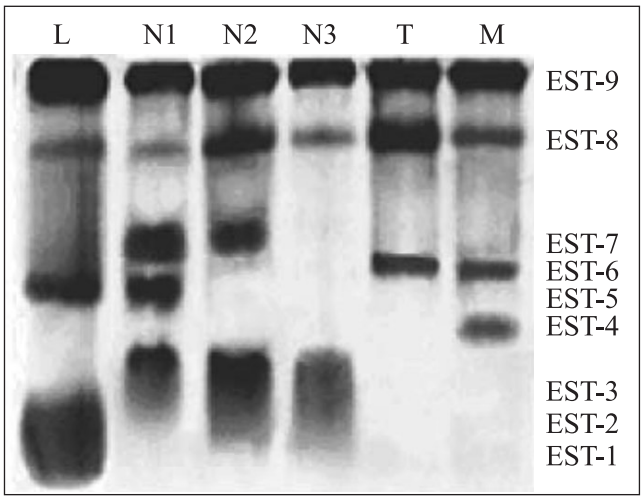

Figure 1. Esterase pattern during the postembryonic development of *Rhipicephalus microplus*. L: larvae; N1: nymphs; N2: semiengorged nymphs; N3: semiengorged females; T: engorged females; M: engorged males.

cuticular wall synthesis and with regulation of juvenile hormone levels (Sparks et al., 1979). These enzymes can also act as alternative phosphorilation sites during the process of intoxication by organophosphate acaricides (Watson & Chambers, 1996).

Differences in the esterase patterns between stages reflect differential gene expression in the production of proteins, which are important not only for maintenance of the stage itself, but also for the synthesis of proteins that trigger processes of metamorphosis. The electrophoretical analysis showed differences in the activities of these enzymes in the postembryonic development phases of *R. microplus*.

In larvae, five regions with esterase activity were detected: EST-9, EST-8, EST-5, EST-2 and EST-1, with the esterase activity EST-5 extending up to nymphs (N1). The bands corresponding to the enzymes EST-1 and EST-2 were detected only in larvae. These esterases EST-1 and EST-2 were considered larvae-specific and were classified as AChE. In this stage of development, larvae migrate along the plant in search of a host and are therefore an easy target for acaricides. AChE associated with organophosphates have already been detected in larvae of *R. microplus* and related to acaricide resistance (Wright & Ahrens, 1988; Baxter & Barker, 2002).

The nymph stage was subdivided into three substages, according to the degree of engorgement and size, which were dubbed N1 (first stage nymphs), N2 (second stage nymphs, or semiengorged nymphs), and N3 (third stage nymphs, or semiengorged females). First stage nymphs showed five regions of esterase activity: EST-9, EST-8, EST-7, EST-5 and EST-3. Region EST-7 was also present in N2, and EST-5 was shared with larvae. Region EST-3 showed activity only in the three nymph substages, and was considered nymph-specific. Four esterases occurred in N2: EST-9, EST-8, EST-7 and EST-3, and the regions corresponding to the activity of EST-9, EST-8 and EST-3 enzymes were detected in third stage nymphs, or semiengorged females.

The EST-5 enzyme, classified as an AChE based on assays with inhibitors, may be related to functional requirements at the beginning of development, since it was identified only in larvae and first stage nymphs. The esterase EST-7, classified as a CaE, was present only in the initial nymphal stages (N1 and N2), and may be related to requirements during the maturing process of nymphs. EST-3, observed in the stages of nymph, semiengorged nymphs and semiengorged females, was classified as an AChE and, in view of its intensity, this enzyme is presumably related not only to metamorphic requirements but also to the development of adult structures.

The stages of larvae and N1 nymph presented the highest esterase activity, which was indicated by both the number of variants and the intensity of expression of the gene product. Esterase requirements can be higher in these initial phases of development than in the other stages, due to the high level of food ingestion, juvenile hormone metabolism, and intense mitotic activity. EST-5, which begins to be expressed in larvae, maintains its activity only up to the N1 nymphs, indicating that some juvenile characteristics are related to this enzyme. This is, therefore, a probable target for acaricides.

The number of regions with esterase activity was lower in completely engorged females, with only three esterases detected: EST-9, EST-8 and EST-6. Four regions were detected in adult males, with the regions of esterases EST-9, EST-8 and EST-6 shared with engorged females, and EST-4 present only in males (sex-specific). EST-6 occurred only in engorged females and males, and was considered adult-specific.

Table 1. Effects of inhibitors on the esterases, detected during the development of *Rhipicephalus microplus*[(1)].

Esterases	Copper	pCMB	Malathion	Eserine	PMSF	Classification[(2)]
EST-1	-	-	+++	+++	+++	AChE
EST-2	-	-	+++	+++	+++	AChE
EST-3	-	-	+++	+++	+++	AChE
EST-4	-	-	+++	+++	+++	AChE
EST-5	-	-	+++	++	+++	AChE
EST-6	-	-	+++	++	+++	AChE
EST-7	-	-	+++	-	++	CaE
EST-8	-	-	+++	-	++	CaE
EST-9	-	-	+++	-	++	CaE

[(1)](-) absence of inhibition; (+, ++, +++) increasing levels of inhibition. [(2)]AChE: acetylcholinesterase; CaE: carboxylesterase.

Adult engorged females and adult engorged males were found to share three regions of esterase activity, EST-9, EST-8 and EST-6. These esterase enzymes are probably involved with digestion, physiology of the nervous system, reproduction, maturing of the external reproductive apparatus, and gonadal development. EST-6, considered adult-specific, may be involved with transmission of the nervous impulse. It would be interesting to analyze how this enzyme behaves in response to acaricides. Depending on the outcome of such an analysis, it would be possible to propose the inhibition of this esterase by a drug that uses a molecular method of the protein product inhibition, as an alternative control method. Baffi et al. (2005) identified an acetylcholinesterase related to pyrethroid resistance, in adult engorged females in a Brazilian strain of *R. microplus*.

In adult engorged males, in addition to the EST-9, EST-8 and EST-6 esterases, it was found the EST-4 esterase, which was present only in males and was considered sex-specific. These esterases have already been detected in some species of *Drosophila*. Kambysellis et al. (1968) described the F esterase as a product of an autosomic gene, whose expression is limited to sex in *D. aldrich*. In *D. melanogaster*, EST-6 has been related to mating behavior, with the male's seminal fluid triggering and influencing the speed of the formation of an aphrodisiac pheromone in the female (Richmond et al., 1986). The same role can be attributed to the EST-4 of *R. microplus*, classified as an AChE. This hypothesis is based on the fact that the female, after copulating, detaches herself from the host to oviposit, after which she dies, while the male mates with several females. A control mechanism of the parasite via sterile males could be the subject of research, starting from the manipulation of this esterase expression.

Conclusion

There are differences in the esterase pattern among the different postembryonic development stages of the life cycle of *Rhipicephalus microplus.*

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