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Relationship between glutathione *S*-transferase, catalase, oxygen consumption, lipid peroxidation and oxidative stress in eggs and larvae of *Boophilus microplus* (Acarina: Ixodidae)☆

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Abstract

Glutathione *S*-transferases (GSTs) are enzymes that act in excretion of physiologic and xenobiotic substances, protecting cells against chemical toxicity and stress. In this work, we characterized the enzymatic activity of GST in eggs and larvae of cattle tick *Boophilus microplus*, on different days after oviposition and eclosion. The results showed that the GST activity varied depending on the time elapsed after oviposition and eclosion. Molecules involved in mechanism of protection from oxidative stress are correlated with the increase in GST activity. The oxygen consumption kinetics showed a positive correlation with the increase in GST activity during embryogenesis. A high content of thiobarbituric acid reactive substances were observed in egg and larva extracts, indicating that ticks face high oxidative stress during embryogenesis and aging. In eggs and larvae, GST activity can be correlated to kinetic parameters of oxidative stress such as catalase and glutathione. In addition, GST activity showed strong positive correlation with lipid peroxidation, an indication that it plays a role in oxidant defences in eggs.

Keywords: *Boophilus microplus*; Embryonic development; Catalase; Glutathione *S*-transferases; Larval aging; Lipid peroxidation; Oxidative stress; Total glutathione

1. Introduction

Ticks (Arachnida) are blood-sucking arthropods that infest a wide array of species (Sauer et al., 1995), including humans and almost every animal of economic importance, and cause significant losses to livestock production (Bowman et al., 1996). The tick *Boophilus microplus* is one of the most important cattle ectoparasites, and causes great economic losses due to the diseases it transmits, along with bovine weight loss and the related high control costs (Jamroz et al., 2000). Studies about ecology, behaviour and physiology of ticks afford a better understanding of these organisms and therefore become important tools to develop new control methods. Due to the

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rapid increase in pesticide-resistant tick populations (Davey and George, 1998), the study of tick physiology has gained increasing importance as regards the mechanisms involved in detoxification of toxins (Hemingway, 2000; Kostaropoulos et al., 2001). These mechanisms, in general, not only act against specific toxic molecules, but also help in the maintenance of physiologic homeostasis, avoiding oxidative damage generated by reactive oxygen species (ROS). There are two antioxidant systems: non-enzymatic and enzymatic systems. The non-enzymatic antioxidant system use scavenger molecules as GSH (Sies, 1999), α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Dandapat et al., 2003), apart from other smaller molecules; the enzymatic antioxidant system is represented by enzymes such as SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase) (Rikans and Hornbrook, 1997), and GSTs (glutathione *S*-transferases) (Ketterer et al., 1983).

Glutathione *S*-transferases form a group of ubiquitous enzymes that catalyze the conjugation between glutathione and several molecules, and play the most important role in the cellular detoxification mechanism of xenobiotic and endogenous compounds (Agianian et al., 2003). There are at least two ubiquitously distributed groups of GSTs, microsomal and cytosolic. A third group of GSTs, structurally distinct from the microsomal and cytosolic GSTs, form the Kappa class and were described in mammalian mitochondria and peroxisomes (Morel et al., 2004; Robinson et al., 2004). This superfamily is subdivided into several classes, such as the five cytosolic mammalian classes Alpha, Mu, Pi, Theta and Sigma (Widersten and Mannervik, 1995); some cytosolic classes of GSTs, such as the Omega (Sheehan et al., 2001) and Zeta (Board et al., 1997) classes are represented in a wide range of species, including mammals (Hayes et al., 2005) In rodents and humans, cytosolic GST isoenzymes within a class typically share >40% identity, and those between classes share <25% identity (Hayes et al., 2005). The Epsilon class is present in insects (Ding et al., 2003), and the Phi and Tau classes are present in plants (Dixon et al., 2002).

The chemical exposure of insects is a classical event that selects pesticide resistance, and has been related with a high GST activity (Ketterman et al., 2001; Wei et al., 2001). It has been suggested that the pesticide may conjugate to glutathione by GST and that the compound obtained may therefore act as a detoxification mechanism in arthropods (Beall et al., 1992; Wei et al., 2001). GSTs also perform other physiological roles such as sequestration and transport of endogenous hydrophobic compounds, which include hormones, steroids, heme, bilirubin, bile acids and their metabolites (Salinas and Wong, 1999). The characterization and molecular cloning of GST of *B. microplus* from larvae (He et al., 1999) and from partially engorged female salivary glands have been reported (Rosa de Lima et al., 2002). In our work we identified a relationship between GST and physiologic stress by measuring the oxygen consumption and lipid peroxidation in eggs and larvae, through the analysis of GSTs enzymatic activity. The data obtained were compared with levels of others known antioxidants, CAT and GSH. Based on the correlation between GST activity and oxidative stress during larva senescence and starvation, we proposed the involvement of GST in oxidative stress protection.

2. Materials and methods

2.1. Ticks

Fully engorged females of *Boophilus microplus* (Acarina, Ixodidae) (Porto Alegre isolate) were experimentally maintained in the laboratory at 28 °C and 85% relative humidity, and their parasitic life completed in calves housed in individual pens on slatted floors.

2.2. Eggs and larvae

Eggs and larvae were collected every 5 days up to the 20th day of oviposition and egg eclosion, respectively. The materials were stored at −70 °C or in liquid nitrogen.

2.3. Extract preparation

Egg and larva extracts (100 mg/mL buffer) were macerated in 100 mM Tris–HCl 5 mM EDTA buffer pH 8.0, centrifuged at 15,000×*g* for 5 min. Next, the supernatants were strained through a 0.45-μm pore size filter to remove fat present in the supernatant after centrifugation. The extracts obtained were incubated in ice bath and then immediately tested for the determination of the enzymatic activity with a specific substrate for GST, or for the determination of catalase activity, lipid peroxidation or GSH content.

2.4. Biochemical analysis

The GST activity of the extracts was measured as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) as substrate. About 90 μL of the reaction mixture, consisting of 50 mM CDNB in methanol, 5 mM glutathione in 100 mM Tris–HCl pH 7.5, and 10 μl of extract in 100 mM Tris–HCl pH 7.5 were tested in a 96-well plate, according to Da Silva Vaz et al. (2004). Buffer without enzyme was used as negative control. The concentration of the product formed was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for *S*-(2,4-dinitrophenyl glutathione) (Widersten and Mannervik, 1995). The protein concentrations of extracts were measured using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. Each assay was run in duplicate and the results were expressed as the calculated mean and standard deviation of three separate experiments.

Oxygen consumption units were assayed using a Clark-type electrode (YSY, model 5775, Yellow Springs, OH, USA), calibrated to 100% with air-saturated buffer at 28 °C, according to Logullo et al. (2002). Reactions were carried out with 200 mg of eggs or larvae in 2 mL of 20 mM PBS buffer pH 7.4, 0.1 mM EDTA, for 60 min. 1 mM KCN was added to inhibit respiration in control samples. Eggs and larvae at 1, 5, 10, 15 and 20 days of age were tested. Assays were carried out in three independent experiments, each in duplicate.

The TBARS (thiobarbituric acid reactive species) assay was used as an index of lipid peroxidation in egg and larva extracts based on the formation of lipid peroxidation products during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the egg and larva extracts prepared were mixed with trichloroacetic acid 10% (v/v), 1 mM butylated hydroxytoluene (BHT), and thiobarbituric acid 0.67% (v/v), and then heated in a boiling water bath for 15 min. TBARS were determined by absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents (nmol/mg protein). Assays were carried out in three independent experiments, each in duplicate.

The catalase (CAT) activity assay was determined in egg and larva extracts as described by Aebi (1984). Briefly, 20 μL of egg or larva extracts were added to 80 μL of 10 mM Tris–HCl pH 8.0 and 900 μL of 9 mM H_2O_2, to a final volume of 1 mL. CAT activity was determined spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm, using the extinction coefficient of 43.6 M^{-1} cm^{-1} (Aebi, 1984). The results were expressed as U/mg protein. Assays were carried out in three independent experiments, each in duplicate.

The CAT activity in egg and larva extracts was inhibited using aminotriazole, a specific inhibitor or catalase, adapting the method described by Wagner et al. (2005). Briefly, the egg and larva extracts incubated for 30 min with 3-aminotriazole (Sigma), at different concentrations. The catalase activity was based on H_2O_2 degradation, using its extinction molar coefficient, 43.6 M^{-1} cm^{-1}. The results were expressed as U/mg protein. Assays were carried out in three independent experiments, each in duplicate.

The glutathione (GSH) determination was carried out as described by Akerboom and Sies (1981). Briefly, an equal volume of 2 M $HClO_4$, 4 mM EDTA solution was added to egg and larva extracts, and the precipitated proteins were separated by centrifugation for 5 min at 5000×*g*. The supernatant was neutralized with 2 M KOH in 0.3 M Mops solution in water, centrifuged and assayed. Total glutathione – the sum of reduced glutathione and oxidized glutathione (GSH and GSSG) was determined in the supernatant by a kinetic assay. In the assay, catalytic amounts of GSH and glutathione reductase caused a continuous reduction in 5,5-dithiobis-2-nitrobenzoic acid (DTNB) by nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). The DTNB reduction rate was measured spectrophotometrically at 412 nm. Total glutathione was expressed as μmol/mg protein. Assays were carried out in three independent experiments, each in duplicate.

2.5. Statistical analysis

All data values were expressed as mean±S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed. The *t*-tests were used when comparing data between two groups. Significance was set at $p<0.05$. Regression analyses were also performed to compare the changes of various parameters tested for embryonic development and larval aging (Swinscow, 1996).

3. Results

3.1. Specific enzymatic activity

A gradual increase in GST activity was observed during embryo development. The highest GST activity was found on day 20 after oviposition (0.19 μmol/min/mg protein). GST activity 1 day after egg eclosion decreased significantly and remained approximately so until day 5. On day 10 an increased level was observed and the highest activity in larva was found on day 20 after eclosion (0.24 μmol/min/mg protein) (Fig. 1). GST activity took place exclusively in embryo and larva tissues, since no activity remained in egg shells (data not shown).

3.2. Oxygen consumption

To test the relationship between GST activity and oxidative stress, we determined oxygen consumption (Fig. 2) in egg and larva extracts at the same time point used for GST enzymatic activity determination.

In eggs, oxygen consumption increased according to the time elapsed after oviposition. A gradual increase was observed in eggs upon day 20, when the highest level was observed (23.18 μmol O_2/min mg egg). In contrast, oxygen consumption in larvae was high on day 1 after egg eclosion (18.33 μmol O_2/min mg larva) and decreased significantly until the last day tested, in particular in 15- and 20-day-old larvae (8.4 μmol O_2/min mg larva and 10.2 μmol O_2/min mg larva).

3.3. Catalase activity and inhibition assays

CAT activity was measured in egg and larva extracts at the same time point used for GST enzymatic activity determination (Fig. 3). In 1–5- and 10-day old eggs CAT activity remained at a lower level when compared to 15- and 20-day-old eggs (0.78 U/mg protein and 1.49 U/mg protein, respectively). CAT activity was significantly lower in 1-day-old larvae (0.642 U/mg protein) than in 20-day-old eggs, but remained at higher level on all days after eclosion as compared to 1- to 10-day-old eggs

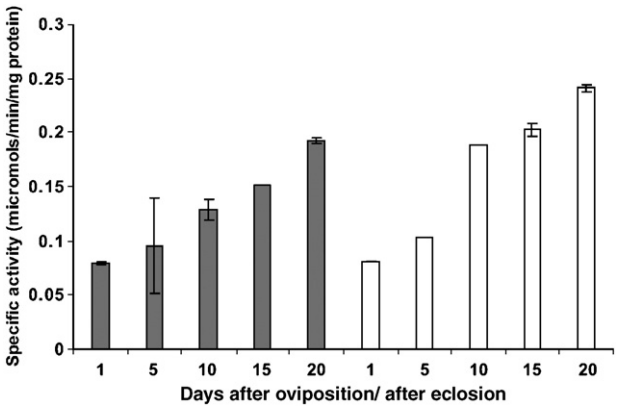

Fig. 1. Enzymatic activity of GST in eggs and larvae of *B. microplus*. Specific enzymatic GST activity in eggs and larvae on different days after oviposition/eclosion. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates.

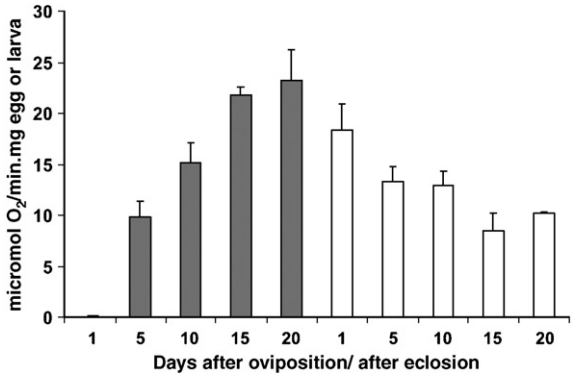

Fig. 2. Oxygen consumption in eggs and larvae. Oxygen consumption was monitored using a Clark-type oxygen electrode. Three assays of 200 mg of eggs or larvae were made in cuvettes containing 2 mL of 20 mM PBS, pH 7.4, 0.1 mM EDTA. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates.

(Fig. 3A). The specificity of CAT activity to degrade H_2O_2 in egg and larva extracts was confirmed by inhibition of the activity by aminotriazole at several concentrations, a compound that is a specific catalase inhibitor (Fig. 3B).

3.4. Glutathione determination

The levels of GSH in eggs increased significantly during embryo development. The highest GST activity was observed in 20-day-old eggs (92.23 μmol/mg protein). A decrease in GSH levels was observed in 1-day-old larvae (2.33 μmol/mg protein) when compared to the days before egg eclosion. Until day 5 after eclosion, GSH concentration was low and gradually increased thereafter until day 20 (Fig. 4).

3.5. Lipid peroxidation

A significant increase in lipid peroxidation in 20-day-old eggs (3.50 nmol MDA/mg protein) was observed when compared to 1-day-old eggs (2.86 nmol MDA/mg protein). In 1-day-old larvae (3.33 nmol MDA/mg protein) and 5-day-old larvae (2.82 nmol MDA/mg protein) the lipid peroxidation was maintained at the same level as in 20-day-old eggs, but decreased significantly in 10-day-old larvae (2.05 nmol MDA/mg protein). Afterwards, a significant increase was observed in 15- and 20-day-old larvae (2.32 and 2.74 nmol MDA/mg protein) in comparison to 10-day-old larvae, but the concentration of MDA produced in 15- and 20-day-old larvae was significantly lower than that observed for 1- and 5-day-old larvae (Fig. 5).

3.6. Correlation between GST activity and other biological parameters

A correlation between GST activity and other biological parameters involved in oxidative stress was established to evaluate the GST role in physiologic oxidative stress. The kinetics of GST activity in eggs and larvae was compared with oxygen consumption, CAT activity and GSH content in its

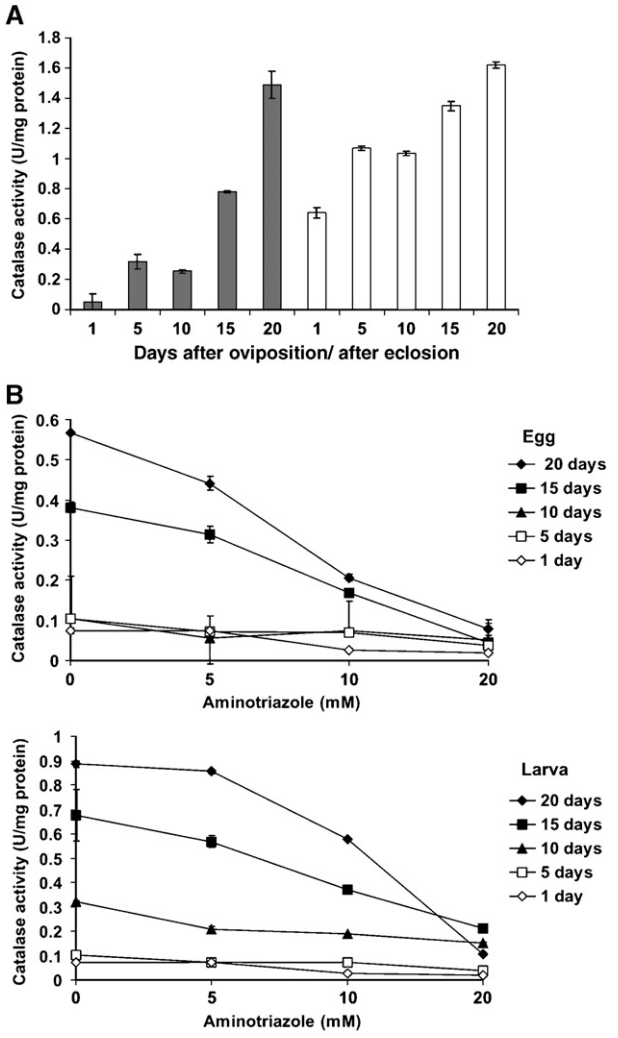

Fig. 3. Catalase activity and inhibition assays. (A) Catalase activity in egg and larva extracts of *B. microplus* in different days. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates (B) Catalase inhibition in egg (upper) and larva (lower) extracts of *B. microplus* on different days, using different concentrations of 3-aminotriazole (Sigma). The catalase activity was based on H_2O_2 degradation, using its extinction molar coefficient, 43.6 M^{-1} cm^{-1}. The results represent mean±S.D. of three independent experiments, in duplicates.

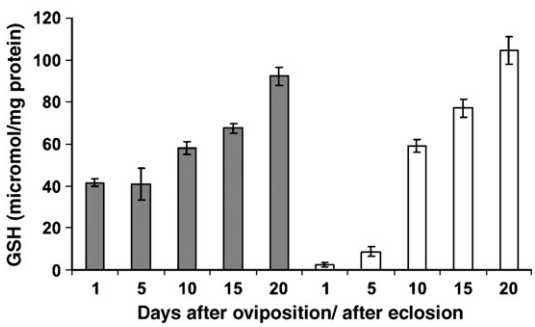

Fig. 4. Determination of total GSH content in egg and larva extracts of *B. microplus*. The GSH content was expressed as μmol/mg protein. In grey bars, eggs; in white bars, larvae. The results represent mean±S.D. of three independent experiments, in duplicates.

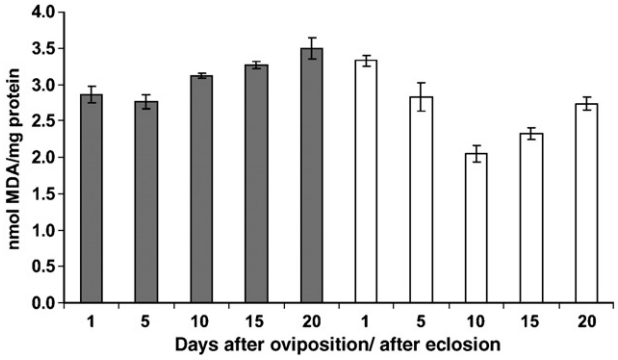

Fig. 5. Determination of thiobarbituric acid reactive substances (TBARS) in eggs and larvae of *B. microplus* on different days. In grey bars, eggs; in white bars, larvae. The results represent mean ± S.D. of three independent experiments, in duplicates.

respective life stages. A positive correlation was observed between GST activity and O_2 consumption in embryos ($r=0.930$), and a negative correlation in larvae ($r=-0.832$). There were positive correlations between kinetics of CAT and GST activities in eggs ($r=0.941$) and larvae ($r=0.879$). Also, positive correlations were found between GST activity and GSH content for eggs ($r=0.985$) and larvae ($r=0.993$). The correlation between kinetics of GST activity and lipid peroxidation in eggs was positive ($r=0.944$), whereas in larvae the correlation was negative ($r=-0.703$).

4. Discussion

GSTs are present in almost all eukaryotes and in most of them this happens in multiple isoenzymic forms, constituting a significant intracellular mechanism of detoxification (Papadopoulos et al., 2004). Detoxification is reached through transport and subsequent excretion of toxic organic compounds. Other functions, not associated with detoxification, include repair of macromolecules oxidized by reactive oxygen species, regeneration of *S*-thiolated proteins, and biosynthesis of physiologically important metabolites (Armstrong, 1997; Sheehan et al., 2001).

In order to understand the role of GST in the tick development, we analyzed its physiological variation during the life stages of eggs and larvae.

The enzymatic GST activity had a significant increase during embryonic development (Fig. 1). The O_2 consumption increases progressively and reached its peak when the embryo is about to hatch (Fig. 2). This increase can be explained by the increased metabolism during the embryonic development in *B. microplus*. We observed strong correlation between O_2 consumption and GST activity.

The enzymatic GST activity increased during the senescence of larvae (Fig. 1). By analyzing the kinetics of GST activity and O_2 consumption during larva aging a strong negative correlation was observed. This decrease in O_2 consumption can be explained by the observation that the larva keeps a high metabolic activity only during the first 5 days after hatching, in order to mature and thus acquire the ability to infest a host. The optimum period for the Porto Alegre *B. microplus* isolate to infest the bovine is approximately between the 5th and the 7th day post-eclosion (Da Silva Vaz, personal communication). Ogunji and Dipeolu (1977) showed the feeding of larval stages of *Amblyomma variegatum*, *Hyalomma rufipes* and *Boophilus decoloratus* in sheep to be more effective when larvae were fed within 1–4 days of hatching. Also, similar observations were described for other ixodid ticks (Sonenshine, 1991). After this time, the metabolic activity of *B. microplus* larvae decreased because the larva is senescent; however, GST activity remains high, since this enzyme is important to protect against oxidative stress to which the larva is submitted, as shown by the equally high CAT activity and GSH content (Figs. 3 and 4).

One of the consequences of elevated O_2 in the cellular environment is the elevation of ROS levels that could cause oxidative damage, such as nuclear DNA and protein breakdown, as well as lipid peroxidation (Mackay and Bewley, 1989; Barata et al., 2005). The antioxidants CAT and GSH tested presented a positive correlation with GST activity in eggs and in larvae. In fact, a high content of thiobarbituric acid reactive substances was observed in egg and larva extracts, indicating that ticks face high oxidative stress during embryogenesis and aging (Fig. 5). It is possible that the high level of lipid peroxidation verified during embryonic development occurs due to a high concentration of lipids, which are used by the egg as energy source (Logullo et al., 2002; Atella et al., 2005). Since the kinetic parameters of GST and catalase as well as GSH content during embryo development were modulated during different stages, and taking into account the fact that these parameters presented a positive correlation with lipid peroxidation, it is possible to suggest a role of GST in oxidative stress process.

The decreased oxygen consumption during larval aging may be related to metabolic depression and starvation. Phillips et al. (1995) showed that the metabolic rates of *Ornithodoros turicata* adults and larvae were affected by starvation and that oxygen consumption was lower than fed ticks. Some animals maximize their survival time using metabolic depression in response to low concentrations of oxygen available, as a common strategy through which they reduce their metabolism (Lutz and Storey, 1997; Hochachka and Lutz, 2001). Several authors have reported that the level of endogenous antioxidant defences is increased in several species during metabolic depression as a means to protect against oxidative stress. Oliveira et al. (2005) reported an increase in GST activity in gills of *Chasmagnathus granulata* during a metabolic depression induced by anoxia. At low temperatures, the larvae of *B. microplus* (Davey and Cooksey, 1989) and of other ticks (Labruna et al., 2003; Estrada-Pena et al., 2004) reduced their metabolism and managed to survive for long periods without feeding. These data are in accordance with the experiments conduced by Papadopoulos et al. (2004), which demonstrated that adult bees kept at low temperatures and starvation had GST activity increased, suggesting that *B. microplus* larvae decrease metabolism and increase GST activity in order to survive longer in the environment, until a host is found.

The results of this work help to elucidate the role of GST in tick development and assist in the understanding of the importance of GST in the excretion of toxic compounds.

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