

Ecdysteroid levels changed by permethrin action in female *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks

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ABSTRACT

As recent studies have shown that ecdysteroids may play a major role in the regulation of vitellogenesis in Ixodidae, the present study quantified, by means of a radioimmunoassay, the levels of ecdysteroids present in the hemolymph of semi-engorged females of *Rhipicephalus sanguineus* ticks obtained from control females (exposed to distilled water) and those exposed to increasing concentrations of permethrin. The levels of ecdysteroids decreased significantly as the concentration of permethrin increased, suggesting that this compound could be an inhibitor of ecdysteroids secretion, and consequently interfering with the reproductive ability of these ticks, since this hormone is responsible for the synthesis and incorporation of vitellogenin by oocytes. This study complements the previous results with *R. sanguineus* semi-engorged females, showing that permethrin is a potent agent causing major morphological changes in tick oocytes, such as the appearance of large vacuoles in the cytoplasm, reduction in the amount of yolk granules and a decrease in oocyte size, thus culminating in cell death and consequently reducing or preventing reproduction in treated females. The findings that permethrin leads to a decrease in ecdysteroid titers could represent an entry step into this scenario.

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1. Introduction

Specific studies on cell biology of ticks of medical-veterinary importance are scarce, especially with respect to Ixodidae. The tick of the domestic dog, *Rhipicephalus sanguineus* is the main vector of canine babesiosis, ehrlichiosis (Flechtman, 1973), and hepatozoonosis (O'dwyer and Massard, 2001), as well as of tularemia in humans (Walker, 1994). Studies performed by the Brazilian Central of Studies on Ticks Morphology (BCSTM) research group on the influence of natural and chemical products on the morpho-physiology of vital organs of ticks (Oliveira et al., 2008, 2009; Pereira et al., 2009; Roma et al., 2009, 2010a,b, 2011; Denardi et al., 2010; Arnosti et al., 2011), have generated information that contributes to the development of control methods that are less aggressive and less toxic to non-target organisms, including hosts.

As in all animals, the functionality of the reproductive system vital for the biological success of these ticks, and focusing on females, Friesen et al. (2003) showed that the synthesis of the main yolk precursor protein, vitellogenin and its uptake by oocytes begins within a few days of engorgement on a blood meal. Although

compared to other arthropods, especially insects, rather little is known about the hormonal regulation of vitellogenin synthesis in ticks, current evidence strongly denotes ecdysteroid as a vitellogenetic hormone in the Ixodidae ticks, such as *Ixodes scapularis* (James et al., 1997), *Dermacentor variabilis* (Sankhon et al., 1999), and *Amblyomma hebraeum* (Friesen and Kaufman, 2002), and in the Argasidae tick *Ornithodoros moubata* (Taylor et al., 1997). In *A. hebraeum* engorged females, the ecdysteroid concentration in the hemolymph increases in parallel with yolk accumulation in the ovaries (Kaufman, 1991) and with the increase of the vitellogenin concentration in the hemolymph (Friesen and Kaufman, 2002).

Given the apparent importance of the ecdysteroids in vitellogenesis in ticks, this study aimed at analyzing changes in the hemolymph ecdysteroid titer of *R. sanguineus* semi-engorged females exposed to the pyrethroid permethrin. Permethrin is a synthetic acaricide widely used to control ticks and causes nerve impulse disorders, as a result of disturbed sodium exchange in cell membranes. Thus, ectoparasites suffer excitement, indicated by tremors and spasms followed by paralysis and death (Mencke et al., 2003). Preliminary studies already demonstrated that it acts on germ cells of this tick species causing major morphological changes, compromising reproduction of this ectoparasite (Roma et al., 2010a,b, 2011).

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2. Material and methods

2.1. *Rhipicephalus sanguineus* ticks

In the present study, 60 semi-engorged females of *R. sanguineus*, weighing 27 mg on average (about 5 days of feeding) were used throughout the experiment. These were taken from a colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) at the Biosciences Institute of São Paulo State University, Rio Claro, SP, Brazil. The ticks were kept under controlled conditions ($28 \pm 1^\circ\text{C}$, 80% relative humidity and 12 h photoperiod) in an Eletrolab EL 202 BOD incubator and blood fed on New Zealand White rabbits (protocol approved by Research Ethics Committee and Scientific Merit – UNIARARAS, Grant no. 011/2009).

Details on feeding and maintenance of *R. sanguineus* ticks on the hosts are given by Bechara et al. (1995). Briefly, ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm wide and 3 cm high) glued to the shaved back of the hosts with a non-toxic and non-lesive preparation (Britannia Adhesives-Unit 4, UK) on the day prior to feeding.

2.2. Dilution assays for permethrin (CAS no: 52645-53-1)

Permethrin (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations were based on LC_{50} of 2062 ppm determined previously in a pilot study by Roma et al. (2009). The doses used corresponded to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1031 ppm) and the normal LC_{50} (2062 ppm). The control group was exposed only to the placebo (distilled water).

Rhipicephalus sanguineus females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Subsequently, 45 females were divided into three groups of 15 females each and immersed for 5 min in Petri dishes containing the different concentrations of permethrin. The control group also consisting of 15 females was immersed in distilled water for the same period. The ticks were then dried on absorbent paper and placed in the BOD incubator ($28 \pm 1^\circ\text{C}$, 80% relative humidity and 12 h photoperiod) for 7 days to observe the action of permethrin on the tick physiology. The observation period was established because frequently the effect of acaricides is not immediate, but acts slowly on the physiology.

2.3. Ecdysteroid quantification

2.3.1. Collection of hemolymph

Tick survival was determined at the time of dissection. If during hemolymph collection the gut was found to be ruptured, the respective tick was discarded. The *R. sanguineus* females were maintained in the refrigerator (5 min) for thermal shock anesthesia. Cooling inhibits gut contraction, thereby reducing the risk of piercing the delicate gut wall and contaminating the hemolymph (Warren and Gilbert, 1986). A small lateral incision was made in the tegument and the extruding hemolymph was collected by means of a calibrated glass microcapillary. In this experiment the hemolymph of 15 females per treatment group (control and permethrin-treated) was collected. Each sample for ecdysteroid analysis consisted of 0.3–5.0 μL hemolymph pooled from 2 or 3 ticks (thus approximately 5 samples for each treatment group). Immediately after collection, the hemolymph was dumped into a 100 \times volume of methanol and stored at -20°C until processed for radioimmunoassay (RIA) analysis.

2.3.2. Ecdysteroid radioimmunoassay

The antiserum used in this study had been produced in rabbits injected with a hemisuccinate derivative of ecdysone conjugated at

C-22 (Warren and Gilbert, 1986). This serum was used at a final concentration of 0.05%. [23,24- $^3\text{H}(\text{N})$] ecdysone (NEN, spec. act. 102 Ci/mmol) served as labeled ligand, at 6000 cpm per 100 μL borate buffer. Standard curves were set up by using 20-hydroxyecdysone (20E) (Simes, Milan) as nonradioactive ligand and covered a range from 25 to 2000 pg.

The methanol treated samples were centrifuged ($14,000\times g$, 4°C , 10 min) to pellet precipitated hemolymph proteins before the supernatants were transferred to 1 mL glass vials. The methanolic extracts were dried by vacuum centrifugation before adding 100 μL of the radioactive ligand solution and 100 μL of the antibody solution. After mixing, the samples were incubated overnight at 4°C . The equilibrium reaction was terminated by precipitating antibody-bound ligand by adding 50% ammonium sulfate (final concentration). The precipitate was pelleted by centrifugation ($7500\times g$, 4°C , 15 min), and the pellet washed by addition of an equal volume of 50% ammonium sulfate. Following a second centrifugation step, the pellet was dissolved in 40 μL of distilled water before adding 5 mL of HiSafe3 Optiphase LSC cocktail (Wallac-Packard) for liquid scintillation counting in a Beckman LS 6000 system. Assay conditions and cross reactivity factors for quantification of ecdysteroids and other compounds have previously been described (Feldlaufer and Hartfelder, 1997). Since standard curves were established by using 20-hydroxyecdysone as the nonradioactive ligand, all results were expressed as 20E equivalents. Standard curve and sample values (in cpm) were entered into a four-parameter non-linear logarithmic regression model provided as an Excel spreadsheet (Immunoassay calculations v. 4 BACHEM).

2.3.3. Statistical analysis

Results are expressed as mean \pm standard deviation. Statistical significance was determined using ANOVA ($p = 0.04$) and test for linear trend ($p = 0.053$). This test was performed in order to verify significant differences in levels of ecdysteroids among the groups treated with permethrin.

3. Results

The results showed that ecdysteroids levels (20E equivalent) in the hemolymph of semi-engorged females of *R. sanguineus* decreased significantly (linear reduction of the means) as the concentration of permethrin increased (Slope = -17.40) (Fig. 1).

4. Discussion

In insects, the roles of ecdysteroids (20-hydroxyecdysone) and juvenile hormone have been well characterized with respect to

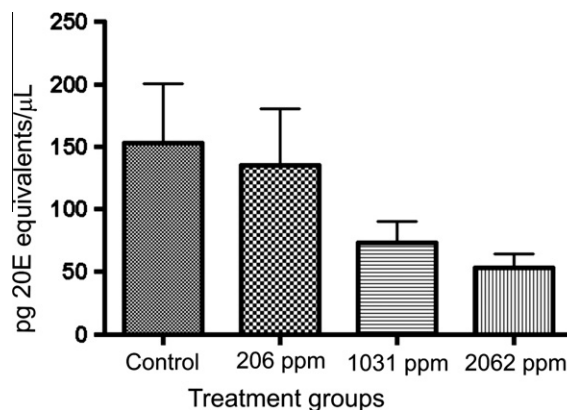


Fig. 1. Ecdysteroid levels (pg 20E equivalents/ μL) in semi-engorged females of *R. sanguineus* ticks from a control group and groups exposed to 206, 1031 and 2062 ppm of permethrin. The results are expressed in mean \pm standard deviation.

development regulation and reproductive physiology (Raikhel et al., 2005). In contrast, little such information is available for ticks. Preliminary studies performed by Pound and Oliver (1979) and Connat et al. (1983) reported that juvenile hormone might be involved in the processes that controls the synthesis of vitellogenin in ticks; however, recent studies using high resolution physicochemical instrumentation, such as gas chromatography and mass spectrometry did not show evidence of the presence of this hormone or of a juvenoid-like substance in ticks (Neese et al., 2000).

According to Horigane et al. (2007) and Ogiwara et al. (2007) the ecdysteroids which are present in the hemolymph of Argasidae ticks would be performing the functions of a vitellogenic hormone. Furthermore, Lomas et al. (1998), Mao and Kaufman (1998, 1999) and Friesen and Kaufman (2002) reported that ecdysteroids play a fundamental role in the process of salivary gland degeneration, as well as in the vitellogenesis of these ectoparasites. In view of the scarce knowledge about the hormonal control of the vitellogenesis in ticks, and based on previous results that showed that permethrin impairs female reproduction in *R. sanguineus* by causing major morphological changes in the germ cells (Roma et al., 2010a,b), the present study proposed to quantify hemolymph ecdysteroid levels in *R. sanguineus* females exposed to permethrin, to determine whether this compound is also capable of interfering in the metabolism of this hormone.

Permethrin significantly reduced the levels of ecdysteroids (20E equivalent) as the concentrations of this acaricide increased. These data suggest that permethrin may impair the synthesis and/or the release of this hormone into the hemolymph of the female ticks. This might either occur through a direct action on the epidermis, which was demonstrated to be a site of ecdysone synthesis in *Ornithodoros parkeri* (Zhu et al., 1991) or by indirect action via the nervous system, since the permethrin has neurotoxic activity (Mencke et al., 2003) and thus might reduce the synthesis of an ecdysiotropic synganglionic factor driving ecdysteroid synthesis (Chinzei et al., 1992). Confirmation of which mechanism occurs in *R. sanguineus* has not been addressed here and will require further studies.

The link between the permethrin-induced decrease in hemolymph ecdysteroid levels and the impairment of ovary activity indicated by morphological changes in the germ cells (Roma et al., 2010a,b, 2011) will also need to be addressed. This acaricide, in addition to acting on the tick nervous system (possibly preventing the synthesis and/or release of an ecdysiotropic factor) might also impair the function of other organs which are dependent on sensorial information, including the reproductive system. Once entering the system, permethrin circulating in hemolymph might be taken up by developing oocytes or pedicel cells, impairing the reproduction of these ectoparasites. A more probable mode of action, however, could be the impairment of vitellogenesis, as indicated by the reduction in yolk granules in the oocytes of the females exposed to permethrin (Roma et al., 2010a). By diminishing circulating ecdysteroid levels, permethrin might inhibit vitellogenin synthesis and, thus, limit the availability of this yolk precursor protein to oocytes induced to grow after a blood meal. Though being the most probable explanation for the observed morpho-functional effects of permethrin on the *R. sanguineus* ovary, and supported by data from other ticks showing that ecdysteroids promote vitellogenin synthesis and release into the hemolymph (Horigane et al., 2007; Ogiwara et al., 2007), a vitellogenic effect of ecdysteroids in the dog tick *R. sanguineus* still remains to be demonstrated.

According to Weiss and Kaufman (2001), the synthesis and the release of ecdysteroids into the hemolymph of the tick females is stimulated by (a) the amount of blood ingested and consequent weight gain, and (b) external factors from the males transferred to the females during copulation (Lomas and Kaufman, 1992). Furthermore, Weiss and Kaufman (2004) consider that a male factor

may act on the synganglion stimulating the production of a peptide which would stimulate the epidermis to synthesize ecdysteroids (Lomas et al., 1997). As the presence of males during the feeding cycle is an essential factor for the engorgement and complete development of germ cells in *R. sanguineus* females, an attractive hypothesis for the observed results is that the neurotoxic effects of permethrin might affect synganglion function and consequently cause impairment of the reproductive physiology of females, despite positive stimuli, such as a blood meal and the presence of males.

The results of the present study corroborate data obtained by Lunke and Kaufman (1992) and Friesen and Kaufman (2003) on females of *A. hebraeum* exposed to avermectin and cypermethrin, respectively. These authors also observed a considerable reduction in 20E levels in the hemolymph of tick females, as well as a decrease in the amount of vitellogenin in their oocytes. These and our results stand in contrast to those obtained by Taylor et al. (1991) who reported that, in females of *O. moubata*, cypermethrin stimulates vitellogenesis via a neuropeptide of the synganglion.

Another point to be analyzed in more depth is the dual role of ecdysteroids which, in addition to promoting vitellogenesis, also act in the degeneration of the tick salivary glands (Charrois et al., 1996). Thus, if the concentration of the levels of ecdysteroids were reduced, the glands would not degenerate, which would be in accordance with studies by Friesen and Kaufman (2003) who observed that in females *A. hebraeum* exposed to cypermethrin and the salivary glands did not degenerate. However, in semi-engorged females of *R. sanguineus* exposed to permethrin, the salivary glands undergo precocious degeneration (Nodari et al., 2011), indicating a direct action of permethrin on the salivary glands of *R. sanguineus* females, independent of reduced ecdysteroid levels that would favor their persistence.

5. Conclusions

We have shown that permethrin treatment significantly reduces hemolymph ecdysteroids levels of *R. sanguineus* females in a dose-dependent manner, complementing our previous results (Roma et al., 2010a,b) that this pyrethroid is a powerful chemical agent that compromises the reproductive success of *R. sanguineus* females. The data implicate ecdysteroids as a mediating factor in the action of this pyrethroid.

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