More about the role of 2,6–dichlorophenol in tick courtship: identification and olfactometer bioassay in *Amblyomma cajennense* and *Rhipicephalus sanguineus*

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This study aimed to identify 2,6-dichlorophenol (2,6-DCP) in Amblyomma cajennense *and to evaluate its role in* A. cajennense *and* Rhipicephalus sanguineus *courtship. Hexanic extract from attractive females was purified by solid phase extraction and the phenol was identified by the single ion monitoring method using GC/MS. In an olfactometer, the responses of* A. cajennense *and* R. sanguineus *males to females, control rubber septa or rubber septa impregnated with 2,6-DCP at 50, 500, and 5000 ng, respectively, were studied. 2,6-DCP was identified in* A. cajennense *extract and the males oriented themselves toward the concentration of 500 ng. These septa and the females were recognized as copula partners. The septa treated with 2,6-DCP did not attract and were not even recognized by the* R. sanguineus *males, whereas the females were recognized. Due to the presence of 2,6-DCP in* A. cajennense *and the results of biological bioassays, it was concluded that this compound acts as an attractant and mounting sex pheromone in this tick, but it does not play any role in* R. sanguineus *courtship.*

Key words: 2,6-dichlorophenol - sex pheromone - Ixodidae - olfactometer bioassay - identification

Mate-finding and courtship behavior in ticks are largely regulated by pheromones. According to Sonenshine (1991), the hypothetical behavioral stages that occur during *Dermacentor variabilis* (Say) courtship can be described as follows. The feeding female secretes a volatile attractant sex pheromone, exciting the male to detach himself. The sexually excited male commences searching behavior, orients to the emitting source, and approaches the pheromone-secreting female. Male contacts the female, detects the mounting sex pheromone, mounts, turns and crawls over the female's opisthosoma to the ventral surface. The male locates the female's gonopore and following probing and successful identification of the genital sex pheromone, the spermatophore is formed and copulation ensues.

Since the pioneer studies identifying 2,6-dichlorophenol (2,6-DCP) as an attractant sex pheromone in *Amblyomma americanum* (Linnaeus) (Berger et al. 1971, Berger 1972) this same compound has been identified in 17 species of Ixodidae (Mayer & Mclanglin 1991, Bruyne & Guerin 1994, Liu et al. 1998, Borges et al. 2002). Although identified in several species, confirmatory studies showing its role as an attractant sex pheromone were conclusive in seven species: *D. variabilis*, *Dermacentor andersoni* Stiles (Sonenshine et al. 1976), *A. americanum*, *Amblyomma maculatum* Koch (Kellum & Berger 1977), *Hyalomma dromedarii* Koch, *Hyalomma anatolicum excavatum* Koch (Silverstein et al. 1983) and *Dermacentor nitens* Neumann (Borges et al. 2002). In *D. variabilis*, *D. andersoni* (Hamilton & Sonenshine 1988) and *H. dromedarii* (Sobbhy et al. 1994), males attracted to 2,6-DCP placed on inanimate objects failed to recognize these objects as potential mates and left quickly even if they made physical contact with them. In these cases cholesteryl esters were necessary for the males to recognize the objects as sexual partners. Although this model is traditionally accepted, Borges et al. (2002) observed that *D. nitens* males are attracted, mount and proceed to ventral positioning on dummies impregnated with 2,6-DCP. They concluded that in this species, 2,6-DCP acts as an attractant and mounting sex pheromone.

Rhipicephalus sanguineus (Latreille) and *Amblyomma cajennense* (Fabricius) are three-host species ticks widespread in Brazil. The main host of *R. sanguineus* is the dog which can suffer from babesiosis, erlichiosis, hepatozoonosis and mycoplasmosis, all transmitted by the tick (Woldehiwet & Ristic 1993). This tick can also parasitize human beings (Dantas-Torres et al. 2006, Louly et al. 2006) and it is the vector of *Rickettsia conorii* in Europe and *Rickettsia rickettsii* in Arizona, USA (Woldehiwet & Ristic 1993, Demma et al. 2005). *A. cajennense* especially parasitizes equids, but it may infest other mammals such as bovids, cervids, wild and domestic canids, birds and even man. In Brazil, *A. cajennense* is considered the main vector of *R. rickettsii*, the agent of Rocky Mountain spotted fever (Dias & Martins 1939, Lemos et al. 1997, Horta et al. 2004, Guedes et al. 2005).

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Chow et al. (1975) identified 2,6-DCP in *R. sanguineus* female extract and observed that males responded to this compound by waving their legs, and detaching from the host, but attraction to this compound was not confirmed. Sobbhy et al. (1994) identified cholesteryl esters in *R. sanguineus* female extracts, but no bioassay was taken to confirm if these substances acted as mounting sex pheromone in this tick. Rechav et al. (1997) affirm that *A. cajennense* females do not emit sex pheromones and the production shifts to males, although no studies have been conducted for identification or attraction to 2,6–DCP. It is known that in this species the males produce a pheromone that induces males and females to attach. However, the aggregation of an individual on the host which occurs in other species of *Amblyomma* does not occur in *A. cajennense*. Thus, it is not known how the sexes would meet. This study was aimed to identify the presence of 2,6-DCP in *A. cajennense* females and to evaluate its role in A. *cajennense* and *R. sanguineus* courtship.

MATERIALS AND METHODS

Ticks - *A. cajennense* and *R. sanguineus* engorged nymphs were collected from infested horses and dogs, respectively, and incubated in constant conditions (27ºC, RH > 80%, 24h dark) until ecdysis. Then adults were fed on rabbits for six days to guarantee sexually mature males and females. The rabbits were infested with a maximum of 20 adults of *A. cajennense* and 30 of *R. sanguineus*. After one infestation with the first species and two or three with the second one, the rabbits were donated. During the infestations, the rabbits were clinically examined every day and showed no symptoms due to the tick parasitism.

Chemicals - Hexane, dichloromethane, ethyl acetate and methanol were Ultra-Resi Analysed grade (Mallinckrodt Baker, NJ, USA). Deionised water was purified through a MilliQ BioCell system from Millipore (Bedford, MA, USA). 2,6-DCP was the standard product (Sigma, St. Louis, USA). Membrane PVDF (0.45 µm) Millex syringe filter units (Millipore) and an AccuBond ODS-C18 solid-phase extraction (SPE) column (100mg/ml) (J&W Scientific, Folsom, CA, USA) were mounted on a vacuum-filtration adapter (Aldrich).

Pheromone extraction - The pheromone was extracted by immersing 50 six-days fed *A. cajennense* females in 3 ml of hexane and exposing them to ultrasound for 15 min. The suspension was filtered by a syringe filter and the solvent was evaporated under a nitrogen gas stream. One aliquot was dissolved in 1 ml of methanol, poured into the AccuBond C18 SPE column and forced to pass through it at 1 ml/min by applying reduced pressure. The column had previously been activated and conditioned with 2 ml of ethyl acetate, 2 ml of methanol, and 2 ml of deionized water to avoid drying out. After application of the sample, the column was dried by a stream of air for 10 min. The analytes were then eluted with 4 ml of ethyl acetate and the eluate was evaporated to dryness under a stream of nitrogen. The dry extract was reconstituted with hexane and 0.5 µl of the extract was injected into the gas chromatograph.

Pheromone identification - The sample analyses were performed on a GC-MS Shimadzu QP5050A instrument under the following conditions: a CBP-5 (Shimadzu) fused silica capillary column (30 m long x 0.25 mm i.d. x 0.25 µm film thickness composed of 5% phenylmethylpolysiloxane) was connected to a quadrupole detector operating in full scan and in selective ion monitoring mode with electron impact ionization at 70 eV; the carrier gas was He (1 ml/min; 56.7 kPa) in constant flow mode. Injector and interface temperatures were 250°C and 270°C, respectively. Injections were in split mode with a split ratio of 1:5. The oven temperature was programmed as follows: the initial temperature was held for 1 min at 60°C and was then raised to 270°C at 40°C/min, and maintained for 3.75 min (total time of 10 min). The ion-fragments m/z 126 [M^+–Cl_2], 162 [M^+], 164 [M^++2] and 166 [M^++4] were recorded for 2,6-DCP (retention time at 5.03 min). Phenol identification was based on matching between the mass spectrum of the peak at retention time of 2,6-DCP in hexane extract and that of the authentic sample.

Olfactometer bioassay - The males were tested against different stimuli: sexually mature females, rubber septa treated with increasing concentrations (50, 500 and 5000 ng) of 2,6–DCP diluted in hexane and septa treated with hexane (control). The septa were the size of a mature virgin female. In a glass arena olfactometer with the bottom covered with a grid paper (1 x 1 cm) (Borges et al. 2002), the *A. cajennense* and *R. sanguineus* males were released individually at 5 and 2.5 cm respectively from the stimulus source. 15 to 20 males of each species were tested against each odor source. The females were attached to the paper by using a hypodermic needle. The total number of ticks (20 to 30 males) tested in one day were divided between the treatments. The grid paper was always changed along with the odor source. Before the beginning of an experimental day, the arena was cleaned using detergent, rinsed with distilled water, hexane and acetone. To avoid any interference of the observer in the behavior of the ticks, he stayed behind and around 30 cm from the arena, and followed the displacement of the tick on another grid paper (3 x 3 cm). The percentages of males that oriented, showing directional movement toward the stimulus, and their tracks were recorded. The ticks' tracks were divided into sections, each of which was three times the length of the male tick. Two consecutive points were linked by a straight line and the angle was calculated between this line and the direction of the wind (angle 0º). The trajectories displayed by ticks were analyzed by means of circular statistics (Zar 1999). The mean angle displayed by each animal was calculated over the pathways they formed. Subsequently, for every experimental group a mean angle and the length of the resultant mean vector (r) was calculated and the statistical differences between groups were evaluated through the Watson U^2 test. The percentage of males that recognized the odor source and proceeded to mounting and ventral positioning was also recorded. The ventral positioning was reached when some mounted males crawled over the septa, located themselves between the septa and the

arena, and lifted the septa with the aid of their legs. The percentages of male ticks that oriented and mounted to different odor sources were recorded and compared using the Kruskal–Wallis test (Sampaio 1998). The significance level was $p < 0.05$ in both tests.

RESULTS

Pheromone identification - The retention time (R_t = 5.03 min) and elution characteristics on the CBP5 column were determined for the authentic sample of 2,6-DCP (Fig. 1a). Observations of retention time and the characteristic cluster of peaks at 126, 162, 164, and 166 m/z in the SPE-purified hexanic extract of attractive *A. cajennense* females were accepted as evidence that 2,6-DCP was present (Fig. 1b).

Olfactometer bioassay - The *A. cajennense* males oriented toward 2,6-DCF at 500 ng concentration. The septa with this concentration and the females were recognized by the males through mounting in 67% and 73% of cases respectively, and ventral positioning in 42% and 62.5% (Table). The displacement angles indicate that 2,6-DCP in all concentrations tested attracted the male ticks: more angles between 0º and 10º were observed in relation to the different odor sources (59% to 80%) than in the control (47%) (Fig. 2). No statistical differences were found among these groups ($0.044 > U^2 > 0.083$) except between control and 500 ng concentration ($U^2 = 0.268$). The percentage of *R. sanguineus* males that reached the different stimulus sources was similar for all odors tested, including the control (Table). The displacement angles also indicate that no source was attractive, because similar results were observed with the control (35% of angles between 0º and 10º) and the other stimuli (47 to 56% of angles between 0º and 10º) (Fig. 3) and no statistical differences were found among these groups ($0.031 > U^2 > 0.083$). The septa treated with 2,6-DCP were not recognized by the males as mounting behavior did not occur, but 42% of the males that reached the females mounted and 20% of them proceeded to ventral positioning (Table).

DISCUSSION

In the present study we add *A. cajennense* to the extensive list of ticks that produce 2,6-DCP (Mayer & Mclanglin 1991, Bruyne & Guerin 1994, Liu et al. 1998, Borges et al. 2002). Leahy and Booth (1976) presumed that 2,6-DCP was a sex pheromone of *A. cajennense*, based on the aggregation of male ticks in a Petri dish arena treated with

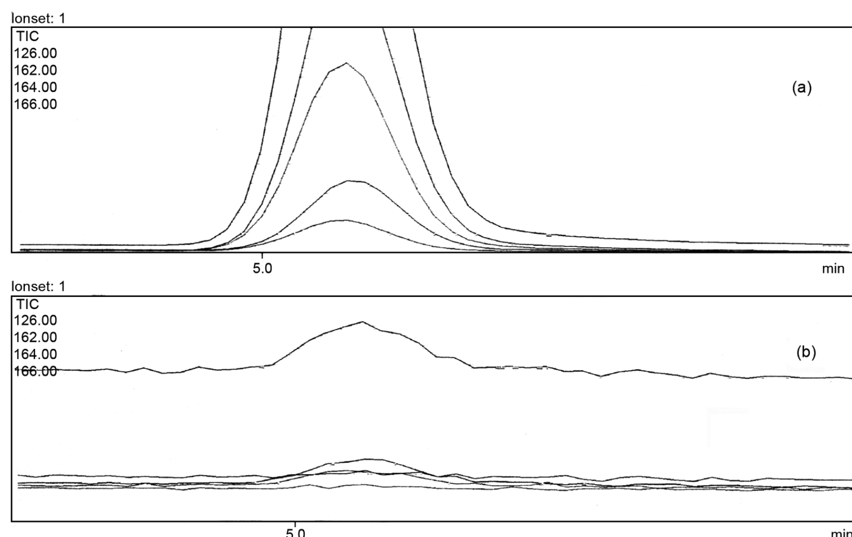

Fig. 1: total ion chromatogram (TIC) of authentic samples of 2,6-dichlorophenol (2,6-DCP) (a) and of the SPE-purified hexane extract of *Amblyomma cajennense* showing the 2,6-DCP in retention time at 5.03 min (b). Numerical value refers to monitored ion-fragments.

TABLE

Percentage of orientation (O), mount (M) and ventral positioning (VP) of the odor source by males of *Rhipicephalus sanguineus* and *Amblyomma cajennense* when exposed to different stimulus, in an olfactometer

	R. sanguineus			*A. cajennense*		
Odor source	Orientation	Mount	Ventral positioning	Orientation	Mount	Ventral positioning
Control	35[a]	0[a]	-	30[a]	0[a]	-
Female	60[a]	42[b]	20	55[a]	73[b]	62.5
50 ng 2,6-DCP	60[a]	0[a]	-	53[a]	0[a]	-
500 ng 2,6-DCP	60[a]	0[a]	-	90[b]	67[b]	42
5000 ng 2,6-DCP	60[a]	17[a]	0	47[a]	0[a]	-

a, b: different letters within the same column indicate significant differences by Kruskal-Wallis test ($p < 0.05$).

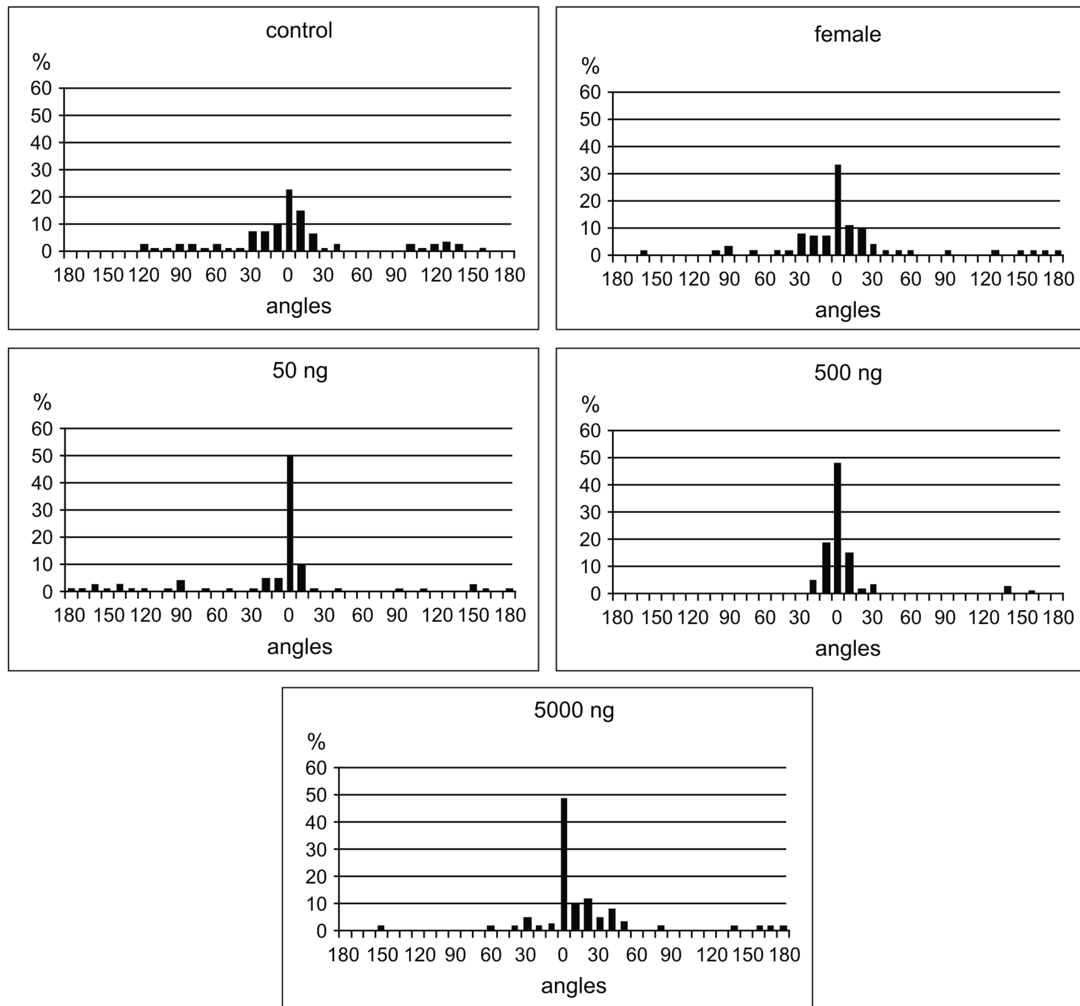

Fig. 2: frequencies of displacement angles of *Amblyomma cajennense* males to different odor sources in an olfactometer bioassay.

that compound; however, no attempt was made to identify the presence of the phenol in tick extracts.

When developing bioassays to evaluate the activity of pheromones or possible pheromones, the methodology must allow the expression of the characteristic behavior believed to be regulated by the pheromone (Sonenshine 1991). The present olfactometer bioassay allows the observation of the attraction, orientation and recognition of the odor source. The first two behaviors are regulated by an attractant sex pheromone and the last one by a mounting sex pheromone. When we used that bioassay for the first time (Borges et al. 2002) we did not expect to observe mounting of the rubber septa treated with 2,6-DCP as that behavior is traditionally guided by cholesteryl esters (Hamilton et al. 1989, Sobbhy et al. 1994). However, the observation of this unusual behavior stimulated us to evaluate the same bioassay with other tick species.

In *A. cajennense* the attraction and recognition (mounting and ventral positioning) of the septa impregnated with 2,6-DCP suggest that this compound acts as an attractant and mounting sex pheromone of this tick. Rechav et al. (1997) say that *A. cajennense* females do not produce sexual pheromones. In the genus *Amblyomma* the presence of aggregation-attachment pheromone produced by fed males has been widely reported (Sonenshine 1991). As demonstrated by Rechav et al. (1997), the males of *A. cajennense* produce a pheromone which promotes the attachment of unfed males and females, but not their aggregation. Given these points, how would the sexes meet in this species? The results obtained here suggest that courtship in *A. cajennense* occurs in the same way as in *D. nitens* (Borges et al. 2002), but is different from what was observed in *D. variabilis*, *D. andersoni* (Hamilton et al. 1989) and *H. dromedarii* (Sobbhy et al. 1994). Males of those last species were attracted to 2,6-DCP which was placed on inanimate objects but failed to recognize them as potential mates and needed cholesteryl esters as a cue for that. It is possible that compounds playing a role as mounting sex pheromones, such as cholesteryl esters strengthen the response of 2,6-DCP in *A. cajennense*, but further studies are needed to address this question.

The septa treated with 2,6-DCP did not attract and was not even recognized by the males of *R. sanguineus*. For this reason, it is probable that 2,6-DCP does not act

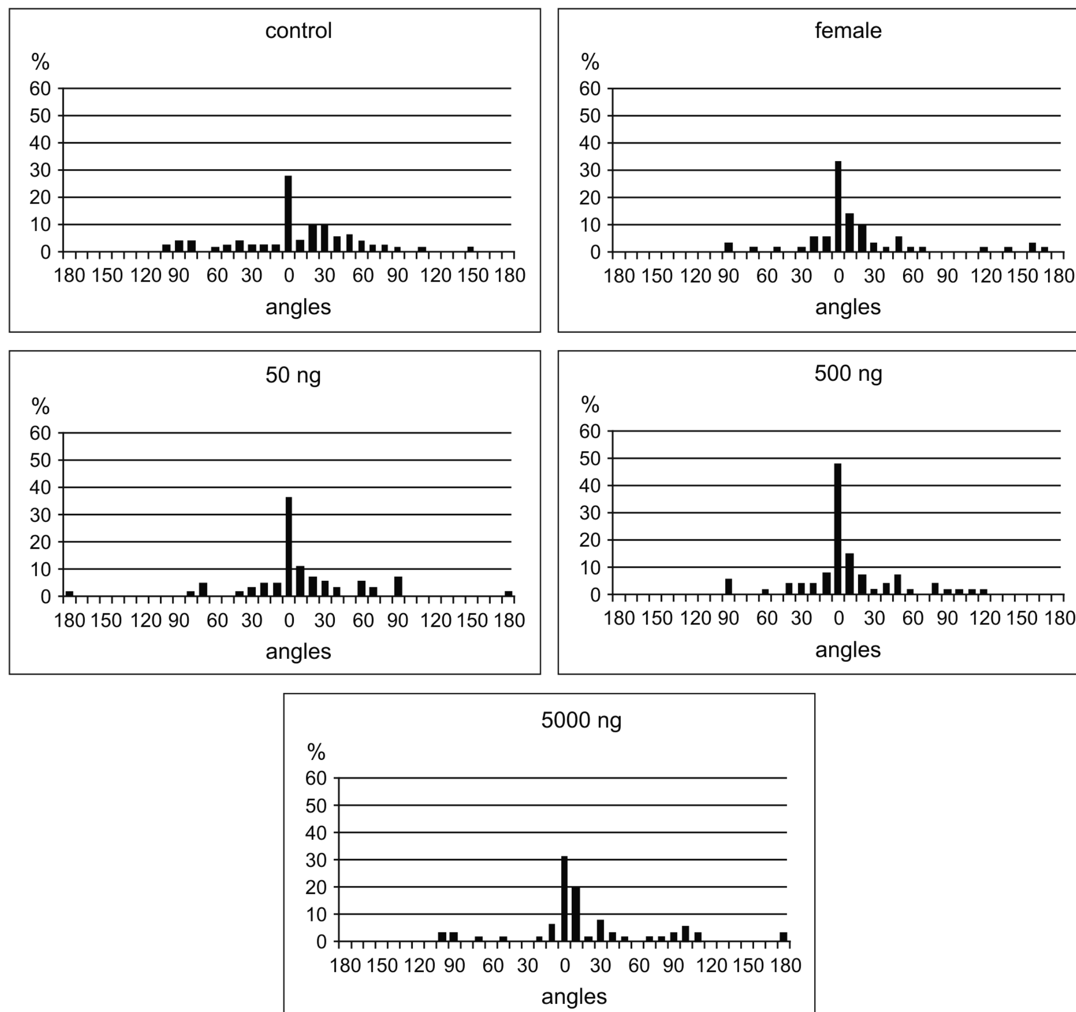

Fig. 3: frequencies of angles of *Rhipicephalus sanguineus* males to different odor sources in an olfactometer bioassay.

as an attractant or mounting sex pheromone in this tick. The non attractiveness of this compound is uncommon among metastriate Ixodidae, since several authors have reported its role as an attractant sex pheromone (Sonenshine et al. 1976, Kellum & Berger 1977, Silverstein et al. 1983, Borges et al. 2002). Bruyne and Guerin (1994) did not observe a behavioral response of *Rhipicephalus microplus* (Canestrini) males to 2,6-DCP. However, Cardoso et al. (2000) observed a little attraction to this compound, suggesting that physiological differences among the ticks used in the different assays would be responsible for the different behavioral responses. On the other hand, the non recognition of the septa treated with 2,6-DCP is more common and has already been reported in *D. variabilis*, *D. andersoni* (Hamilton & Sonenshine 1988) and *H. dromedarii* (Sobbhy et al. 1994).

The higher orientation responses of *A. cajennense* to impregnated 2,6-DCP septa at 500 ng concentration in comparison to females probably occurred because the concentrations tested were higher than those released by females. The maximum amount of 2,6-DCP detected in *Amblyomma* females is around 65 ng (Kellum & Berger 1977), therefore justifying the results. The decreasing responses to the highest concentration may have occurred because of the saturation of tick receptors.

Borges et al. (2006) tried to associate the aspect of the female dorsal fovea of four species of Ixodidae with the role of 2,6-DCP in their courtship. Based on fovea aspects the ticks were divided in two groups, with (*D. nitens* and *A. cajennense*) and without (*R. microplus* and *R. sanguineus*) secretions on the fovea. At that time it was known from the literature that 2,6-DCP played a role as a mounting sex pheromone in *D. nitens* (Borges et al. 2002), but not in *R. microplus* (Bruyne & Guerin 1994, Cardoso et al. 2000). For this reason, they hypothesized that 2,6-DCP would be a mounting sex pheromone in *A. cajennense* but not in *R. sanguineus*. Thus, the results of the bioassays described here corroborate the hypothesis suggested in that study.

2,6-DCP has previously been shown to control metastriate ixodid ticks by confounding males or mimicking females (Ziv et al. 1981, Sonenshine et al. 1985, Abdel-Rahman et al. 1998, Sonenshine 2006, Borges et al. 2007). Considering the results obtained here, it is reasonable to

suggest the use of this compound to intercept *A. cajennense* courtship; however its use in *R. sanguineus* is not recommended because it is not an attractant for this species of tick.

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