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Differences in the behavior of *Rhipicephalus sanguineus* tested against resistant and susceptible dogs

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Abstract To ascertain whether brown dog tick *Rhipicephalus* sp. infests resistant (beagle) and susceptible (English cocker spaniel) dogs differently, five animals of each breed were maintained in a kennel whose walls were infested with 7,000 larvae, 320 nymphs, 80 males and 80 females, in 3 infestations, at 10-day intervals. Five times more ticks were found on cocker spaniels (498) than on beagles (96). Substances were collected by rubbing pieces of clean flannel on the dogs for 15 min and these were tested for arrestment and attractiveness of ticks. Three choices were offered: cocker extract vs. control; beagle extract vs. control, and cocker extract vs. beagle extract. When allowed to choose between substances rubbed from dogs and a control, more ticks were arrested by extracts from the cockers than from beagles. In the arrestment tests with only a choice between substances from dogs of each breed, more ticks were arrested by cocker substances. To test for attraction, capsules containing adsorbent were used and the tests were carried out in a Y-olfactometer. Fifteen males and 15 females were tested, for each treatment. In the olfactometer, the ticks were not attracted to the odor of either breed, however the odor of the Beagle was apparently repellent. These results indicate that *R. sanguineus* can use substances from the dogs to differentiate susceptible English Cocker Spaniels from resistant Beagles.

Keywords Kairomones · Arrestants · Attractants · *Rhipicephalus sanguineus*

Introduction

Rhipicephalus sanguineus (Latreille) is the most widely distributed tick species around the world and has the dog as its preferential host. In Brazil, this species is rarely found parasitizing other hosts, such as equine, bovines and humans, and when it happens this parasitism is usually associated with a direct contact of these hosts with the dog (Labruna and Pereira 2001; Louly et al. 2006). However, in other parts of the world it is sympatric with *Rhipicephalus turanicus* and *Rhipicephalus camicasi* which are very closely related to

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each other and morphologically similar, constituting what is known as the *R. sanguineus* group. Besides *R. sanguineus*, there are reports of these species parasitizing several mammals and even ground foraging birds, but due to difficulties in identification there is no consensus on the parasitic specificity of these species (Soulsby 1966; Uspensky et al. 1997; Guglielmone et al. 2006).

For the dog, *R. sanguineus* causes direct damage such as blood spoliation, dermatitis, discomfort and paralysis provoked by neurotoxins liberated by the tick during feeding, and indirect damage such as babesiosis, hepatozoonosis and erlichiosis transmission (Woldehiwet and Ristic 1993; O'Dwyer and Massard 2001.

For 1 year Louly et al. (2007) studied natural *R. sanguineus* infestation of English cocker spaniels and mongrel dogs, maintained at the same kennel. Cockers were infested by up to 10 times more *R. sanguineus* than the mongrels, suggesting that this race is more susceptible to this tick. Later, through artificial infestations and associated biological parameters, Louly et al. (2009) confirmed that cocker spaniels were more susceptible to *R. sanguineus* than beagles and that successive infestations affected the development of the tick, especially in the resistant individuals.

Studies about the sensorial capacity of the ticks demonstrate that these arthropods are capable of responding to a great variety of stimuli such as mechanical, thermal, chemical, optical and olfactory. However, few studies exist about the response of *R. sanguineus* to the stimuli produced by the dog, except for the studies confirming the attraction of this tick to CO_2 (Stella et al. 1988; Louly 2003). To find a host, ticks can perceive volatile and non-volatile substances liberated by it, or excretion products as feces and urine (Waladde and Rice 1982; Carroll 2000). *R. sanguineus* is a nidicolous species, meaning that it breeds in the immediate environment of the host and can actively hunt to obtain a blood meal, or even use the ambush strategy (Labruna and Pereira 2001; Uspensky 2002).

Substances produced by the host acted as arrestants for *Ixodes scapularis*, *Amblyomma americanum* and *Dermacentor variabilis* (Carroll 1998, 1999). *Rhipicephalus evertsi evertsi* and *Rhipicephalus appendiculatus*, two species of ticks that parasitize bovines, are capable of differentiating preferential sites of attachment on the same host, through chemical substances (Wanzala et al. 2004). Waladde and Rice (1977) suggested that the resistant animals present substances on the skin or in the serum, that when perceived by gustatory sensilla, inhibit the conclusion of a blood meal by the tick, in this way contributing to the selection of the host, and thus in the manifestation of resistance.

There is a difference of susceptibility between the beagle and cocker spaniels breeds to the *R. sanguineus* (Louly et al. 2009). There is a possibility that chemical substances produced by the hosts as a signal of its resistance (Waladde and Rice 1977). Based on these facts, this study aimed to evaluate the behavior of *R. sanguineus* exposed to resistant (beagle) and susceptible hosts (cocker). Therefore, animals of the two breeds were artificially infestated and the chemical substances which they produced were evaluated through behavioral assays.

Materials and methods

Experiment 1: location of the host in artificial infestation

Colony of ticks

Engorged females of *R. sanguineus* were harvested from naturally infested mongrel dogs for establishment of a colony. The ticks were maintained in an acclimatized chamber (27°C

and 80% of R.H.) and they were fed on rabbits (*Oryctolagus cuniculus*) to obtain stages to be used in the dog infestations. The rabbits were infested with a maximum of 30 adults, 200 nymphs or 1,000 larvae and after two or three infestations were substituted. During the infestations the rabbits were examined daily and none showed symptoms of damage due to tick parasitism.

Animals

Five animals cocker spaniels and five beagles with previous contact with *R. sanguineus* were used. These dogs were previously evaluated through artificial infestations (Louly et al. 2009) with the cockers showing the highest susceptibility to *R. sanguineus*. Both groups had two males and three females and the animals' ages varied from 90 to 105 days. The dogs had not been treated with acaricide for at least 30 days before the beginning of the study.

During the experiment the dogs were fed twice a day (Max Filhote–Total Alimentos), following the amount recommended by the manufacturer and received water *ad libitum*. The animals were maintained at the kennel of the Veterinary Hospital at the Veterinary School of the Federal University of Goiás. The kennel had a covered area, with 12 individual stalls (1 × 1.5 m) and an open area with a solarium (3 × 5 m). The floor was cemented, and the walls of the patio and of the stalls were tiled or covered with synthetic enamel to facilitate hygienic cleaning. One month before the infestations the kennel was sprayed, using an aerosol bomb, with amitraz 12.5% (Bayer), diluted following manufacturer's instructions and was cleaned with a fire broom weekly until the beginning of the experiment.

Infestations

Seven thousand larvae, 320 nymphs and 160 adults (80 males and 80 females) in 3 infestations at 10 day intervals were released from breeding tubes on the walls of the kennel. The open tubes were positioned close to the walls at 30 cm from the floor and the ticks leave them spontaneously. The dogs of both groups were kept in this kennel. The cockers had their pelage cut to be the same length as that of the beagles, offering the same conditions for tick infestation. Two days after the first infestation, the dogs were inspected, twice a day, 0900 and 1700 hour, for 40 days. The ticks found were counted and harvested for identification. None of the dogs exhibited clinical symptoms due to tick infestation.

Experiment 2: arrestment and attractiveness tests

The arrestment and attractiveness tests were conducted in an acclimatized room ($T = 27°C$, RH = 70% and 12:12 light:dark), located in the Arthropods Laboratory of the Parasitology Center of the Federal University of Goiás. Samples were collected from all dogs, being them free of ticks and each dog of the same breed was randomly numbered in a sequence from 1 to 5. The dogs with the same number were tested simultaneously. In both tests the treatments were: 1-cocker versus control; 2-beagle versus control and 3-cocker versus beagle.

Arrestment tests

Substances from the skin of the dogs were collected using a modification of the technique described by Carroll (2002a, b), substituting the glass rods with pieces of cotton flannel.

The pieces of cotton flannel were washed with detergent, rinsed with distilled water, autoclaved, dried at room temperature, immersed in hexane, dried and immersed in acetone then later dried at 100°C for 1 h. The dogs were left for 15 days without a bath and for 30 days without contact with acaricides. The flannels were rubbed on the backs, heads and external parts of the ears of the dogs, for approximately 15 min (5 min in each area). Samples were collected from all the dogs, sealed in plastic bags, identified and stored in a freezer, at −20°C.

For the tests, white rectangular trays (20 × 40 × 8 cm) were used, containing distilled water. A rectangle of Styrofoam (15 × 30 × 2 cm) was placed on top of water in the center of a tray ringed with double face tape at the borders to prevent the escape of the ticks. On the opposite extremities of the Styrofoam rectangle, two plates of Styrofoam (5 × 5 × 1 cm) were placed, each bearing a piece of cotton flannel, one with the odor of cocker or beagle and the other a clean flannel as control. Five males and five females of *R. sanguineus*, all unfed, aged 7–21 days, were released in the center of the Styrofoam. After 1, 18 and 24 h of the release, the number of ticks arrested at each sample was recorded. Ten repetitions of each treatment were done with the substances from each dog.

Attractiveness tests

The odor of the dogs was collected using a modification of the technique described by Gikonyo et al. (2000). A rectangular capsule (10 × 8 cm) similar to an envelope with one side made of clean aluminum foil and the other of wire-mesh stainless steel (100 μm) contained a filter paper sachet (9 × 7 cm) (Whatman no. 1 Qualitative), with activated charcoal (350 mg, 40 μm Fluka–Sigma Aldrich) and Octadecyl bonded silica-C18 (450 mg, particle size 40 μm JT Baker–Hexis-Sigma Aldrich) as adsorbents. Two capsules were fastened on each dog's dorsum, with the steel face in contact with the skin, and left for 12 h. The sachets were stored in a freezer at −20°C until extraction. Adsorbents from two sachets were pooled and transferred to an ODS cartridge (Accu Bond, Solid Phase Extraction-J&W Scientifc), eluted with 4 ml of dichloromethane HPLC grade (IN Science-Merck) and later concentrated to 150 μl under a gentle stream of clean nitrogen, and stored at −20°C.

The tests were conducted using a glass Y olfactometer (20 cm), connected by the two arms to silicon hoses to the front exit of a glass Kitasato flask containing distilled water (300 ml). The superior entrance of the Kitasato was connected by a silicon hose to the exit of an air compressor (Diapump/Fanem). The humidity inside the olfactometer and the speed of the wind were 80% and 30 cm/s, respectively. The odor sample (10 μl) from each dog was applied to a filter paper (5 × 5 mm) and as a control the filter paper received an equal volume of dichloromethane and both filter paper were dried at room temperature during 1 min. Fifteen adult ticks of each sex, aged between 7 and 21 days, were used in each treatment.

Each tick was released in the body of the Y-olfactometer and observed for 1 min to see for which arm they opted. Ticks that did not choose either of the arms were removed and released again. Each tick was allowed three opportunities, if it failed to choose either of the arms it was substituted.

Statistics

The data from the artificial infestations were analyzed using the ANOVAs and compared by the Student's *T* test. The results from the arrestment and attractiveness tests were

analyzed using Chi Square test. All tests were evaluated at the significance level of $P < 0.05$.

Results

Experiment 1: location of the host in artificial infestation

Of a total of 21,000 larvae released in the kennel, only 87 larvae (0.4%) were recovered. The number of larvae was significantly larger in the cocker group than in the beagle group in the first two infestations, but there was no difference among the groups in the third. Of the 960 nymphs released at the kennel, 276 (29%) were recovered. The number of nymphs recovered in the cocker group was significantly higher than in the beagle group in the three infestations. Of a total of 460 adults released at the kennel 230 (50%) were recovered. The total number of males and females was significantly higher in the cocker group (98 and 80) than in beagle group (35 and 17), and higher in all three infestations (Table 1).

Arrestment tests

Combining the results obtained for all the dogs, a significant number of ticks was already arrested after 1 h of exposure on the flannels treated with the substances from the dogs, with 67% for the cockers and 47% for the beagles, comparing to 17 and 16% in the control for each breed, respectively. From 18 to 24 h there was a slight increase in the number of ticks arrested, reaching, in the last observation, with 77% in the cockers' flannels and 55% in the beagles, and 10 and 13% in the control flannels, respectively (Fig. 1).

Due to the large numbers of ticks arrested after 24 h, the breeds were compared in this period. When the substances of the two breeds were tested comparing with the control, the ticks always preferred the dogs substances to the control, especially in the Cocker substances. In this breed there was >70% arrestment with the substances of all the tested animals, while in beagles this arrestment only reached this level in one of the tested animals. In both breeds, some ticks were not arrested, staying on the Styrofoam or being retained in the double face tape or in the water. In the cocker group the number of ticks

Table 1 Total number of larvae, nymphs, males and females harvested from five dogs of the beagle breed and five dogs of the English cocker spaniel breed, after three infestations on the walls of the kennel

Group	Infestation	Larvae	Nymph	Male	Female	Total
Cocker	1st	19	24	43	33	119
	2nd	52	38	35	32	157
	3rd	3	184	20	15	222
	Total	74	246	98	80	498
Beagle	1st	1	10	19	6	36
	2nd	9	5	9	8	32
	3rd	3	15	7	3	28
	Total	13	30	35	17	96

For the same infestation, the number of ticks was higher in the cocker group than in the beagle group, using ANOVAs and Students T test (χ^2) ($P < 0.05$), with the exception of the number of larvae in the third infestation that was the same in the two groups

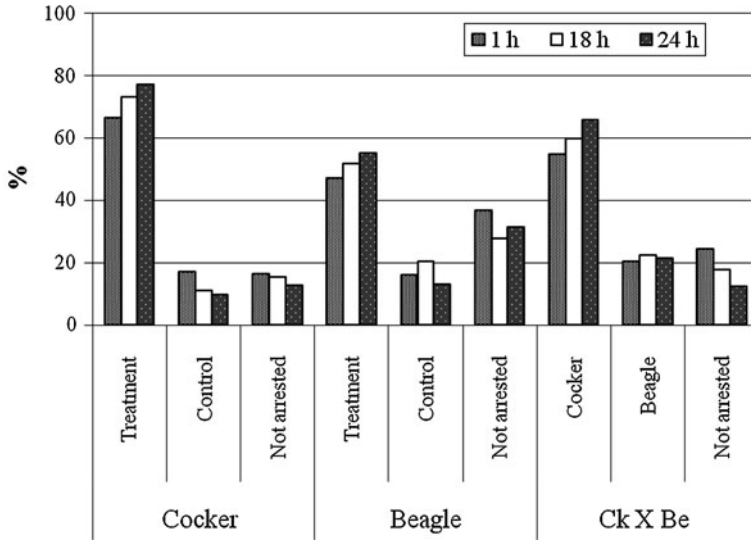

Fig. 1 Percentage of arrestment of *Rhipicephalus sanguineus* adults after 1, 18 abd 24 h in the presence of odors collected from two breeds of dogs, English cocker spaniel and beagle

Table 2 Number of adults of *Rhipicephalus sanguineus* arrested in substances collected from two breeds of dogs, beagle and English cocker spaniel

	Animal tested														
	1			2			3			4			5		
Choice:	T	C	N	T	C	N	T	C	N	T	C	N	T	C	N
Ck vs. Control	86	0	14	78	8	14	79	10	11	72	20	8	72	11	17
Be vs. Control	47	4	49	66	13	21	45	18	37	60	19	21	58	12	30
Ck vs. Be	64	6	30	68	24	8	75	21	4	54	38	8	68	19	13

T treated with the substance of one breed or in the line Ck vs. Be with substance of cocker

C control with cleaned flannel or in the line Ck vs. Be with substance of beagle

N, not arrested ticks; Ck, cocker; Be, beagle

In all treatments the number of ticks was higher in the treated flannel than in control. In the cocker versus beagle, the number of ticks was higher in all cocker substances except in animal four ($P < 0.05$—Chi-square test)

which were not arrested was always smaller than the ticks arrested, but in the beagle group it was sometimes higher. When the two breeds were tested simultaneously, a significantly higher ($P < 0.05$) number of ticks was arrested in the flannels treated with the substances of cockers than of beagles, in four of the five tested dogs (Table 2).

There was no significant difference ($P > 0.05$) between the number of male and female ticks found in the treated flannels in any of the three treatments. In tests with a control, the total number of ticks on the flannels treated with odor of cocker consisted of 49% males and 51% females and on the flannels treated with odor of beagle 53% were males and 47% were females. When the two races were tested simultaneously, 53% were males and 47%

were females on the cockers flannel, and 66% were males and 34% were females on the beagle's flannels.

Attractiveness tests

The combined results obtained for all the tested dogs showed that neither the males (45%) nor the females (55%) of *R. sanguineus* were attracted to the odor of cocker. When exposed to the odor of beagle, only 31% of the males and 21% of the females went to the treated arm. These results seem to indicate that the adults of *R. sanguineus* are repelled by the scent of beagle. When odor samples from the two breeds were tested simultaneously there was no difference in the percentage of males going toward the scent of any one of the breeds, with 43% for the scent of beagle and 57% for the scent cocker. Similar results were observed with the females (Tables 3, 4).

When the dogs were analyzed individually, there was no significant difference ($P > 0.05$) in the response of males and females of *R. sanguineus* to the scents of any of the five cockers compared to the control. When analyzing the response to the odor of beagle, in four out of five dogs, the female ticks chose the control. When the two breeds were tested simultaneously, the males and females *of R. sanguineus* did not discriminated

Table 3 Number of females and males of *Rhipicephalus sanguineus*, on the treated arm of a Y-olfactometer, testing the odor of one breed, beagle or English cocker spaniel dogs and control

Animal	Beagle		Cocker	
	F	M	F	M
1	3*	4	8	5
2	4	5	9	8
3	3*	4	7	8
4	3*	4	9	4
5	3*	6	8	9
Total (%)	16 (21)*	23 (31)*	41 (55)	34 (45)

A total of 15 males and females was tested against each animal odor

* Statistically different from the control untreated arm ($P < 0.05$—Chi-square test)

Table 4 Number of females and males of *Rhipicephalus sanguineus,* on the arm of a Y-olfactometer treated with the odour of beagle or English cocker spaniel dogs

Animals	Females		Males	
	Beagle	Cocker	Beagle	Cocker
1	7	8	8	7
2	3*	12	9	6
3	4	11	8	7
4	7	8	3*	12
5	7	8	4	11
Total (%)	32 (43)	43 (57)	32 (43)	43 (57)

A total of 15 males and females was tested against each animal odor

* Statistically different ($P < 0.05$—Chi-square test)

between the odors of the two tested breeds, except the highest number of females on cocker #2 and males on cocker #4 (Tables 3, 4).

Discussion

The results obtained from the experiments of artificial infestations and from the behavioral tests indicate that *R. sanguineus* might be able to use chemical substances from the dogs to differentiate between the susceptible breed, English cocker spaniel and the resistant one, beagle.

The total number of larvae recovered from the two groups was low, representing less than 1% of the total of larvae released in the kennel. Even if we considered the losses that are expected, for example, due to desiccation or predation, this percentage was very low. One of the factors that might have contributed to this is the grooming done by the animals themselves as no preventive measures were taken to stop this behavior. Grooming can remove from 30 to 80% of the ticks that climb and attach to the animal and this removal is more intense in the most resistant animals. The larvae, due to specific characteristics, such as their reduced size and the behavior of attaching and detaching several times on the host before beginning the blood meal, are the most susceptible stage for mechanical removal by the host (Rechav 1992; Mooring et al. 2004). The results obtained here reinforce this explanation, because with the cockers almost six times more larvae were counted than in the beagles.

Higher number of ticks in other stages, were also counted in cocker group than in beagles with the number of adult ticks almost five times higher on cockers. It could be argued that these effects were due to the grooming behavior, however, the response of the adults to the substances of the two breeds indicates that *R. sanguineus* could distinguish between them and use this information to choose susceptible individuals among a group of sensitive and resistant dogs before acquiring a host.

Ticks use substances liberated by the animals to locate a host in order to obtain a blood meal (Carroll 1998, 1999, 2002a, b), as well as, to identify more appropriate places on the host in which to feed (Wanzala et al. 2004). Waladde and Rice (1977) suggested that the resistant animals might present substances on the skin or in the serum, that when perceived by gustatory sensilla may inhibit the conclusion of the blood meal by the tick, been related as much in the selection of the host, as in the manifestation of the host resistance. Aside from this supposition, we are aware of no other reports confirming this idea.

When tested with a control, the ticks showed arrestment by the substances from the dogs of both breeds. However, this arrestment was more conspicuous in the substances from cockers. On the other hand, when the ticks had to choose among the substances of the two breeds, they clearly preferred, those from cockers. When the odors from the dogs of the two breeds were tested in the Y-olfactometer, attractiveness was not observed for either of them but in the tests with the odor of beagle and the control, the adults of *R. sanguineus* exited the olfactometer. This behavior was not observed with the cocker odor, suggesting that there are repellent chemicals in the Beagle odor. These results need to be confirmed, since according to Dautel (2004) the Y-olfactometer is not the most suitable biological assay for repellency evaluation. Perhaps, the mixture of the odor that occurs in the olfactometer can neutralize the repellent substances that might be present in the beagle odor. This deficiency of the test could explain why when the two breeds were evaluated simultaneously, in the attractiveness tests, there was almost no repellency.

The species *R. sanguineus* is known as a hunter tick (Labruna and Pereira 2001), although the ambush strategy cannot totally be dismissed (Uspensky 2002). Based on our results, we suggest that benefits *R. sanguineus* to avoid parasitizing resistant dogs, like beagles, and this avoidance is facilitated by perceiving and being repelled by volatile substances associated with such dogs. The arrestant substances could be sensed after the tick climbs onto the host inciting it to begin the blood meal. They could still be used for the detection of vantage points if this tick uses the ambush strategy. In this case the substances left by the dogs in the environment would indicate to the tick the most appropriate places to position itself and to find a host as demonstrated by Carroll (2001) with *Ixodes scapularis*.

Even if it has been demonstrated that the chemical substances affect of the parasitism of *R. sanguineus* in resistant animals, this strategy is not totally effective, since beagles were also artificially infested. There was arrestment in response to the substances of this breed and yet it is known that this breed is infested by this tick in natural situations (Inokuma et al. 1997; Jittapalapong et al. 2000a, b). It is worth noting that *R. sanguineus* is strongly attracted by CO_2 (Stella 1998; Louly 2003), and Sonenshine (1993) reported a response of this tick to the bark of dogs. These factors probably contributed to the artificial infestations observed in the present study, as well as the natural infestations already reported in the literature.

The development of resistance by the host causes several changes in the skin of the animal such as an increase in temperature, migration of defense cells and liberation of substances that stimulate the grooming behavior of the host such as histamine (Engracia Filho et al. 2006). Those changes could result in alterations in the chemical substances produced by the dogs that would have been perceptible to the tick, enabling it to differentiate the resistant animals from the susceptible ones. As we used animals previously exposed to *R. sanguineus* we cannot say that the difference in the behavior observed would be present if those substances had been collected from naïve animals. Tick themselves may have caused those alterations in the dogs' substances. Further studies are necessary to address this question.

A choice of hosts is advantageous to *R. sanguineus* for its survival, since it allows to the tick to escape the deleterious effects caused when feeding on a resistant individual which were demonstrated by Louly et al. (2009) who observed a decline in the biological viability of ticks fed on beagles.

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