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INTERSPECIFIC CUTICULAR HYDROCARBON VARIATIONS AND TENTATIVE HYBRIDS OF *RHIPICEPHALUS SANGUINEUS* AND *R. PUSILLUS* TICKS (ACARI: IXODIDAE) IN NATURE

A. ESTRADA-PEÑA, C. GORTAZAR, C. CALVETE

SUMMARY

The interspecific variation of *Rhipicephalus sanguineus* and *R. pusillus* ticks is studied by means of cuticular hydrocarbon gas chromatography, from specimens collected in nature. Several compounds able the effective separation of « pure » strains of both species, together with tentatively determined hybrid specimens, collected exclusively on foxes. Cuticular hydrocarbon features are compared with morphological observations of the hybrid ticks. Those show a variable morphology with mixed characters of both *R. sanguineus* and *R. pusillus,* as size and scutal punctations. Also, chromatographic pattern is intermediate between the typical one of the two species. The possibility of hybridation of the two species in nature is discussed, bearing in mind the ecological and biological requirements of both hosts and parasites.

RÉSUMÉ : **Variations interspécifiques des hydrocarbones cuticulaires des tiques *Rhipicephalus sanguineus* et *R. pusillus* (Ixodidae) et de spécimens naturels supposés hybrides.**

Étude de la variation interspécifique entre deux espèces de tiques *(Rhipicephalus sanguineus* et *R. pusillus)* à l'aide de la chromatographie gazeuse des hydrocarbones cuticulaires. Différents composés permettent de séparer les souches « pures » des deux espèces des spécimens supposés hybrides récoltés uniquement sur les renards. Les caractéristiques des hydrocarbones sont comparées avec la morphologie des tiques supposées hybrides. Ces hybrides ont une morphologie variable avec un mélange des caractéristiques des deux espèces en particulier la taille et les ponctuations de l'écusson. La formule chromatographique est intermédiaire entre celles des deux espèces. La possibilité d'une hybridation dans la nature est discutée en tenant compte des nécessités écologiques et biologiques des hôtes et des parasites.

INTRODUCTION

One of the most controversial groups in the genus *Rhipicephalus* is the *R. sanguineus* groups. Although the type-specimens of *R. sanguineus* has been lost and little is known of its origin, the species remains the type of the genus and the baseline for this group (Pegram *et al.,* 1987a). Because it is the most widely distributed tick species in the world, it also exhibits a considerable morphological variation (Paperna and Giladi, 1974). On the other hand, *R. pusillus* Gil-Collado was described from specimens collected in Spain; currently it is known from Spain, Portugal, France, Morocco, and Tunisia as a parasite of Warren rabbits *(Oryctolagus cuniculus algirus)* and foxes (Estrada-Peña *et al.,* 1987; Santos Dias, 1987). Although the species was first included in the *R. capensis* group (Zumpt, 1942), Morel and Vassiliades (1963) placed it in the *R. sanguineus* group. These authors also noted the resemblance of *R. pusillus* to dwarf specimens of species in the group.

In the captures of rhipicephalines from dogs and rabbits in Spain, there is commonly not doubt about the specific determination of both *R. sanguineus* and *R. pusillus;* however, specimens collected on foxes always exhibits a considerable degree of morphological variation. Specific assignation may be difficult because the specimens share structural features of both *R. sanguineus* (greater size, elongate adanal plates in male) and *R. pusillus* (scutum rough, heavily punctate); in some cases, « pure » *R. pusillus* ticks can be recorded, together with small *R. sanguineus* displaying a rough punctation only at some places of the scutum.

Cuticular hydrocarbon analysis is one of the new biochemical techniques used to identify the adults of sibling species in Diptera (Phillips *et al.,* 1988) but its use on ticks has been restricted only to one work on American *Amblyomma* (Hunt, 1986). In a previous paper on *Rhipicephalus* (Estrada-Peña *et al.,* in press), the validity of the

Unidad de Parasitología, Facultad de Veterinaria, C/Miguel Servet, 177, 50013-Zaragoza, Spain.

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method for species discrimination was demonstrated with some specimens collected in the Ebro river valley, at the Northeast of Spain.

The purpose of this study was to determine if the method is also applicable to the easy determination of allopatric populations of *R. sanguineus* and *R. pusillus,* collected from several provinces in Spain, as well as for assignation of bizarre specimens. Chromatographic patterns are used for the cluster of batches following their similarities. Data of gas chromatography are also compared with morphological peculiarities, with special atention focused on specimens sharing features from both species.

MATERIAL AND METHODS

Ticks. Adults of two *Rhipicephalus* species are included here: *R. sanguineus* Latreille (132 males, 89 females) and *R. pusillus* Gil Collado (91 males, 55 females). Several specimens (47 males, 39 females) were included as « unassigned » because of the presence of morphological features from both species in the same individual. Ticks were collected by both standard flagging and captures on domestic and wild hosts at several areas of Spain. Semiengorged or replete females were avoided because of variation of cuticular hydrocarbon pattern with engorgement was observed. *Figure 1* displays the collecting zones in several Spanish provinces, the number of batch as referred herein, and the primary specific status of specimens based only on morphological appreciation. Specimens were assigned to batches. All ticks belonging to the same species and sex, and collected on the same host, are here considered as a batch. Data for hosts are also included in the *Figure 1.*

Extraction. All hexane was redistilled in glass, and all glassware was rinsed with hexane before use. Ticks were placed individually in test tubes to which approximately 500 microlitres of hexane was added. Test tubes were gently swirled periodically for 10 minutes, after which hexane was decanted to 1 ml scintillation vials. This sample was evaporated to 100 microlitres. Hydrocarbons were isolated by elution from small columns (7 cm by 0.5 cm i. d.) of BioSil A (Bio-Rad Labs.) with 7 ml of hexane. Such a procedure eliminates more polar lipids removed by the extraction with hexane. Alkanes and alkenes were separated by chromatography of the hydrocarbons on a column (7 cm by 0.5 cm i. d.) of 20 % (wt/wt) silver-nitrate impregnated BioSil A (Nelson *et al.,* 1981). Alkanes were eluted with 7 ml of hexane and alkenes with 7 ml of chloroform. The eluted samples were again evaporated to 100 microlitres.

Gas chromatography (GC) analysis was performed with a Hewlett-Packard Model 5890A equipped with a flame ionization detector, on an RSL-150 fused silica column (10 m by 0.53 mm i. d.) 0.25 micrometers film thickness, temperature programmed from 150 to 300° C at 10°/min, injector 325° C, detector 325° C. Samples were injected using a packed column injector with injection insert. Peaks were provisionally identified from their retention time and numbered so that the same peak in different individuals can be monitored. N-alkane and n-alkene standards (Sigma Biochemical Co., St. Louis, MO.) were used as internal standards to calculate equivalente chain lengths (ECLs) for peaks (Miwa, 1963). The final identification of each compound was performed by mass spectrometry, following the methods of Lockey (1988). Relative abundance values were obtained as follows. The peak with the maximum concentration in each run was referred as « concentration 100 » and the area for other compounds compared as a portion of the maximum concentration; this procedure also standardizes runs by eliminating differences in injection amount of sample because the size of the specimen.

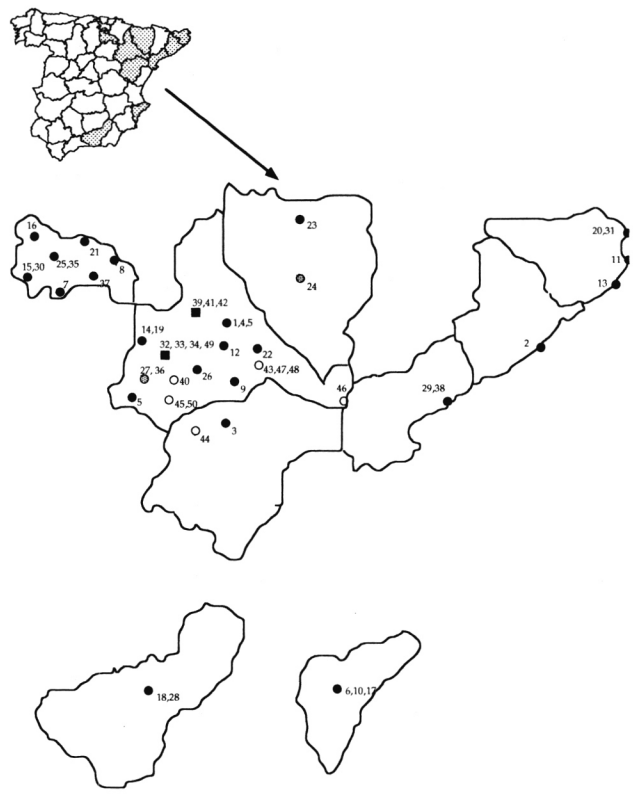

FIG. 1. — Map of Spain, showing the collection localities of ticks. Shaded provinces are magnified in the drawing below. Each point is labelled with the number of batch for each collection. Black circles: collection of only *R. sanguineus* « pure » specimens. White circles: *R. pusillus* « pure » specimens. Shaded circles: Both « pure » *R. pusillus* and primarily unassigned specimens. Black squares: « Pure » *R. sanguineus, R. pusillus,* and primarily unassigned ticks. Hosts for the batches are as follows: *Canis familiaris* (batches 1, 2, 5, 6, 7, 8, 10, 11, 13, 15, 16, 20, 21, 22, 23, 25, 28, 29, 30, 31, 35, 37, 38, 39), *Oryctolagus cuniculus* (batches 40, 43, 44, 45, 46, 47, 48, 50), *Vulpes vulpes* (batches 3, 4, 9, 12, 14, 17, 18, 19, 24, 26, 27, 32, 33, 34, 36, 41, 42, 49).

Statistical studies were performed only with the peaks that provide the greatest separation between *R. sanguineus* and *R. pusillus.* A Principal Component Analysis (PCA) was performed with peaks eluting at 10.3 (n-triacontane), 11.0 (2-methyltriacontane), 11.2 (n-hentriacontane), 11.7 (3-methylhentriacontane), 11.9 (2-methylhentriacontane), 12.1 (n-dotriacontane) and 13,0 (n-tritriacontane), with an Orthotran-Varimax transformation. Computations were done with the SYSTAT version 5.0 for the Apple Macintosh computer. As mentioned by Estrada-Peña *et al.* (in press) other compounds can be used for the effective separation of « pure » *R. sanguineus* and *R. pusillus* but unassigned specimens can not be adequately separated by them. Also, no attempts were done to separate samples by sex nor by geographic origin.

RESULTS

Figure 2 shows the results of the PCA; the batches of ticks are displayed in the figure following their coordinates

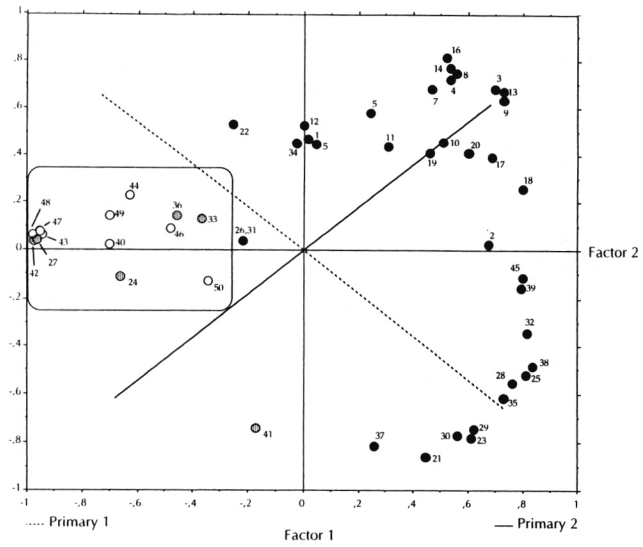

FIG. 2. — Principal Components Analysis carried out with the compounds resolved as mentioned in *Table I* and « Material and Methods » section. Black circles: *R. sanguineus;* Withe circles: *R. pusillus;* Shaded circles: tentative hybrid specimens. The area at which « pure » *R. pusillus* specimens clust, together with some hybrids is enclosed by a square.

after the first and second principal axes. The Orthotran-Varimax transformation allows to the rotation of the primary axes to improve the partition of the sample lots into well defined components. In such a way, « true » *R. pusillus* ticks (empty circles in *Figure 2*) are grouped along the first quadrant, while *R. sanguineus* representatives (full black circles) are located along the remaining three quadrants. The use of the cuticular compounds with retention times as mentioned above supports the effective separation of populations.

Primarily unassigned specimens are represented by gray shaded circles in the *Figure 2.* Two samples (lots 27 and 42; 17 females and 10 males, respectively) are closely located to pure *R. pusillus* ticks in the factorial space representation, three ones more (lots 24, 33, and 36; 8 females, 19 males and 14 females, respectively) are slightly moved away from this main group; one sample (lot 41; 18 males) is located in an intermediate position between *R. sanguineus* and *R. pusillus* positions. *Table I* includes the data for the relative amount of the seven compounds considered as valid for species and populations discrimination. Unassigned specimens lay on an intermediate position between *R. sanguineus* and *R. pusillus.*

Almost all the samples included in the computations as unassigned consist of ticks with a size very to that of *R. sanguineus,* twice as much as the usual for *R. pusillus.* However, variations of other body structures have been observed, as follows. Males for lot 41 *(Fig. 3 to 5)* have a scutal punctation pattern with many minute punctures,

TABLE I. — *Relative amount of the compounds used to resolve determination between* R. sanguineus *and* R. pusillus *(average for all the specimens in each group). Ret. time means for retention time of each peak at the chromatographic conditions mentioned in the Material and Methods section.*

Ret. time	R. sanguineus		R. pusillus		unassigned	
	male	female	male	female	male	female
10.3	0.26±0.02	0	5.11±1.11	1.07±0.23	9.72±1.25	9.12±1.58
11.0	11.78±0.89	17.23±0.21	1.07±0.56	4.45±0.56	0	0
11.2	1.51±0.11	3.05±0.51	5.08±0.99	1.63±0.25	6.48±1.86	0
11.7	5.03±0.45	5.55±0.89	0.92±0.01	0.95±0.02	0.24±0.01	5.79±2.53
11.9	5.95±0.59	14.98±1.21	4.62±0.16	7.70±0.52	0	4.92±1.12
12.1	1.38±0.09	0.26±0.01	6.70±0.98	4.05±1.10	9.24±2.58	0
13.0	1.19±0.12	1.70±0.12	9.03±1.10	4.78±1.04	17.25±4.25	20.12±5.25

with several rough punctations symmetrically located; margin lines are very deep and full of unsmooth cavities; adanal and spiracular plates are outlined in the *Figures 4 and 5.* Morphological variations in females (lots 24, 27 and 36) lay always on the size and shape of punctations of scutum, from very small and closely located, to rough and unevenly distributed. *Figure 6* displays the most commonly observed scutal punctations in primarily unassigned female specimens. Genital aperture (not illustrated) was typical for *R. sanguineus* in all unassigned females. However, spiracular plate shows a well defined, rounded and wide dorsal prolongation *(Fig. 7)* in almost all the females. Males from the groups 42 and 33 *(Fig. 8 to 10)* are closely related to pure *pusillus* males, having a well-defined scutal pattern composed by many rough punctations; margin lines are superficial and not covered by dense puncture. Adanal plate is almost as long as broad, and spiracular plate has a wide, rounded dorsal prolongation. The size of these specimens ranges from 3.9 to 5.1 millimeters.

DISCUSSION

Phillips *et al.* (1988) outlined that the analysis of cuticular hydrocarbons in insects has been used to tackle a variety of taxonomic problems, besides its use as a taxonomic tool. The technique is also applicable to ticks, with a widening field in the problems of determination of sibling species. As demonstrated in our results, the method is a useful tool in the separation of populations,following their chromatographic pattern.

The results obtained in this paper allow us to consider the existence in Spain of several well characterized populations of *R. sanguineus* and *R. pusillus* ticks, easily separated by their cuticular mixtures, and outlined by the Principal Component Analysis. There is also a third group of specimens that shares morphological affinities with the previous two species, without correlation between the different body structures. There are two possible explanations

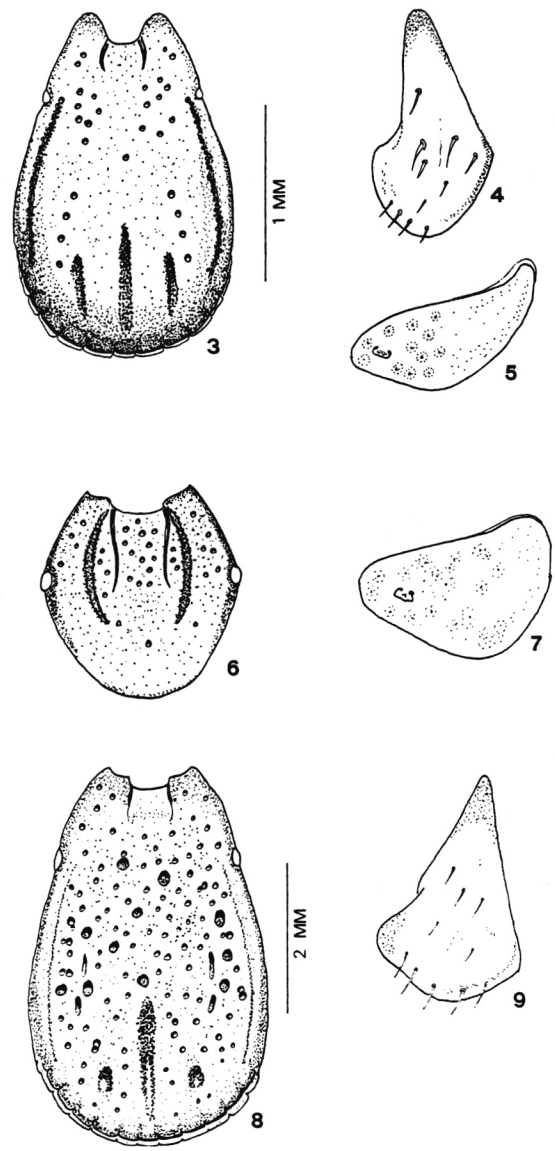

FIG. 3 to 9. — Morphological observations of several tentatively determined hybrid specimens. *Figures 3 to 5:* males from lot 33. Notice the very small size and deep margin lines; both adanal and spiracular plates are typical for *R. sanguineus. Figure 6 and 7:* females from lot 24. *Figures 8 and 9:* males from lot 27. The size is between the range of *R. sanguineus,* but notice the deep dorsal punctations and wide spiracular plate; margin lines are very shallow.

for our combined morphological and chromatographic results. First, primarily unassigned ticks may belong to subpopulations of pure *R. pusillus* from both fox and rabbit. However, several batches did not exhibit the typical facies for the species. It could be expected that an intermediate, intraspecifically crossed subpopulation, would display morphological figures typical for the species, leaving aside the size. Furthermore, the chromatographic mixture would be approximately that of the species. In such a way, no significative differences have been found between *R. pusillus* collected on fox and those collected on rabbit, but unassigned specimens are very separated from both « strains ». Moreover, statistical clustering would result in a near proximity of batches, but unassigned specimens are randomly distributed in the reduced space, close to the clust of *R. pusillus* « pure » ticks.

Our results, as a whole, show the lack of homogeneity in the batches of primarily unassigned specimens, from both morphological and chromatographic points of view. These unfixed figures lead us to think in the presence of hybrid *R. sanguineus-R. pusillus* ticks. These populations not seem to constitute a yet undescribed *Rhipicephalus* species, because the absence of stable features leading to the determination, as well as an extreme variability in the specimens. As mentioned, the most striking feature of the tentative hybrids is the presence of a very variable chromatographic pattern, associated to a bizarre morphology. In laboratory experiments in which heterogamic crosses between *Argas polonicus* and *A. vulgaris* have been carried out (Estrada-Peña and Dusbábek, in press), the hybrids displayed a chromatographic mixture with low similarities to those of their parents. In these hybrids, the most prominent features were the detection of many compounds not previously observed in the parental species, as well as the absence of hydrocarbons typical for both *A. polonicus* and *A. vulgaris.* In the results obtained for this paper, tentative hybrid specimens show the absence of compounds consistently detected in both *R. sanguineus* and *R. pusillus;* moreover, the variation in the peaks already detected is greater than in the « pure » specimens.

The behavior of foxes and rabbits seems to be responsible for the appearance of bizarre specimens. *R. pusillus* is an endophilic species, specimens being collected from the first 2-3 meters of burrows, walking on the sand, and between the pebbles of the lair opening. On the other hand, foxes inhabit dens only in the reproduction season (from approximately mid February to late June in Spain). At this time, foxes build their own tunnel or employ another one already in use by rabbits. In such a way, the *R. sanguineus* ticks carried by the fox can drop into the den, and mate with *R. pusillus* already present inside the gallery. Such a conclusion is supported by the date of collection of the tentative hybrids, which have been recorded only on foxes in the spring; pure *R. pusillus* are collected all around the year on rabbits.

In their Ethiopian studies, Pegram *et al.* (1981) noted that some *Rhipicephalus* specimens had structural features of both *R. senegalensis* (male and female scutae) and *R. cliffordi* (female genitalia). In our specimens, the only *pusillus* character is the scutal punctations, and, at some degree, the relative size of the adanal plates; spiracular plates and female genitalia are the *sanguineus* features commonly found in the hybrid ticks. In some areas of Eastern

and Southern provinces of Zambia *Rhipicephalus appendiculatus* and *R. zambeziensis* are sympatric, and numerous intermediate forms as well as typical *R. appendiculatus* and *R. zabeziensis* are found (Zivkovic *et al.*, 1986). The two species cross-breed under laboratory conditions and a fertile hybrid is produced by *R. zambeziensis* females and *R. appendiculatus* males.

Intensive morphological and biological studies on reared materials and critical restudy of collections from foxes are essential to resolve the determination of these specimens. Also, dwarf *R. sanguineus* specimens must be biochemically examined. In future ecological studies there should be opportunity for further clarification on the distribution of the specimens considered in this paper. Comprehensive integrated studies on the biology, ecology, and morphology, supported by chromatographic findings are required to define species. Cuticular hydrocarbons seem to be a promising tool in such as investigations.

REFERENCES

Estrada-Peña A., Lucientes J., Sánchez C., Cutiérrez J., Ocabo B. : Nuevos datos relativos a la distribución de los lxódidos en España (II). *Rev. Ibér. Parasitol.*, 1987, *46,* 431.

Estrada-Peña A., Dusbábek F. : Cuticular hydrocarbon composition of Argas *(A.) polonicus, A.(a.) vulgaris* and their hybrids. Proceedings of the Second EURRAC Symposium, Krynicka, Poland, September 1972, in press.

Estrada-Peña A., Estrada-Peña R., Peiró J. M. : Differentiation of *Rhipicephalus* ticks (Acarina: Ixodidae) by cuticular hydrocarbons gas chromatography. *J. Parasitol.*, in press.

Hunt L. M. : Differentiation between three species of *Amblyomma* ticks (Acari: Ixodidae) by analysis of cuticular hydrocarbons. *Ann. Trop. Med. Parasitol.*, 1986, *80,* 245-249.

Miwa T. K. : Identification of peaks in gas-liquid chromatography. *J. Am. Oil Chemist. Soc.*, 1963, *40,* 309-313.

Morel P. C., Vassiliades G. : Les *Rhipicephalus* du groupe *sanguineus* : espèces africaines (Acariens: Ixodoidea). *Rev. Élev. Méd. Vét. Pays Trop.*, 1963, *15* (nouvelle série), 343-386.

Nelson D. R. J., Dillwith J. W., Blomquist G. J. : Cuticular hydrocarbons of the house fly *Musca domestica. Insect Biochemist.*, 1981, *11,* 187-197.

Paperna I., Giladi M. : Morphological variability, host range, and distribution of ticks of the *Rhipicephalus sanguineus* complex in Israel. *Ann. Parasitol. Hum. Comp.*, 1974, *49,* 357-367.

Pegram R. G., Hoogstral H., Wassef H. W. : Ticks (Acari: Ixodidea) of Ethiopa. I. Distribution, ecology, and host relationships of species infesting livestock. *Bull. Entomol. Res.*, 1981, *71,* 339-359.

Pegram R. G., Clifford C. M., Walker J. B., Keirans J. E. : Clarification of the *Rhipicephalus sanguineus* group (Acari, Ixodoidea, Ixodidae). I. *R. sulcatus* Neumann, 1908 and *R. turanicus* Pomerantsev, 1936. *Systematic Parasitol.*, 1987a, *10,* 3-26.

Phillips A., Milligan P. J. M., Broomfield G., Molyneux D. H. : Identification of medically important Diptera by analysis of cuticular hydrocarbons. *In: Biosystematics of Haematophagous Insects.* M. W. Service Ed., Systematics Association Special Volume No. 37, pp. 39-59. Clarendon Press, Oxford, 1988.

Santos Dias J. A. T. : Ticks (Acarina: Ixodoidea) parasites of the fox *Vulpes vulpes* Linnaeus, 1758 in Portugal. *García de Orta Ser. Zool.*, 1987, *12,* 43-48.

Zivkovic D., Pegram R. G., Jongejan F., Mwase E. T. : Biology of *Rhipicephalus appendiculatus* and *R. zambeziensis* and production of a fertile hybrid under laboratory conditions. *Exp. Appl. Acarol.*, 1986, *2,* 285-298.

Zumpt F. : Zur Kenntnis Afrikanischer *Rhipicephalus* arten. V. Vorstudie zu einer Revision der Gattung *Rhipicephalus. Z. Parasitenk.*, 1942, *12,* 479-500.