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DEVELOPMENT OF *BORRELIA BURGDORFERI* IN *IXODES RICINUS* FEMALES DURING BLOOD FEEDING

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SUMMARY

The development and modes of transmission of *Borrelia burgdorferi* during blood feeding of *Ixodes ricinus* were evaluated. Our results show that during the slow engorgement phase, the Lyme disease spirochete, *B. burgdorferi* ingested two hours before a blood meal or transmitted transstadially from the nymphs, multiplies in the midgut lumen of *I. ricinus* females. The deeper parts of the midgut diverticula appeared to be the preferred sites for this spirochete. In some gut-infected ticks, a few spirochetes can penetrate the midgut wall and induce a systemic infection, while the overwhelming majority of spirochetes persist in the midgut. Presence of *B. burgdorferi* in hemocoel, and in both acini and ducts of the salivary glands, leads us to suppose a salivary transmission of this spirochete by *I. ricinus* females.

RÉSUMÉ: **Développement de *Borrelia burgdorferi* dans la femelle d'*Ixodes ricinus* durant le repas sanguin.**

Nous avons étudié le développement de *B. burgdorferi* dans la femelle de la tique *I. ricinus* durant le repas sanguin. Les modes de transmission possibles ont été évalués. Nos résultats ont montré que le spirochète se multiplie dans l'intestin des femelles durant la phase lente du repas sanguin, soit pendant les premiers jours alors que la prise de sang est minime. Ce phénomène s'observe aussi bien chez les tiques infectées artificiellement 2 heures avant le repas sanguin que chez les tiques ayant acquis l'infection après une transmission transstadiale de la nymphe à l'adulte. Les parties profondes des diverticules de l'intestin semble être des localisations de choix pour les borrélies. Chez quelques tiques, de rares spirochètes peuvent traverser la paroi intestinale et provoquer une infection systémique alors que la majorité d'entre eux persistent dans l'intestin. La présence de *B. burgdorferi* dans l'hémocoele, ainsi que dans les acini et les conduits des glandes salivaires, laisse supposer que cette borrélie peut être transmise à l'hôte vertébré via la salive des femelles d'*I. ricinus*.

INTRODUCTION

Borrelia burgdorferi, agent of Lyme borreliosis, was first discovered in the United States in the midgut of *Ixodes dammini* (Burgdorfer *et al* 1982) and soon afterwards in Europe, in *I. ricinus* from Switzerland (Burgdorfer *et al* 1983).

Although in unfed ticks the spirochete is generally limited to the midgut (Burgdorfer 1984; Burgdorfer *et al* 1982, 1983, 1985, 1988), it may be observed in other tissues including hemolymph and salivary glands (Burgdorfer *et al* 1983, 1985, 1988). In blood feeding *I. dammini, B. burgdorferi* was also found in the hemolymph (Benach *et al* 1987; Ribeiro *et al* 1987) and in the saliva (Matuschka *et al* 1986; Ribeiro *et al* 1987). In a recent study, both unfed and fed nymphs of *I. dammini* were found to be systemically infected with borreliae in all tissues including the lumen and ducts of salivary glands (Zung *et al* 1988).

In order to evaluate the development of *B. burgdorferi* in blood feeding *I. ricinus* and the possible mode of transmission of the spirochete to the host, a series of histological experiments have been carried out. In this paper, we report our study on the behavior of *B. burgdorferi* in *I. ricinus* females during the blood meal. An account of our results has been presented at the 13th World Association for the Advancement of Veterinary Parasitology Conference held on August 7-11, 1989, at Berlin/GDR.

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MATERIAL AND METHODS

Origin and cultivation of B. burgdorferi : B. burgdorferi (strain NE4) was isolated from *I. ricinus* from the Swiss Plateau (Staatswald). Spirochetes were cultivated in BSK-II medium (Barbour 1984). A culture with a concentration of 10^5-10^6 cells/ml (Helber count cell chamber) was used to infect the ticks.

Source of I. ricinus : Uninfected nymphs and adults of *I. ricinus* are regularly bred in our laboratory for many years (Graf 1978). Periodic controls by direct immunofluorescent test (Peacock *et al* 1971) and dark field microscopy have shown that the ticks are free of *B. burgdorferi* infection. It must be stressed here that transovarial transmission of *B. burgdorferi* in *I. ricinus* is low (2-3 %) (Zhioua *et al* 1988).

Infection modes and blood feeding of I. ricinus:

Three groups of ticks were used:

Group I: pre-feeding infected adults. To obtain pre-feeding *B. burgdorferi* infected females, a modified capillary method was

employed (Chabaud 1950). Female ticks were fastened, abdomens upwards, by double-sided adhesive tape and then allowed to feed in spirochete culture containing capillary glass tubes (Model 6C-120 TF-10, Science Products Trading GmbH, Frankfurt). Ticks were allowed to ingest spirochete culture for 2 hours at 35° C and 95 % RH. The midgut infection was evaluated by direct immunofluorescence. Two days after spirochete ingestion, 68/90 females (75.6 %) were positive. Two hours after infection they were placed on rabbits (Graf 1978).

Group II : transstadially infected adults. Uninfected nymphs were fed in plastic capsules on uninfected gerbils (Graf 1978). One day after attachment, 0.3 ml of spirochete culture medium was injected subcutaneously at the feeding sites of ticks. After drop off, ticks were kept in tubes at room temperature until they moulted (Graf 1978). The infection rate of the resulting adults was evaluated as for ticks of Group I. Six from 13 ticks (46.2 %) were positive.

Group III : uninfected adults (negative controls). Uninfected adults were also used as negative controls.

Blood feeding of adults of all three groups was carried out on ears of uninfected New Zealand white rabbits which were controlled by indirect immunofluorescence test against *B. burgdorferi* antigen (Graf 1978).

PREPARATION FOR HISTOLOGY :

Female ticks were sampled daily until the 6th day of attachment for Group I and at day 1, 3 and 5 after attachment for Groups II and III. Whole ticks were fixed in Karnovsky's fixative (Karnovsky 1965; Agbede *et al* 1986) and embedded in plastic (JB-4 Embedding Kit; Polysciences, Warrington, Pa). For the rapid penetration of the fixative, tick legs were cut off and several knife-cuts were made all around the tick body. These dissections were carried out in cold fixative. In addition, a prolonged time for fixation and infiltration (overnight) and a temperature of 4° C were used.

Transverse sections of plastic embedded ticks were cut at a thickness of 4 or 5 μm using an ultramicrotome (Sorvall, MT 2-B Ultra Microtome, DIGITANA AG). Dieterle silver stain was employed to visualize the spirochetes (Van Oden and Greer 1977). For JB-4 plastic sections a prolonged developing time of about 12 minutes proved necessary.

RESULTS

Group I: pre-feeding infected females *(table I)*. Two of the 12 ticks proved to be negative (no 40 and 46). Spirochetes were observed in the midgut lumen only in 4 ticks (no 1, 16, 17 and 28), in the midgut lumen and midgut epithelial cells in 2 (no 31 and 52). In four ticks the infection was systemic, extending to hemocoel and salivary glands (no 27, 39, 45 and 51) *(table I and figs 1, 2, 3, 4)*.

Spirochetes were first found in midgut epithelial cells and hemocoel at 3 days, and in salivary glands at day 5 after attachment. They were present only in ticks harbouring borreliae in their midgut lumen, midgut epithelia cells and hemocoel respectively *(table I)*.

No spirochetes were found in tissues other than those mentioned above. The synganglion was considered a preferred site for different borreliae species (Burgdorfer 1951; Sarasin 1957; Benach *et al* 1987). In our study, by contrast, neurons and other fibrilar materials in the synganglion were dyed by silver stain and it seemed very difficult to visualize spirochetes in this organ (Benach *et al* 1987).

In systemically infected ticks, spirochetes were found to be much more abundant in the midgut lumen than in other tissues *(table I)*. Clusters of spirochetes were often found in the midgut lumen or in spaces between midgut epithelial cells. In other tissues, spirochetes were limited in number, 6-16 in midgut epithelial cells, 6-15 in salivary glands and about 3 to 7 in hemocoel per *I. ricinus* female *(table I)*.

Although the number of observed ticks is low, an increased spirochetal burden in the midgut lumen was evident after 3 to 4 days on rabbits. Spirochetes were more numerous in the deeper parts of the midgut diverticula than in others. Some borreliae were found to attach themselves close to

TABLE I.

	Group I										Group II									
Blood feeding time (day)	1	2		3			4		5		6		1		3			5		
Tick no	1	16	17	27	28	31	39	40	45	46	51	52	101	102	116	117		140		
Midgut:																				
- Lumen	*	**	*	**	**	***	**	-	***	-	***	**	-	-	***	-		**		
- Epithelial cells	-	-	-	⊛6	-	8	10	-	16	-	11	7	-	-	14	-		-		
Hemocoel	-	-	-	3	-	-	5	-	7	-	4	-	-	-	5	-		-		
Salivary glands:																				
- Acinus I	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-		-		
- Acinus II	-	-	-	-	-	-	-	-	8	-	4	-	-	-	7	-		-		
- Acinus III	-	-	-	-	-	-	-	-	4	-	2	-	-	-	4	-		-		
- Ducts	-	-	-	-	-	-	-	-	2	-	-	-	-	-	1	-		-		

*,**,***: About 5, 10 and more than 10 spirochetes within a visual field at lo x 100 magnification

⊛ : Total number of spirochetes observed in an organ or tissue of a tick

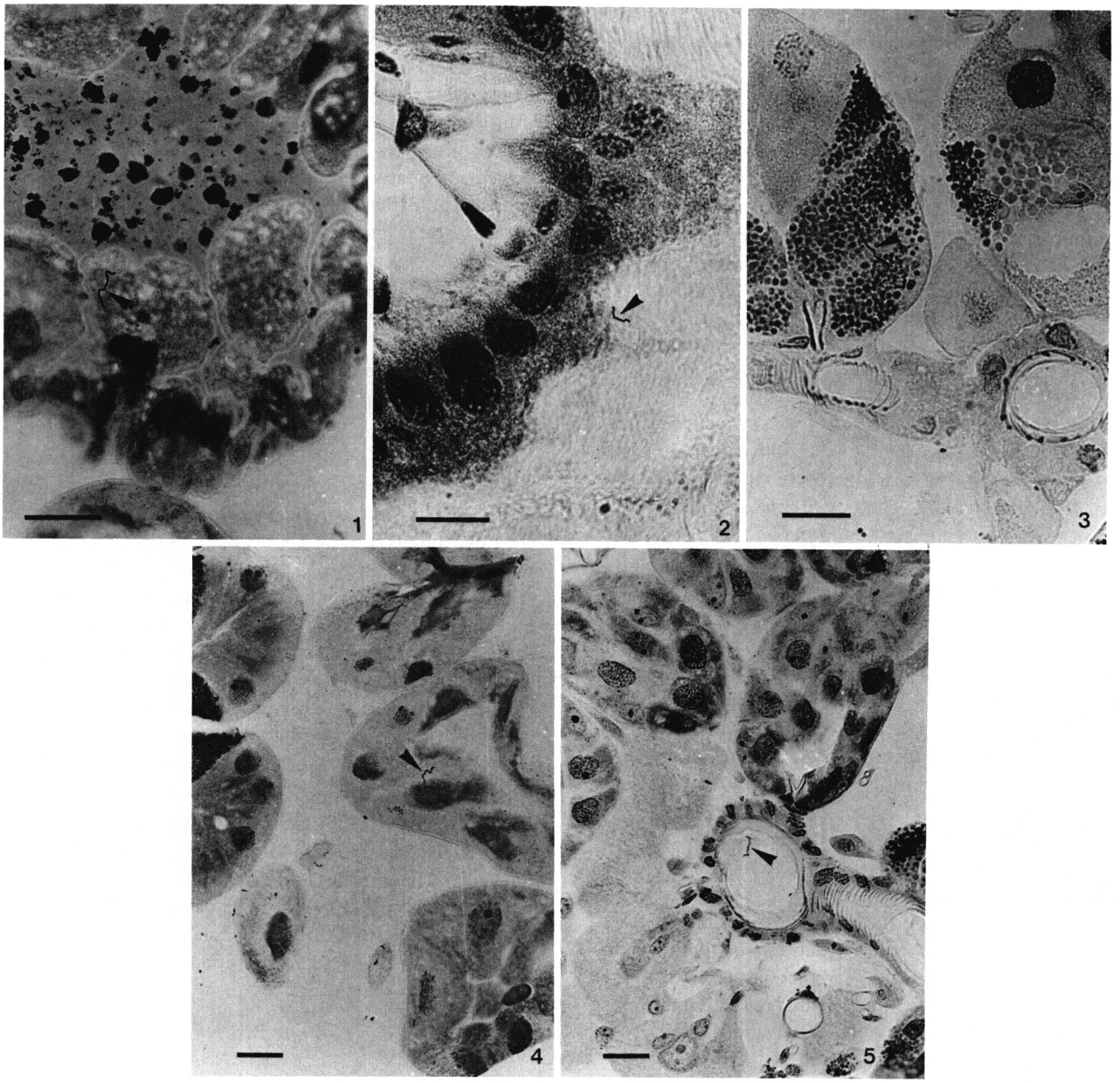

FIG. 1. — *B. burgdorferi* (arrow) in a midgut epithelial cell of tick 51 of Group I examined 6 days after the start of the blood feeding on rabbit (Dieterle staining. Bar = 25 μm).

FIG. 2. — *B. burgdorferi* (arrow) in hemocoel of tick 51 of Group I examined 6 days after the start of the blood feeding on rabbit (Dieterle staining. Bar = 20 μm).

FIG. 3. — *B. burgdorferi* (arrow) in an acinus II of salivary glands of tick 51 of Group I examined 6 days after the start of the blood feeding on rabbit (Dieterle staining. Bar = 20 μm).

FIG. 4. — *B. burgdorferi* (arrow) in an acinus II of salivary glands of tick 45 of Group I examined 5 days after the start of the blood feeding on rabbit (Dieterle staining. Bar = 20 μm).

FIG. 5. — *B. burgdorferi* (arrow) in a duct of salivary glands of tick 116 of Group II examined 3 days after the start of the blood feeding on rabbit (Dieterle staining. Bar = 20 μm).

the epithelial midgut cells, while most of them seemed to be distributed randomly in the same section of diverticulum or stomach.

Group II: transstadially infected females. Three from 5 ticks (no 101, 102 and 117) were negative. In one tick, spirochetes were seen in the midgut lumen only (no 140) and a systemic infection involving midgut lumen, midgut epithelial cells, hemocoel and salivary glands (acini and

ducts) was observed in the remaining tick (no 116) *(table I and fig. 5)*. In this systemically infected female, no spirochetes were detected in tissues other than those mentioned above. Many spirochetes were observed in the midgut lumen, especially in the deeper parts of the midgut diverticula of the two gut infected ticks (no 140 and 116), while only a few could be found in the infected tissues other than the midgut of the systemic infected tick (no 116).

Group III: uninfected ticks (negative control). In none of the 6 control ticks were spirochetes revealed in any tissues by the silver stain.

DISCUSSION

Recently, increased percentages of systemic infected female ticks from less than 4 % in unfed ticks to about 19 % in engorged and spent ones were reported in *I. dammini* (Burgdorfer *et al* 1988). In *I. ricinus,* a similar phenomenon was also observed by Monin *et al* (1989). Above findings suggest that gut penetration and systemic infection of *B. burgdorferi* occurs during the blood meal and/or after engorgement in both tick species.

In several studies dealing with the development and the modes of transmission of *B. burgdorferi* (Matuschka and Spielman 1986; Benach *et al* 1987; Ribeiro *et al* 1987; Zung *et al* 1988), systemic infections were recorded in both nymphal and female *I. dammini* during blood feeding activity, and salivary transmission of *B. burgdorferi* to the host was suggested.

Our present study shows that during the slow engorgement phase (Lees 1952; Kheisin and Lavrenko 1956; Aeschlimann and Grandjean 1973), *B. burgdorferi,* ingested two hours before attachment (Group I) or transstadially transmitted from nymphal stage (Group II), multiplies in the midgut of *I. ricinus* females. In some gut-infected ticks a few spirochetes can penetrate the midgut wall and induce systemic infections, while the overwhelming majority of borreliae persist in the midgut lumen. This observation confirms our first description of *B. burgdorferi* infection in *I. ricinus* ticks (Monin *et al* 1989). The presence of spirochetes in epithelial cells of the midgut wall, in hemocoel, in acini and ducts of salivary glands provides evidence for midgut and salivary glands penetrations of *B. burgdorferi* in blood feeding *I. ricinus* females, and strongly supports the hypothesis that this spirochete could be transmitted via saliva of feeding *I. ricinus* females.

Although we failed to detect spirochetes in genital tissues and Malpighian tubules, the possibility of spirochetal infections of these two organs during blood feeding of *I. ricinus* females cannot be ruled out because of the relatively low number of ticks examined. In fact, in another paper, spirochetes have been described in genital tissues of blood feeding *I. ricinus* females (Monin *et al* 1989). On the other hand, it is possible that most of the spirochetes, having entered the hemocoel three days or more post attachment, could get access to the salivary glands helped by the directional current of hemolymph (Benach *et al* 1987) and could invade the synganglion attracted by an organotropic mechanism (Burgdorfer 1951; Sarasin 1957; Benach *et al* 1987) where spirochetes could not be visualized by silver stain. This might greatly reduce the rates of spirochetal infection of organs other than the synganglion and the salivary glands. Interestingly, *B. burgdorferi* still appear quite active in their migration in replete females kept at room temperature (Burgdorfer *et al* 1988) which is not suitable for the *in vitro* cultivated spirochetes (Barbour 1984). The mechanism of gut penetration and systemic infections in an engorged female still remains to be elucidated.

The dynamic of the infection of different tick tissues observed in this study, confirms our first findings (Monin *et al* 1989). Spirochetes remain numerous in the midgut during the whole blood meal while some may invade other tissues 3 days post-attachmant, but always in low numbers. Our observation of spirochetes in salivary glands and according to Ribeiro *et al* (1987) in salivary secretions of *I. dammini,* leads us to suppose that such transmission occurs in *I. ricinus* too. Nevertheless, after engorgement the salivary glands of ixodid ticks degenerate and atrophy completely (Vitzhum 1943; Till 1961; Kaufmann 1986). The persistence of *B. burgdorferi* in the midgut throughout the blood meal and even in unfed ticks suggest that this organ is a real reservoir for spirochetes in the tick.

Considering the number of spirochetes persisting in the midgut lumen, their transmission by regurgitation of infected gut contents should not be excluded, even if we failed to demonstrate borreliae in the oesophagus and pharynx in the present study. The detection of spirochetes in acini and ducts of the salivary glands of blood feeding *I. ricinus* females gives further support to transmission of *B. burgdorferi* via the saliva.

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REFERENCES

Aeschlimann A., Grandjean O. : Influence of natural and artificial mating on feeding, digestion, vitellogenesis and oviposition in ticks (Ixodoidea). *Folia Parasit. (Praha),* 1973, *20,* 67-74.

Barbour A. G. : Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.,* 1984, *57,* 521-525.

Benach J. L., Coleman J. L., Skinner R. A., Bosler E. M. : Adult *Ixodes dammini* on rabbits: A hypothesis for the development and transmission of *Borrelia burgdorferi. J. Infect. Dis.,* 1987, *155,* 1300-1306.

Burgdorfer W. : Analyse des Infektionsverlaufes bei *Ornithodoros moubata* und der natürlichen Uebertragung von *Spirochaeta duttoni. Acta Trop.,* 1951, *8,* 196-262.

Burgdorfer W. : Discovery of Lyme disease spirochete and its relation to tick vectors. *Yale J. Biol. Med.,* 1984, *57,* 515-520.

Burgdorfer W., Barbour A. G., Hayes S. F., Benach J. L., Grunwaldt E., Davis J. P. : Lyme disease — a tick-borne spirochetosis. *Science,* 1982, *216,* 1317-1309.

Burgdorfer W., Barbour A. G., Hayes S. F., Péter O., Aeschlimann A. : Erythema chronicum migrans — a tickborne spirochetosis. *Acta Trop.,* 1983, *40,* 79-83.

Burgdorfer W., Lane R. S., Barbour A. G., Gresbrink RA, Anderson J. R. : The western black-legged tick, *I. pacificus:* a vector of *Borrelia burgdorferi. Am. J. Trop. Med. Hyg.,* 1985, *34,* 925-930.

Burgdorfer W., Hayes S. F., Benach J. L. : Development of *Borrelia burgdorferi* in Ixodid tick vectors. *Ann. NY Acad. Sci.,* 1988, *539,* 172-179.

Graf J. F. : Copulation, nutrition et ponte chez *Ixodes ricinus* L. (Ixodoidea: Ixodidae) 1re partie. *Bull. Soc. Ent. Suisse,* 1978, *51,* 89-97.

Karnovsky M. J. : A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Cell. Biol.,* 1965, *27,* 137-139.

Kheisin E. M., Lavrenko L. E. : Duration of blood-sucking and diurnal rythm of nutrition and dropping of females of *Ixodes ricinus* L. *Zool. Zh.,* 1956, *35,* 379-383.

Lees A. D. : The role of cuticle growth in the feeding process of ticks. *Proc. Zool. Soc. Lond.,* 1952, *121,* 759-772.

Matuschka F. R., Spielman A. : The emergence of Lyme disease in a changing environment in north America and central Europe. *Exp. Appl. Acarol.,* 1986, *2,* 337-353.

Monin R., Gern L., Aeschlimann A. : A study of the different modes of transmission of *Borrelia burgdorferi* by *Ixodes ricinus.* Lyme Borreliosis II. *Zbl. Bakt.,* Suppl. 18, 1989, 14-20.

Ribeiro J. M. C., Mather T. N., Piesman J., Spielman A. : Dissemination and salivary delivery of Lyme disease spirochetes in vector tick (Acari: Ixodes). *J. Med. Entomol.,* 1987, *24,* 201-205.

Sarasin G. : Zum Organotropismus der Spirochete *B. duttoni* gegenüber der übertragenden Zecke. *Acta. Trop.,* 1959, *16,* 218-243.

Till W. M. : A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* (Neumann). *Mem. Ent. Soc. Sth. Afr.,* 1961, 124 p.

Van Oden, Greer P. W. : Modification of Dieterle spirochete stain. *J. Hist.,* 1977, *1,* 51-53.

Vitzhum H. G. : Acarina. In: Beonn's Klassen und Ordnungen des Tierreichs. 5: IV Abt, 5 Buch Leipzig, *Becker und Erler,* 1943.

Zhioua E., Monin R., Gern L., Aeschlimann A. : Infection of free-living life-stage of *Ixodes ricinus* with *Borrelia burgdorferi* in Switzerland (abstract). *Zentbl. Bak.,* 1988, *306,* 293.

Zung J. L., Lewengrub S., Rudzinska M. A., Telford III S. R., Spielman A. : The ultrastructure of *Borrelia burgdorferi* in *Ixodes dammini.* 63rd Annual Meeting Program and Abstracts. Am Soc Parasit Winston-Salm, North Carolina. July 31-August 4, 1988, p. 52.