to 210°C, at 5° per minute. Compounds were identified by comparisons of retention times (in some cases co-injection) and mass spectra with those of reference compounds in our position. In a few cases reference compounds were synthesized (by J. Bergström, Göteborg University). Percentages were calculated from total ion chromatograms and trace amounts were detected by selective ion monitoring. For further description of techniques for the isolation and enrichment of plant volatiles, see G. Bergstrom *et al.* [*Chem. Scripta* **16**, 173 (1980)].

9. L. B. Thien and G. McPherson, in preparation.
10. G. Gottsberger, *Osterr. Bot. Z.* **118**, 237 (1970); L. B. Thien, *Am. J. Bot.* **61**, 1037 (1974).
11. O. Pellmyr and L. B. Thien, in preparation.
12. Plant voucher specimens (collected by G.M.) are deposited in the herbarium at the Missouri Botanical Garden as follows: *Z. baillonii*, 5833; *Z. bicolor*, 5873; *Z. pomiferum* subsp. *balansae*, 5767; *Z. mackeei*, 5933; and *Exospermum stipitatum*, 5929. Moth specimens, which are not numbered, are stored at the Department of Zoology, University of Wellington, Wellington, New Zealand. L.B.T. thanks J. P. Cherrier, the head of Forests and of Ecology in New Caledonia for permission to work on the island. P. Morat and J. Gutierrez provided research facilities via ORSTOM. P. Raven inspired the research. The following individuals critically read the manuscript: B. Sampson, V. Grant, P. Raven, S. Carlquist, D. Davis, and B. J. D. Meeuse. The National Geographic Society provided a grant making the research possible.

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Transovarial Transmission of Murine Typhus Rickettsiae in *Xenopsylla cheopis* Fleas

Abstract. *It has been generally accepted that infected fleas do not pass on* Rickettsia mooseri, *or indeed any other known pathogen, to their progeny. It is reported here that such transovarial transmission does occur in laboratory-infected* Xenopsylla cheopis *fleas. By means of the direct fluorescent antibody test,* Rickettsia mooseri *was observed in cells of the hemolymph of infected fleas. As many as 11 percent of the adults and 2.9 percent of the larvae of the generation reared therefrom, had demonstrable rickettsiae. Moreover, batches of the F_1 fleas were capable of transmitting the infection to more than 18 percent of the rats they infested. The data support the contention that* Xenopsylla cheopis *fleas play an important role in the maintenance of murine typhus in rats in nature.*

In the classical studies on murine typhus that incriminated fleas, particularly *Xenopsylla cheopis*, as vectors, it was reported that infected fleas do not transmit the etiological agent, *Rickettsia typhi* (referred to here as *R. mooseri*), to their progeny (*1*). This statement has remained unchallenged for more than 50 years (*2, 3*).

It has become generally accepted that all the other microbial pathogens of humans that are transmitted by fleas also remain wholly restricted to the confines of the alimentary canal while resident in the flea, thereby excluding the possibility of transovarial transmission (TOT) (*4, 5*). This so-called gut barrier has even been proposed as the reason why fleas do not serve as true vectors of viruses (*4*), which, like rickettsiae, are intracellular parasites. Since TOT occurs in at least 7 of the 15 arthropod-borne rickettsial diseases of man or domestic animals, it is somewhat surprising that over so many decades no questions have been raised about the purportedly invincible nature of the barrier posed to human pathogens by the gut of fleas. In this report we present evidence that not only may transovarial transmission of *R. mooseri* to the next generation take place, but that the F_1 fleas are capable of transmitting the infection to rats.

Murine typhus is believed to be contracted by contact with the crushed bodies or feces of infected fleas and not by the bite of these insects. In earlier papers (*6, 7*), in which we presented details on methodology, we confirmed that when *X. cheopis* fleas are infected with *R. mooseri*, the rickettsiae penetrate and develop in the cells of the midgut epithelium, the only part of the intestine that lacks a cuticular lining. It seemed likely that any escape of rickettsiae from the gut would have occurred when the infection had become so massive that the lumen of the midgut and hindgut were packed with the organisms. We could even observe rickettsiae in the proventriculus and foregut about 15 days after the fleas had fed on infected rats or later (*7*).

In the present study, 17 percent (10 of 60) of the smears of the hemolymph of fleas examined 19 to 22 days after they had fed on infected rats were positive for intracellular rickettsiae by the direct fluorescent antibody test (DFA), whereas no rickettsiae were observed in 50 fleas examined earlier. Further, as shown in Table 1, not only do some of the progeny of infected fleas harbor *R. mooseri*, but TOT occurred in significantly greater numbers of offspring derived from eggs deposited late in the course of the infection of the parents than from those laid shortly after the infectious meal; for example, 7.5 to 11.3 percent for days 26 and 31, in contrast to 2.9 percent for days 5 to 11. The corresponding data for the minimum number of infected F_1 fleas in the pools tested are 1 in 125 or 132 compared to <1 in 180 (*8, 9*).

Further analysis of the data for the groups 17 to 31 days after infective feeding showed that there were no major differences between males and females or between fed and unfed fleas, even though the fed fleas had an additional 4 to 6 days for the growth of any transovarially transmitted rickettsiae (Table 2) (*10*).

As shown in Table 3, the F_1 generation of fleas was capable of infecting rats after being allowed to infest and feed upon them during a period of 24 hours. The highest rates of transmission (15.5 to 18.4 percent) occurred in fleas that had fed (on uninfected hosts) on one or two previous occasions and were 7 to 18 days of age, suggesting that the more time available for growth of rickettsiae within TOT-infected fleas, the greater the chance for transmission of rickettsiae by those fleas (*11*).

Five of the seven known rickettsial infections in which TOT occurs are transmitted by ticks and two by mites (*12, 13*). This phenomenon has not been demonstrated in the two that are louse-borne. In scrub typhus, the trombiculid vector also serves as the reservoir of the infection because of TOT in "naturally

Table 1. *Rickettsia mooseri* infection rates among F_1 progeny of *X. cheopis* fleas reared from eggs collected at various dates after infection of the parents (*8*).

Days after infective feeding of parental fleas	Direct fluorescent antibody test			Mouse antibody test			
	Number of fleas		Percentage of fleas infected	Number of fleas examined	Number of		Minimum infection rate
	Examined	Positive			Pools tested	Positive pools	
5 to 11*	305	9	2.9	180	15	0	<1 to 180*
17	493	19	3.8	568	29	4	1 to 142
26	252	19	7.5	250	10	2	1 to 125
31	398	45	11.3	395	12	3	1 to 132

*The apparent discrepancy probably reflects the small number of infected fleas present.

Table 2. Transovarial transmission of *R. mooseri* in fed and unfed F_1 adult *X. cheopis* fleas, as arranged by sex and determined by the direct fluorescent antibody test.

Days after infective feeding of parental fleas	Unfed fleas				Fed fleas			
	Males		Females		Males		Females	
	Positive/total	Percentage	Positive/total	Percentage	Positive/total	Percentage	Positive/total	Percentage
17	5/137	3.6	7/143	4.9	3/93	3.2	4/120	3.3
26	3/60	5.0	6/66	9.1	4/60	7.7	6/66	9.1
31	11/99	11.1	11/100	11.0	13/98	13.3	10/101	9.9
Total	19/296	6.4	24/309	7.8	20/251	8.0	20/287	7.0

Table 3. Transmission of *R. mooseri* by F_1 adult *X. cheopis* to uninfected baby rats.

Group	F_1 Adult fleas			Baby rats		
	Number feedings on normal rats	Age of fleas (days)	Average number of fleas per rat	Number of rats		Percentage positive
				Examined	Positive	
A	1	1 to 2	41	26	1	3.8
B	1	3 to 6	56	40	3	7.5
C	2	7 to 12	52	49	9	18.4
D	3	13 to 18	55	45	7	15.5
Infected controls		14 to 17	50	14	12	85.7

infected'' lines (*12, 14*). This is also true in the spotted fevers, but here TOT may also ensue if the ticks feed on a host with a high level of rickettsemia (*12*).

Ticks are notorious not only for the numbers and kinds of human infections they transmit, but also because so many of the agents are transmitted to their progeny. In contrast, TOT of such pathogens in insects has appeared to be rare, and the relatively few instances that have been cited are essentially restricted to mosquitoes and sand flies (*13, 15, 16*). Transovarial transmission in a nondipteran insect is therefore a somewhat unexpected event. However, published statements about the effectiveness of the gut barrier in fleas (*4*) really apply only to pathogens of man, since the sporozoan *Hepatozoon* of rodents develops in the coelomic cavity of the flea vectors (*17*). Furthermore, except for *R. mooseri*, the human pathogens commonly transmitted by fleas (for example, plague and tularemia) develop only in the lumen of the gut rather than intracellularly in the lining of the intestine (*5, 18, 19*). *Trypanosoma lewisi*, a parasite of rats, may at times undergo an intracellular stage in the flea's midgut (*20*).

It is axiomatic that adult F_1 fleas cannot become infected by TOT unless trans-stadial transmission also takes place, and such passage has not been previously observed for any microbial agent in fleas. In the present study, 7 of 240 F_1 second-instar larval *X. cheopis* (2.9 percent) were positive by DFA compared to 6.3 percent of 1448 F_1 adults. In tests for the presence of antibody to *R. mooseri*, 3 of 15 (20 percent) samples of pooled larvae (total: 420 larvae) inoculated into mice proved positive for *R. mooseri*. This is equivalent to a minimum infection rate of 1 per 140. The comparable figures for adults were 166 samples positive (1393 pooled larvae), with a minimum infection rate of 1 per 155.

The relatively small number of infected F_1 progeny suggests that *R. mooseri* only occasionally escape from the gut of the fleas and infect the eggs. This low incidence probably accounts for our earlier failure (*6, 7*) to demonstrate rickettsiae in nonintestinal tissues. Inadequate sampling, testing fleas before the growth level of the rickettsiae permitted penetration of the gonads, and the absence of specific testing procedures are factors that may explain why TOT was not demonstrated in investigations (*1*) on murine typhus undertaken 50 years ago. The widespread impression that fleas are not natural vectors of viral infections (*4*) may ultimately prove to be the result of similar limitations, especially since natural infection with certain pathogenic viruses, and experimental transmission, have been reported in fleas (*5*). Moreover, since *R. mooseri* routinely develops within the epithelial cells of the midgut, it is theoretically possible that some viruses also penetrate and multiply at least in those cells.

The maintenance of murine typhus in nature has not been definitely determined, although commensal rats living indoors are undoubtedly critical factors (*2*). Since fleas infected with *R. mooseri* remain infective for months and their life-span is unaffected, they can infect the next generation of rats and hence help perpetuate the cycle (*1*). Further, the feces of fleas can remain infective for months or years. To these indications that fleas may serve as reservoirs of murine typhus must be added the possibility that fleas that feed on rickettsemic hosts are capable of passing *R. mooseri* infection to the next generation of hosts.

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References and Notes

1. E. T. Ceder, R. E. Dyer, A. Rumreich, L. F. Badger, *Public Health Rep.* **46**, 3103 (1931).
2. R. Traub, C. L. Wisseman, Jr., A. Farhang-Azad, *Trop. Dis. Bull.* **75**, 237 (1978).
3. R. N. Philip, in *CRC Handbook Series in Zoonoses*, J. H. Steele, Ed. (CRC Press, Boca Raton, Fla., 1980), pp. 317–335.
4. V. A. Bibikova, *Annu. Rev. Entomol.* **32**, 23 (1977).
5. R. Traub, in *The Rothschild Collection of Fleas. The Ceratophyllidae*, R. Traub, M. Rothschild, J. Haddow (Academic Press, London, 1983), pp. 202–228.
6. A. Farhang-Azad, C. L. Wisseman, Jr., R. Traub, in *Rickettsiae and Rickettsial Diseases*, W. Burgdorfer and R. L. Anacker, Eds. (Academic Press, New York, 1981), pp. 363–373.
7. A. Farhang-Azad, R. Traub, C. L. Wisseman, Jr., *Am. J. Trop. Med. Hyg.* **32**, 1392 (1983).
8. A single uninfected baby rat in each of eight jars was exposed to 300 infected female *X. cheopis* fleas for 48 hours. Flea eggs were collected 5 to 11 days later. The same infected fleas were fed on normal rats semiweekly and their eggs were collected 17, 26, and 31 days after infective feeding. Recently hatched, unfed larvae were used for rearing the adult F_1 fleas. The immature stages were maintained at 24°C and 70 to 80 percent relative humidity. Pupae were collected by sifting the media. Samples of F_1 larvae and F_1 adults were tested by DFA or mouse seroconversion tests (*6, 7*); that is, three female ICR mice (18 to 20 g, Hilltop Laboratories, Pa.) were inoculated with 0.4 ml of a pooled homogenate of 18 to 30 fleas. Serum samples were tested for the presence of antibody by IFA 28 days after inoculation (*9*). Controls (350 adults and 100 larvae in DFA tests, and 20 pools of 50 adult fleas in antibody tests) were consistently negative for *R. mooseri*.
9. S. Arango-Jaramillo, A. Farhang-Azad, C. L. Wisseman, Jr., *Am. J. Trop. Med. Hyg.* **33**, 1017 (1984).
10. Groups of F_1 fleas that had never fed as adults and that had been collected 5 to 7 days after emergence were compared with batches of F_1 fleas that had emerged 9 to 11 days previously and had been fed once on noninfected baby rats 3 to 4 days earlier.
11. Uninfected baby rats that were littermates, 5 to 7 days of age, were used as hosts and then returned to their mothers after being cleaned with 70 percent alcohol. They were tested by IFA for seroconversion 4 weeks later. After the initial feeding, the fleas were manually removed and counted, and the bulk were saved for reuse as groups C and D, feeding on new littermates. The remainder were frozen for subsequent examination. The fleas used in these tests were from the same lots that provided the specimens reported in Table 2, where the TOT rate in F_1 fleas was 3 to 13 percent. As controls, 12 days after feeding on rickettsemic rats, *X. cheopis* fleas with a known rickettsial titer were similarly exposed to uninfected littermates, which were later checked for seroconversion.

12. W. Burgdorfer, in *CRC Handbook Series in Zoonoses*, J. H. Steele, Ed. (CRC Press, Boca Raton, Fla., 1980), pp. 279–300.
13. R. F. Harwood and N. T. James, *Entomology in Human and Animal Health* (Macmillan, New York, ed. 7, 1979).
14. R. Traub and C. L. Wisseman, Jr., *J. Med. Entomol.* **11**, 237 (1974).
15. A. Spielman, *Ann. N.Y. Acad. Sci.* **266**, 115 (1975).
16. L. Rosen, D. A. Shroyer, R. B. Tesh, J. E. Freier, J. C. Lien, *Am. J. Trop. Med. Hyg.* **32**, 1108 (1983).
17. H. E. Krampitz and V. Wongchari, in *Fleas*, R. Traub and H. Starcke, Eds. (Balkema, Rotterdam, 1980), pp. 349–358.
18. The bacterial agent of listeriosis has been reported as penetrating into the body cavity of *Nosopsyllus consimilis* fleas after developing in the midgut epithelium (*19*).
19. V. S. Vaschenok and P. A. Chirov, *Parazitologia* **10**, 61 (1976).
20. D. H. Molyneux, *Ann. Trop. Med. Parasitol.* **63**, 403 (1969).
21. Supported by NIH grants AI-17828 and AI-04242.

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Hypothalamic Reward Mechanism: Two First-Stage Fiber Populations with a Cholinergic Component

Abstract. *Refractory periods were estimated for fibers of the hypothalamic substrate of brain stimulation reward. Two nonoverlapping populations were evident: a homogeneous fast population and a more heterogeneous slow population. Cholinergic blockade eliminated the contribution of the fast but not the slow fibers, while dopaminergic blockade reduced responding without significantly influencing either directly activated fiber population. These data indicate that the hypothalamic reward substrate is more complex than has been widely appreciated; it contains two or more parallel subsystems, and one of these subsystems has a cholinergic link.*

Electrical stimulation of the medial forebrain bundle (MFB) can have rewarding effects stronger than those caused by natural biological rewards such as food, even under conditions of life-threatening deprivation. Since only a few of the approximately 50 fiber systems passing through this region are likely to play a role in the rewarding effects of stimulation, knowledge of which components of the MFB play causal roles in brain stimulation reward has been elusive (*1, 2*). Pharmacological studies suggest that dopaminergic fibers are involved (*3*). However, a variety of considerations make it clear that dopaminergic fibers are not usually directly depolarized by the rewarding currents used in brain stimulation reward experiments (*2*); rather, it seems likely that directly activated fibers, constituting a "first-stage" system, synapse on dopaminergic cells, which then serve as the second-stage system (*4*). Paired-pulse experiments suggest that the poststimulation excitability cycles and conduction velocities of the directly activated MFB fibers are faster than most monoaminergic fibers (*5*). Furthermore, double electrode experiments, in which propagation of the action potential from a depolarizing pulse at one end of the MFB is impeded by hyperpolarizing the other end of the bundle, indicate that most first-stage fibers carry the reward-relevant signal in a rostral-to-caudal direction—opposite to the direction of conduction of the dopaminergic fibers (*6*).

We now report that the first-stage reward fibers include at least two populations, one of which is cholinergic or is synaptically linked to cholinergic efferents. One first-stage population has short refractory periods that do not overlap the slower refractory periods of the remaining reward-relevant fibers; the contribution of the fast fibers is blocked by the muscarinic receptor antagonist atropine sulfate. The contribution of cholinergic fibers to MFB brain stimulation reward has not been seen in previous studies of this type, partly because refractory periods have been analyzed over a coarser sampling of test parameters and partly because pharmacological studies have usually been designed on the assumption that a single transmitter system carried the reward signal to the second stage neurons. Our data suggest that perhaps 25 percent of the reward-relevant signal is carried by cholinergic

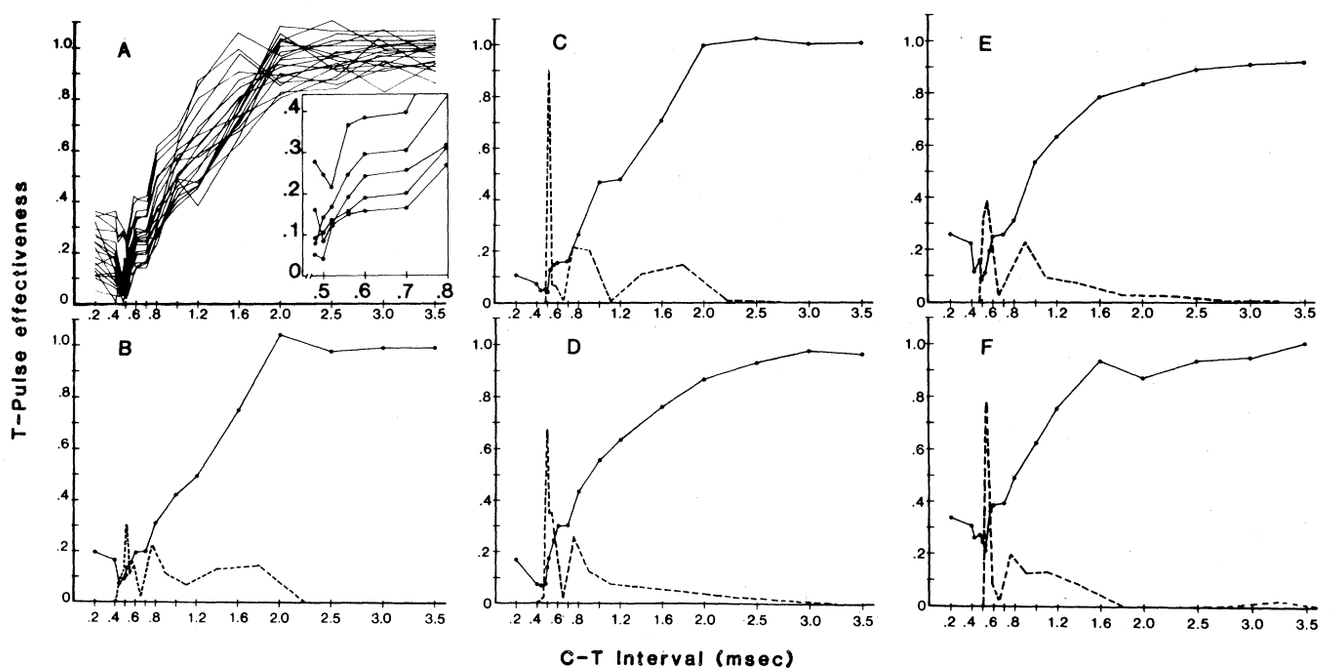

Fig. 1. Relation of T-pulse effectiveness to C-T interval. (A) Data for each of four replications in each of five rats. Note the horizontal step in each curve between 0.6 and 0.7 msec; the inset shows an expanded version of the horizontal step based on mean data for each animal. (B to F) Mean data for each animal (solid lines) along with relative slopes (dotted lines). These slopes (first derivatives) reflect the relative numbers of fibers that have refractory periods within each range of C-T intervals. The slopes fall to zero between 0.6 and 0.7 msec in each case.