

RESEARCH ARTICLE

A Role for Early-Phase Transmission in the
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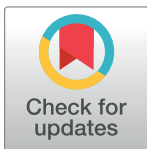
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Abstract

Yersinia pestis, the bacterial agent of plague, is enzootic in many parts of the world within wild rodent populations and is transmitted by different flea vectors. The ecology of plague is complex, with rodent hosts exhibiting varying susceptibilities to overt disease and their fleas exhibiting varying levels of vector competence. A long-standing question in plague ecology concerns the conditions that lead to occasional epizootics among susceptible rodents. Many factors are involved, but a major one is the transmission efficiency of the flea vector. In this study, using *Oropsylla montana* (a ground squirrel flea that is a major plague vector in the western United States), we comparatively quantified the efficiency of the two basic modes of flea-borne transmission. Transmission efficiency by the early-phase mechanism was strongly affected by the host blood source. Subsequent biofilm-dependent transmission by blocked fleas was less influenced by host blood and was more efficient. Mathematical modeling predicted that early-phase transmission could drive an epizootic only among highly susceptible rodents with certain blood characteristics, but that transmission by blocked *O. montana* could do so in more resistant hosts irrespective of their blood characteristics. The models further suggested that for most wild rodents, exposure to sublethal doses of *Y. pestis* transmitted during the early phase may restrain rapid epizootic spread by increasing the number of immune, resistant individuals in the population.

Author summary

The ecology of bubonic plague is complex but depends largely on flea-borne transmission. Certain susceptible rodents experience periodic epizootics that can decimate local populations, but the conditions that lead to these episodes are not fully understood. Fleas can transmit *Yersinia pestis*, the bacterial agent of plague, during two different phases: an early phase within the first few days after their infectious blood meal and again sometime later

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after the development of a *Y. pestis* biofilm in the flea foregut. The relative contribution of these two transmission modes to plague ecology has not been systematically examined. Our results indicate that in most ecological contexts early-phase transmission is too inefficient to drive an epizootic, but instead acts to reduce the number of susceptible individuals in a population, thereby favoring a more stable enzootic state.

Introduction

Plague is a vector-borne zoonotic disease that primarily afflicts rodents, notably members of the Muridae (rats, mice, gerbils) and Sciuridae (squirrels, marmots, prairie dogs), and persists in extensive natural foci throughout the world. *Yersinia pestis*, the plague bacillus, circulates within these host populations via rodent-flea-rodent transmission cycles that involve several flea vector species. Plague exhibits an enigmatic pattern in which dramatic epizootics in highly susceptible rodent populations periodically flare up from the normal relatively quiescent enzootic background state of a plague focus or from reintroduction of *Y. pestis* into a population. The ecological factors and biological mechanisms that initiate and drive these periodic epizootics versus those that tend toward enzootic maintenance have been subjects of long-standing interest [1–5]. Metapopulation structure, fluctuations in population densities of rodents and fleas, and the immune status and degree of susceptibility to overt disease within and among reservoir host species have been theorized to be important [1, 6–12].

One crucial component of plague cycle dynamics is the transmission rate from infected to uninfected hosts. Although it can sometimes be transmitted by direct contact, ingestion, or aerosol, *Y. pestis* has evolutionarily adapted to the flea-borne transmission route on which it now depends. Fleas can transmit *Y. pestis* in three stages following an infectious blood meal. Transmission can occur the very next time they feed during the first week after infection, a phenomenon referred to as early-phase transmission. The second and third stages of transmission are effectuated after *Y. pestis* forms a bacterial biofilm in the proventriculus, a valve between the esophagus and midgut. As the biofilm grows it gradually restricts the passage of blood through the proventriculus and impedes valvular function. In this partially blocked state, fleas are able to ingest some blood but because the proventricular valve is unable to close completely, blood contaminated with bacteria from the flea digestive tract can backflow into the bite site [13, 14]. Eventually, in the final stage, the biofilm can fill the entire proventriculus and completely block the flow of incoming blood into the midgut. When such completely blocked fleas attempt to feed, blood flowing into the esophagus is stopped in front of the blocked proventriculus, the esophagus initially expands, and then the blood, mixed with some of the bacteria washed from the proventricular biofilm, is recoiled back into the bite site [14, 15]. Early-phase transmission was historically referred to as mass transmission, because it is rarely observed unless groups of 5 to 10 or more infected fleas feed simultaneously on a naïve animal. Although long assumed to be a form of mechanical transmission, early-phase transmission also appears to occur via regurgitation of bacteria from a heavily infected proventriculus even though it does not depend on formation of a mature biofilm [14, 16–18]. Thus, bacterial obstruction of the lumen of the proventricular valve and its normal function, to a greater or lesser extent, is the underlying mechanism common to all three modes of transmission [14].

The several rodent flea species implicated in plague transmission cycles vary in their vector efficiency [19]. However, the relative efficiency of the different phases of transmission by individual fleas has not been empirically evaluated in a systematic way. Early-phase transmission

and transmission by blocked fleas have been studied in separate experiments and comparisons made between them, but significant differences in experimental design among the studies make the conclusions problematic. Different infectious doses and blood meal sources, both of which greatly affect flea infection and transmission outcomes, have been employed. Transmission by partially blocked fleas has for the most part been neglected; often the blockage rate has been used as the surrogate indicator for transmission beyond the early phase. Mathematical modeling based on parameterization of these available data has supported a role for early-phase transmission in driving epizootics in some ecological contexts [20, 21]. However, there is a recognized need to reexamine the relative vector competency of different flea species and efficiencies of the different transmission modes using newer standardized, more exacting methods [22].

In this study, we examined transmission of *Y. pestis* by the North American ground squirrel flea, *Oropsylla montana*. Several aspects of transmission dynamics were recorded during a one-month period following a single infectious mouse or rat blood meal, including infection and mortality rates and the incidence of proventricular blockage. The number of *Y. pestis* transmitted by individual fleas during the early phase (2 to 4 days after infection) and after the development of partial or complete blockage was also determined. The data were used to parameterize a simple deterministic susceptible-exposed-infected-recovered (SEIR) model of plague transmission dynamics that we developed. Simulations using the model provided an assessment of the relative contributions of early-phase transmission and biofilm-dependent transmission to disease incidence ensuing from a single cohort of fleas that feed on a highly bacteremic host. The influence of infectious blood source (mouse vs. rat) on plague transmission dynamics was also evaluated.

Results

Quantitative evaluation of flea infection and transmission parameters

Cohorts of *O. montana* fleas were infected by feeding on highly bacteremic mouse or rat blood (containing 5 to 8×10^8 *Y. pestis*/ml) and thus began with a 100% infection rate at an average of 3 to 6×10^4 *Y. pestis* per flea on day 0. Fleas were maintained at 21 °C and provided twice-weekly mouse or rat sterile blood meals for 4 weeks and monitored for mortality, infection, and proventricular blockage status (Fig 1). Host blood source had a large effect on infection rate. Consistent with a previous study [23], over half of the fleas infected using mouse blood had completely cleared the infection by day 3. In contrast, around 90% of fleas infected using rat blood remained infected (Fig 1A). The bacterial load of the chronically infected fleas was similar, however (Fig 1B). In correlation with their higher infection rate, a higher percentage

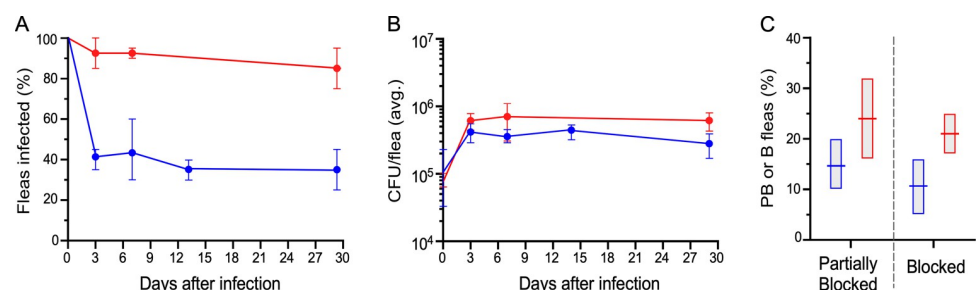


Fig 1. Infection and blockage rates of *O. montana* fleas during a four-week period following a blood meal containing 5 to 8×10^8 *Y. pestis*/ml in mouse blood (blue symbols) or rat blood (red symbols). (A) The percentage of fleas still infected and (B) the bacterial load per infected flea at different times after infection. (C) The percentage of fleas that developed partial or complete proventricular blockage during the four-week period. The mean and range of three independent experiments using mouse blood and two experiments using rat blood (Table 1) are indicated.

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Table 1. Values for flea infection and transmission factors determined in this study.

	mouse blood	rat blood
Fleas cleared of infection by:		
day 3	59% (55–65%)	8% (0–15%)
day 7	57% (40–70%)	8% (5–10%)
day 28	65% (55–75%)	15% (5–25%)
Fleas partially blocked (%)	15% (10–21%)	24% (16–32%)
Days to develop partial blockage	8 (2–30)	12 (3–27)
Life span of partially blocked fleas (days) ^a	7 (1–18)	8 (1–20)
Fleas blocked (%)	11% (5–16%)	21% (17–25%)
Days to develop blockage	11 (6–22)	15 (3–35)
Life span of blocked fleas (days) ^a	5 (1–11)	4 (1–8)
Mortality of unblocked fleas (28 days P.I.)	17% (12–21%)	35% (28–39%)
Transmission efficiency ^b of:		
Early-phase fleas (day 2–4)	3% (1/38)	10% (4/42)
Partially blocked fleas	11% (2/18)	10% (5/48)
Blocked fleas	50% (20/40)	67% (46/69)
Median no. CFU transmitted ^c by:		
Early-phase fleas (day 2–4)	1	17
Partially blocked fleas	1,090	417
Blocked fleas	230	63

The average and range of the results from three independent experiments with *O. montana* fleas infected and maintained using mouse blood (n = 177, 209, 283; 669 fleas total) and two experiments using rat blood (n = 247, 192; 439 fleas total) are shown. Of these, 80–100 were used solely for flea infection rate and bacterial load determinations in each experiment (n = 20 fleas for each of the timepoints); the other 429 (mouse blood experiments) and 279 fleas (rat blood experiments) were used to monitor blockage, mortality, and transmission. The early-phase transmission efficiency (rat blood) was based on two separate experiments (n = 60 and 72 fleas; 132 total) with fleas infected with the *Y. pestis* Δ *hmsH* mutant.

^a Days from diagnosis of partial or complete blockage to death

^b The number of positive transmission events divided by the total number of bites by individual fleas in each category

^c Values for positive transmission events only

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of fleas infected using rat blood developed proventricular blockage during the experiments than fleas infected using mouse blood (21% and 11%, respectively; Table 1, Fig 1C). Fleas that appeared to be partially or even completely blocked were seen as early as 2 to 3 days after infection, although the highest incidence occurred between 1 and 3 weeks after infection (Fig 2).

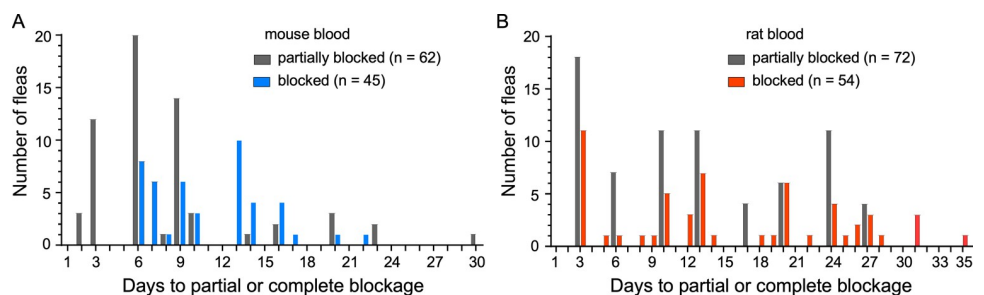


Fig 2. Temporal distribution of the occurrence of partial and complete blockage in *O. montana* fleas following a single infectious blood meal containing 5 to 8×10^8 *Y. pestis*/ml in mouse or rat blood. The cumulative numbers from the three experiments using mouse blood (A) and two experiments using rat blood (B) are shown; see Table 1 for details.

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Transmission efficiency of fleas during the early phase (2 to 4 days after the infectious blood meal) and of partially and completely blocked fleas was determined by allowing individual fleas to feed on a small reservoir of sterile mouse or rat blood for 1 h, and then recovering and plating the blood for CFU (colony-forming unit) count. Fleas were examined for evidence of feeding and for proventricular blockage status immediately after the 1 h access period. The rodent source of the infectious blood meal had a large effect on early-phase transmission efficiency. Only 1 of 38 fleas (3%) infected using mouse blood transmitted during their first blood meal 2 to 4 days later, and only 1 CFU was recovered from the blood that this flea fed upon (Fig 3). In contrast, 8 of 33 (24%) of fleas infected using rat blood transmitted 3 to 2,000 CFU during early-phase transmission trials. Notably, however, 5 of these 8 fleas were observed to be completely blocked and 2 appeared to be partially blocked after this initial post-infection feeding on day 3 or 4. For this reason, we repeated early-phase transmission trials with fleas infected with a *Y. pestis hms* mutant strain, which is unable to block fleas but is fully transmissible in the early phase [17, 24]. To clearly distinguish the two transmission modes, we concentrated on results with this *hms* mutant [4 of 42 fleas (10%) transmitted 3 to 72 CFU (median 17 CFU); Fig 3] to estimate the early-phase transmission efficiency.

Our estimate of early-phase transmission efficiency for fleas infected using rat blood was in line with those reported previously for *O. montana* infected similarly that were used to challenge mice [17, 20, 25–27]. However, our estimate for fleas infected using mouse blood (3%)

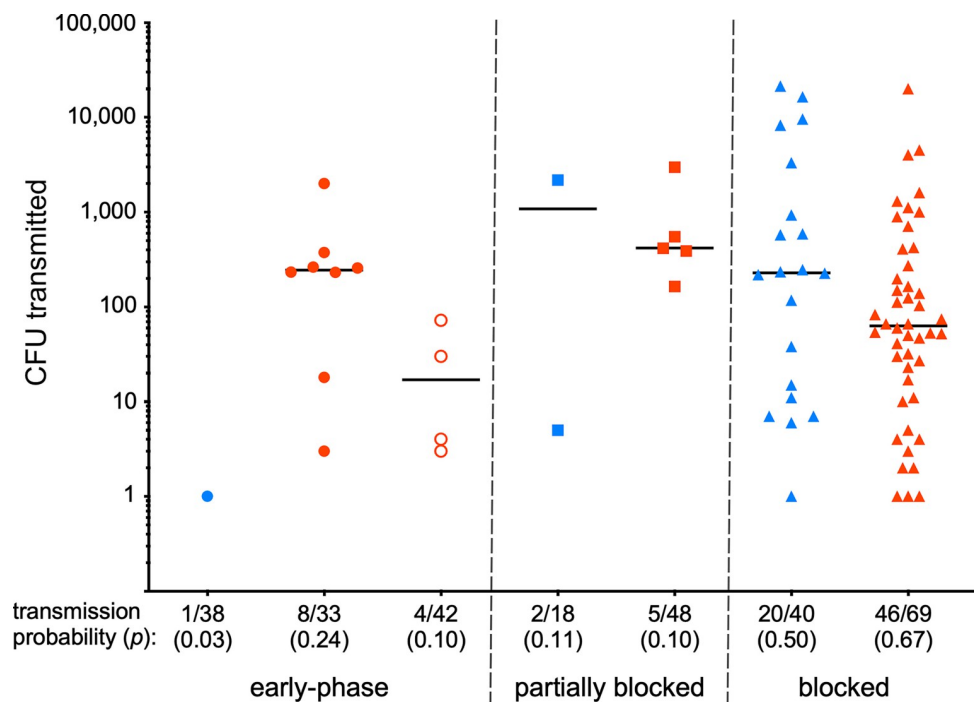


Fig 3. The number of *Y. pestis* CFU transmitted by individual *O. montana* fleas 2 to 4 days after infection (early-phase) and after the development of partial or complete proventricular blockage. Cumulative results from three experiments using mouse blood (blue symbols), two experiments using rat blood (red symbols), and two experiments using rat blood and the *Y. pestis* $\Delta hmsH$ mutant strain (open circles) are shown (see Table 1 for details); bars indicate the median number of *Y. pestis* transmitted per individual flea bite. The transmission probability (number of positive transmissions divided by the total number of trials) is indicated. All early-phase fleas were confirmed to have been infected when they fed for the transmission test. For both the partially blocked and blocked groups, differences in transmission probability and the number of CFU transmitted by fleas infected using mouse blood or rat blood were not statistically significant.

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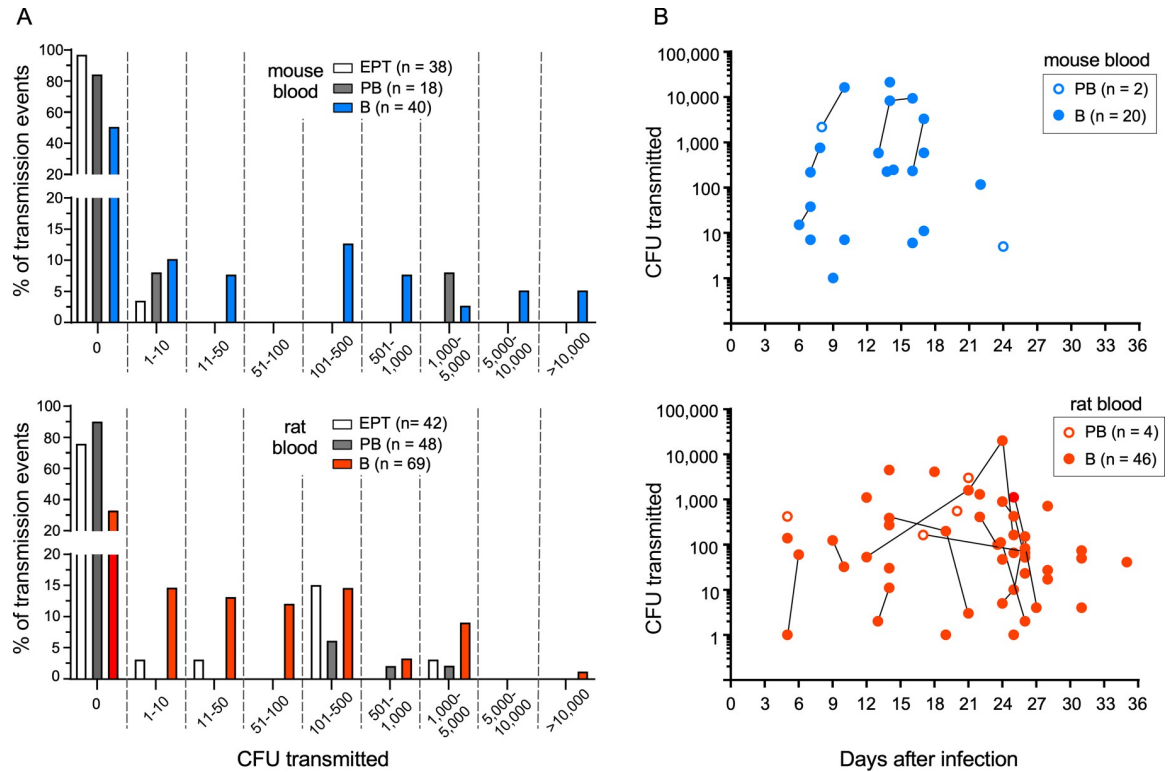


Fig 4. (A) Frequency distribution histogram of the numbers of *Y. pestis* CFU transmitted by individual *O. montana* fleas during the early phase (EPT) and by partially blocked (PB) and completely blocked (B) fleas. (B) Temporal distribution pattern of the number of CFU transmitted by individual partially blocked (PB) and completely blocked (B) fleas (positive transmission events only). Lines connecting data points indicate transmissions by the same flea on successive days. Cumulative results from three experiments using mouse blood (blue symbols) and two experiments using rat blood (red symbols) are shown; see Table 1 for details.

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was much lower than we found with the mouse challenge model (18%; [27]). This may in part be due to a lower sensitivity of the *in vitro* transmission model. The number of CFUs transmitted in the early phase by fleas infected using mouse blood was clearly very low, but some transmissions of one or a few bacteria may have been missed because the efficiency of plating and recovering very low CFU numbers from a blood reservoir is likely not 100%. For the early-phase probability of transmission parameters (p_{ep}) described below, therefore, we chose to use the higher estimates reported for the mouse challenge model (18% and 14% for *O. montana* infected using mouse or rat blood, respectively) [27]. These parameter values were used in conjunction with our empirical data on the number of wild-type and *hms* mutant *Y. pestis* transmitted by individual early-phase fleas infected using mouse or rat blood, respectively (Fig 3).

Transmission by partially blocked fleas was also rather inefficient—only about 10% of feeding events resulted in transmission, regardless of the infectious blood source (Fig 3). Completely blocked fleas had the highest transmission efficiency, and it was similar for fleas infected using mouse or rat blood (50% and 67%, respectively; $p = 0.1$). Transmission tests were repeated periodically for blocked fleas as long as they remained alive, and several of these fleas transmitted on more than one day (Fig 4). In one case, a single blocked flea transmitted four different times over a period of 12 days.

Another aspect of transmission efficiency is the number of bacteria transmitted per bite, which we were able to assess on an individual flea basis. The number of *Y. pestis* transmitted by blocked fleas can be highly variable [28, 29]. Our results confirm that, with a range from 1

to >10,000 CFU transmitted by individual fleas, and also indicate that the number of *Y. pestis* transmitted by early-phase and partially blocked fleas can vary widely (Figs 3 and 4). An exception may be early-phase transmission by fleas infected using mouse blood, which was of such low efficiency that only small numbers are likely to be transmitted (Fig 3). A summary of the results of the flea infection and transmission experiments is given in Table 1.

Model simulations of flea-borne transmission dynamics

We developed a deterministic SEIR model to compare the relative contribution of three transmission stages on plague dynamics in the context of two host blood sources that are known to influence infection of fleas and early-phase transmission [16, 30]. Conceptual design of the model is illustrated in Fig 5. Our experimental results summarized above were used to estimate the parameter values of the flea vector submodel (Table 2). Parameter values for the rodent host submodel (Table 3) are from published sources. We examined different model structures based on fleas infected using bacteremic mouse or rat blood, host susceptibility to fatal plague [lethal dose (LD₁₀₀) of 1, 10, or 100 *Y. pestis* CFUs], and transmission mode (early-phase transmission or biofilm-dependent transmission by blocked or partially blocked fleas). The starting condition was 9 susceptible hosts, 1 infected (highly bacteremic) host, and 50 uninfected fleas; model outputs were recorded over a simulated 100-day period (S1 Fig). Fig 6A shows the predicted mortality for the various scenarios. With all transmission modes operative, plague epizootics (arbitrarily defined as ≥ 50% cumulative mortality during the 100-day simulation) ensued in the most susceptible host population, but at the second tier of susceptibility (LD = 10 CFU) the output was host-blood dependent. Fleas infected using rat blood were predicted to cause an epizootic, but fleas infected using mouse blood were not. In the more resistant population (lethal dose of 100 CFU), enzootic scenarios (<50% mortality) ensued with both sets of fleas. Most of the mortality was attributable to transmission by blocked fleas—when early-phase transmission parameters were set to zero, mortality was equivalent or even slightly

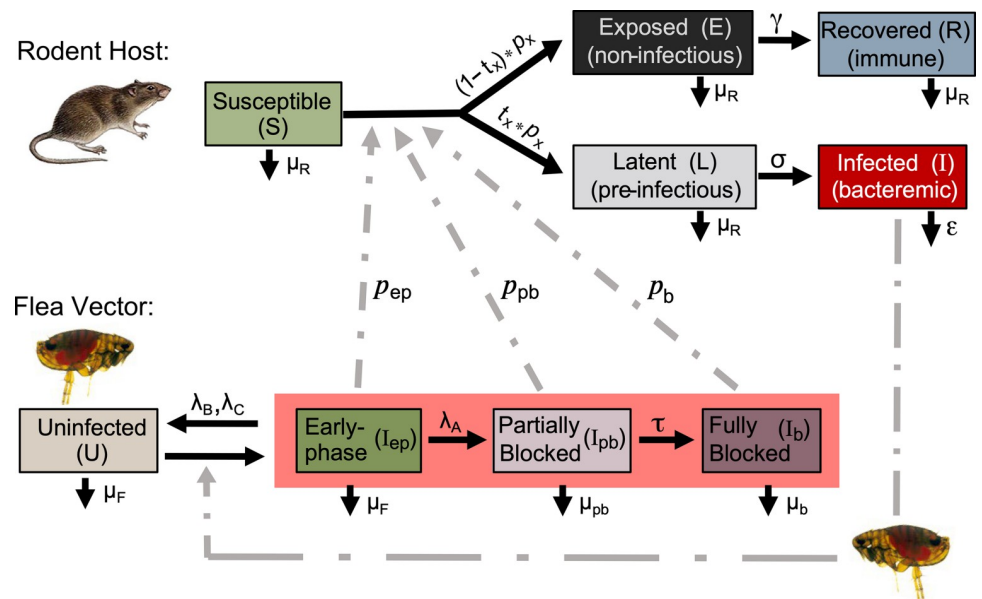


Fig 5. Flow chart of the flea vector-rodent host model. The three transmission-competent stages of flea infection are highlighted, and the different possible outcomes following transmission to the rodent host are indicated. See text and Tables 2 and 3 for details. $t_x = t_{ep}, t_{pb}, \text{ or } t_b$; $p_x = p_{ep}, p_{pb}, \text{ or } p_b$.

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Table 2. Summary of parameter values, flea vector sub-model.

Parameter	Definition	Calculation	Value		Ref.
			Mouse blood	Rat blood	
b	Daily biting rate (uninfected, early-phase, and partially blocked fleas)	$\frac{1}{\text{avg. interval (days) between feeds}}$	0.4	0.4	This study
b_b	Daily biting rate (blocked flea)	$\frac{1}{\text{avg. interval (days) between feeding attempts (B fleas)}}$	> 1	> 1	This study, [15, 28]
α	Proportion of fleas infected from host with high bacteremia	$\frac{\text{no. fleas infected (d0)}}{\text{no. fleas that fed on bacteremic blood}}$	1.0	1.0	This study
λ_A	Rate of developing partial blockage	$\frac{(\% \text{ PB fleas}) \div 100}{(\text{avg. time to PB}) - 3d}$	0.035	0.04	This study
λ_B	Rate of infection clearance	$\frac{(\% \text{ fleas cleared by 3d}) \div 100}{3d}$	0.20	0.02	This study
λ_C	Rate fleas leave infectious population after early phase	$\frac{(\% \text{ fleas still infected after 3d but never PB/B}) \div 100}{(\text{avg. time to PB}) - 3d}$	0.07	0.06	This study
τ	Rate of developing complete blockage	$\frac{1}{\text{avg. days from PB to B}}$	0.39	0.48	This study
μ_f	Uninfected flea mortality rate	$\frac{1}{\text{avg. life span of fleas (days)}}$	0.02	0.02	[31]
μ_{pb}	Mortality rate of partially blocked fleas	$\frac{1}{\text{avg. TTD after becoming PB}}$	0.14	0.13	This study
μ_b	Mortality rate of blocked fleas	$\frac{1}{\text{avg. TTD after becoming B}}$	0.20	0.26	This study
p_{ep}	Probability of early-phase transmission	$\frac{\text{no. transmission events}}{\text{no. pos. feeding attempts (d 2-4)}}$	0.18 (0.86)	0.14 (0.78)	This study, [27]
p_{pb}	Probability of transmission (partially blocked fleas)	$\frac{\text{no. transmission events}}{\text{no. pos. feeding attempts (by PB fleas)}}$	0.11	0.10	This study
p_b	Probability of transmission (blocked fleas)	$\frac{\text{no. transmission events}}{\text{no. pos. feeding attempts (by B fleas)}}$	0.5 (0.75)	0.67 (0.89)	This study
t_{ep}	Proportion of early-phase transmission events ≥ LD of rodent	$\frac{\text{no. events in which CFUs transmitted} \geq \text{LD}}{\text{total transmission events (early phase)}}$	1.0 (1.0) ^a	1.0 (1.0) ^a	This study
0 (0) ^b			0.5 (1.0) ^b		
0 (0) ^c			0 (0) ^c		
t_{pb}	Proportion of transmission events by partially blocked fleas ≥ LD of rodent	$\frac{\text{no. events in which CFUs transmitted} \geq \text{LD}}{\text{total transmission events (by PB fleas)}}$	1.0 ^a	1.0 ^a	This study
0.5 ^b			1.0 ^b		
0.0 ^c			1.0 ^c		
t_b	Proportion of transmission events by blocked fleas ≥ LD of rodent	$\frac{\text{no. events in which CFUs transmitted} \geq \text{LD}}{\text{total transmission events (by B fleas)}}$	1.0 (1.0) ^a	1.0 ^a	This study
0.8 (0.96) ^b			0.8 (0.96) ^b		
0.65 (0.88) ^c			0.41 (.65) ^c		

B, blocked; PB, partially blocked; TTD; time to death.

Values in parentheses adjusted to account for cumulative transmission by simultaneous flea bites as described in Methods section.

^{a, b, c} = Values for LD (lethal dose₁₀₀) = 1, 10, or 100 *Y. pestis* CFU

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Table 3. Summary of parameter values, rodent host sub-model.

Parameter	Definition	Calculation	Value*	Ref.
γ	Recovery rate after infectious transmission	$\frac{1}{\text{avg. time to recovery (14 d)}}$	0.07	[32]
σ	Rate of developing terminal bacteremia after infectious transmission	$\frac{1}{\text{avg. time to terminal bacteremia (4 d)}}$	0.25	[33–38]
μ_R	Mortality rate of uninfected rodents and those with latent or resolved infections	$\frac{1}{\text{avg. life span of rodent}}$	0.002	[39]
ε	Mortality rate of rodents with septicemic plague	$\frac{1}{\text{avg. life span of rodent with terminal bacteremia (2 d)}}$	0.50	[28, 33]

Parameter values are based on data from mouse and rat models of acute plague

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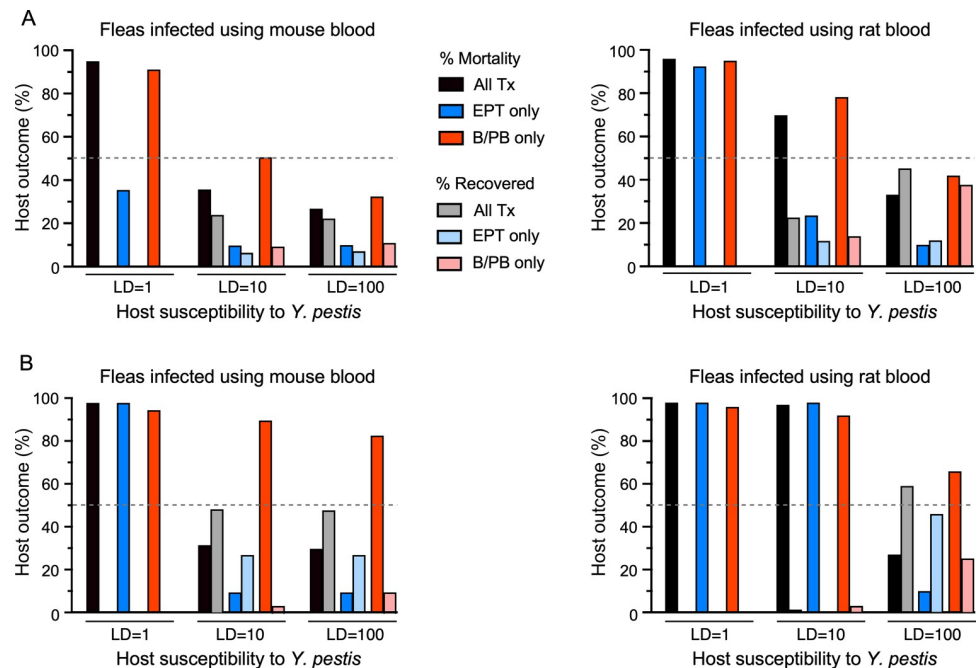


Fig 6. Model predictions of the incidence of infected-dead (% mortality) and infected-recovered (% recovered) hosts in populations with different levels of susceptibility [lethal dose (LD) of 1, 10, or 100 *Y. pestis* CFU]. Separate outcomes produced by fleas infected using mouse blood or rat blood in which both early-phase transmission and biofilm-dependent transmission by partially and completely blocked fleas are operative (All Tx); or in which only early-phase transmission (EPT only) or only biofilm-dependent transmission (B/PB only) are operative are indicated. All simulations were initiated with 9 susceptible hosts, 1 infected (highly bacteremic) host, and 50 uninfected fleas, monitored over a 100-day period. The results from two versions of the model using (A) unmodified parameters and (B) modified parameters for probability of transmission (p) and probability of transmission at or above a lethal dose (t) that account for cumulative transmission by simultaneous flea bites (S1 and S2 Figs). See text for details.

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higher than when both transmission modes were operative. Conversely, when blockage-dependent transmission was removed, predicted mortality was 2.7- to 3.6-fold lower in all cases. The strong influence of the infectious blood source on early-phase transmission (Fig 3) was reflected in the model output (Figs 6A and S1). Early-phase-dependent mortality effected by fleas infected using mouse blood was 36% in the highly susceptible host population (LD = 1 CFU) but nil in the other two populations—the only fatality was the single infected host used to initiate the model simulations. In contrast, early-phase transmission generated higher mortality (93% and 24%) in the two susceptible populations in the rat blood context, although it was still negligible in the more resistant population (LD = 100 CFU).

Recent studies of early-phase transmission efficiency have used a model in which groups of ~10 fleas infected using highly bacteremic rat blood fed simultaneously on highly susceptible laboratory mice ($LD_{50} < 10$ CFU) [20, 40, 41]. To explicitly examine this scenario, we adjusted our early-phase transmission parameters, which are based on an individual flea bite, to reflect the cumulative probability of 10 simultaneous flea bites transmitting a lethal dose. In addition, since a blocked flea makes repeated feeding attempts, we likewise adjusted the blocked flea transmission parameters to reflect the cumulative probability of transmitting a lethal dose in two consecutive feeding attempts. The adjusted parameters are listed in Table 2. In this scenario, early-phase transmission by fleas infected using mouse blood was sufficient to produce an epizootic only in the most susceptible host population, and in populations with the first two levels of susceptibility by fleas infected using rat blood (Figs 6B and S2). High mortality ensued

in the more resistant host population only when transmission by blocked and partially blocked fleas was included in the model. Notably, with both modes of transmission active, mortality in this more resistant population was less than half that (27%) of the mortality predicted when blockage-dependent-transmission operated alone (early-phase transmission parameters set to zero; 66% mortality). Correspondingly, infected but recovered hosts were greater in this population when both modes of transmission were operative than when early-phase transmission was eliminated (59% and 25% recovery rate, respectively; Figs 6 and S2).

Estimation of flea densities required for enzootic and epizootic transmission dynamics

The basic reproduction number (R_0) is defined as the number of secondary cases that ensue from a single case in a naïve population. We used the next-generation matrix method [42] on our SEIR model to estimate R_0 values for the various scenarios and to estimate the flea density per host (m) that would be required to sustain an enzootic or epizootic state (defined here as $R_0 = 1$ and $R_0 = 2$, respectively). With all transmission modes in effect, an m value of five or fewer fleas per host was sufficient to achieve an enzootic state ($R_0 = 1$) for all the scenarios (Fig 7A).

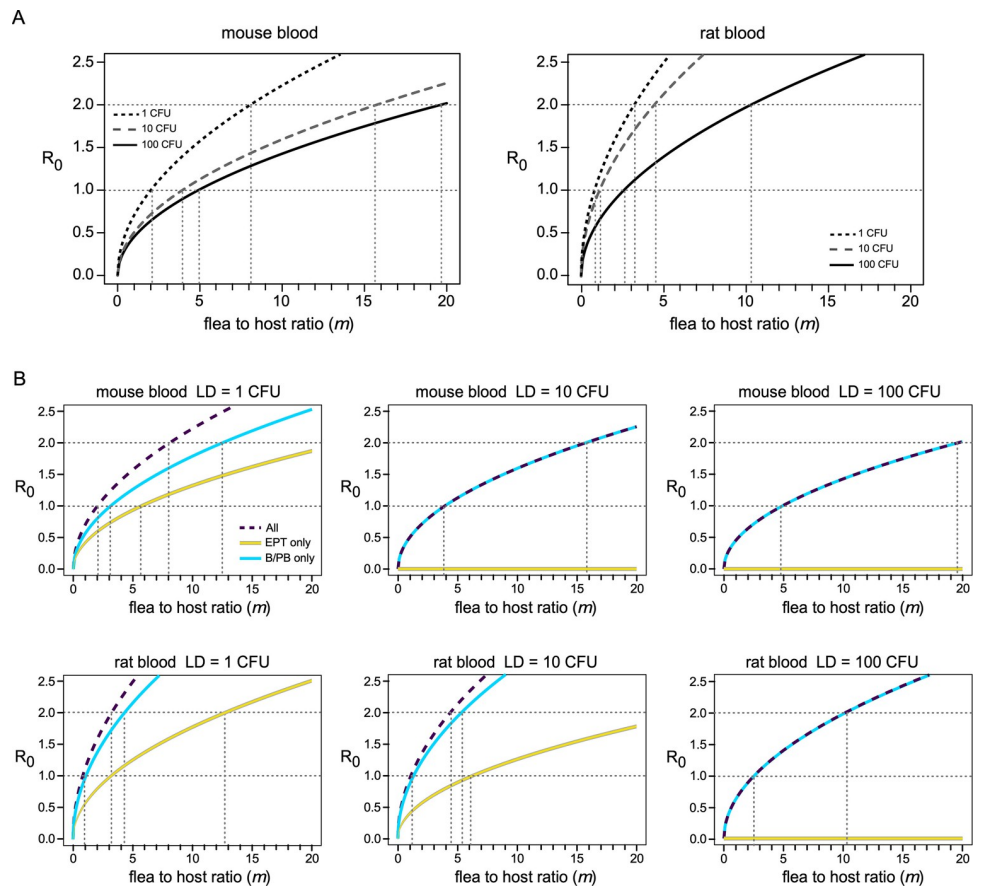


Fig 7. The number of *O. montana* fleas per host (m) required to realize different levels of host-to-host transmission (R_0) predicted by the SEIR model, with all transmission modes operative (A); or (B), with only early-phase transmission (EPT only) or only biofilm-dependent transmission (B/PB only) operative, shown relative to each other and to cumulative transmission by both modes (All). The results for fleas infected using mouse blood or rat blood and for three host populations with different susceptibilities to *Y. pestis* (lethal dose of 1, 10 or 100 CFU) are shown. Dashed lines indicate the intersects of the curves for $R_0 = 1$ (enzootic) and $R_0 = 2$ (epizootic) conditions.

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To reach an epizootic state ($R_0 = 2$), m values of 8, 16, and 20 were estimated from the model, respectively, for fleas infected using mouse blood with the three host populations (LD of 1, 10, or 100 *Y. pestis* CFU). For fleas infected using rat blood, estimated m values required for $R_0 = 2$ were 4, 5, and 11 (Fig 7A).

Flea densities required for enzootic and epizootic conditions were also estimated separately for early-phase transmission only and for transmission by blocked fleas only. The results from the SEIR model are shown in Fig 7B. In all cases, the flea burdens required for a given R_0 level were lower for blocked-flea transmission than for early-phase transmission. For example, in a host population with rat blood characteristics for which the LD = 10 *Y. pestis* CFUs, 6 fleas per host were sufficient to drive an epizootic ($R_0 = 2$) by the proventricular blockage mechanism, whereas early-phase transmission would require ~25 fleas per host. Early-phase transmission alone was not sufficient for even enzootic maintenance—blocked fleas were required (Fig 7B).

Discussion

In this study we systematically examined flea-borne transmission dynamics during a one-month period following an infectious blood meal. Cohorts of *O. montana* fleas were infected uniformly, using two different host blood sources, and then transmission by individual fleas in three progressive stages of transmission competence was evaluated. Two aspects of transmission efficiency were quantified: the probability of transmission and the number of *Y. pestis* transmitted during a single flea bite. The standardized experimental design enabled a more stringent head-to-head comparison of the early-phase and proventricular-blockage dependent transmission modes. Previous comparisons have depended on separate studies that were not standardized as to infectious dose, blood source, or the suitability of the experimental conditions for the general fitness of a particular flea species, all of which can affect infection, blockage rates, and transmission dynamics [16, 23, 29, 30, 33, 40, 43]. Early-phase transmission dynamics following rat blood meals containing $\sim 10^9$ *Y. pestis*/ml have been compared with blocked-flea transmission dynamics following infection by feeding on guinea pigs with a much lower bacteremia level, such that only a minority of fleas (~20 to 32%) that fed on them became infected [44–46]. This low infection rate is consistent with a bacteremia of only about 10^7 *Y. pestis*/ml [29, 33], below the threshold for efficient early-phase transmission [43]. Another problematic area has been the essentially preliminary, and sometimes discordant, available data on blockage rates of different flea vectors, often based on single trials with small sample sizes performed under a variety of conditions and lacking important controls [22, 40, 44]. For example, we recently showed that the prairie dog flea, *Oropsylla hirsuta*, can become blocked at a rate much higher than previously reported [47]. Other studies incorrectly reported that *O. montana* rarely becomes blocked and transmits beyond the early phase [20, 28, 33, 48]. As shown here and in previous studies however, *O. montana* readily becomes blocked and transmits efficiently by the proventricular blockage mechanism [23, 49–51]. Blocked *O. montana* are efficient vectors and can transmit large doses of *Y. pestis* (Fig 3).

The focus of this study was the transmission rate from flea to rodent, which we maximized by infecting the fleas with blood with the high bacteremia level that has been used for early-phase transmission efficiency experiments [20, 52, 53]. Because host blood source can influence infection and early-phase transmission rates [16, 30], we compared fleas infected using mouse blood or rat blood. Fleas in three transmission-competent states were examined individually. Early-phase transmission by fleas infected using mouse blood was rare and inefficient but was much better if the fleas were infected using rat blood. These results are consistent with a previous study that evaluated early-phase transmission by groups of fleas [16]. Rat blood promotes early-phase transmission because it induces a phenomenon termed post-infection

esophageal reflux (PIER), in which a mixture of partially digested blood, hemoglobin crystals, and *Y. pestis* is refluxed into the foregut soon after an infectious blood meal [16]. Mouse blood, characterized by a more soluble hemoglobin molecule and a faster digestion rate in the flea gut, does not engender PIER. As in the previous study [16], some fleas appeared to be completely blocked as early as three days after infection using rat blood, suggesting that early-phase can overlap temporally with biofilm-dependent transmission (Fig 2). For this reason, we based the early-phase transmission estimate on fleas infected with a *Y. pestis hms* mutant, which is incapable of producing proventricular blockage but fully capable of early-phase transmission [17, 24].

A lower percentage of fleas infected using mouse blood developed partial or complete blockage (Fig 1); but this can be attributed to their lower infection rate compared to fleas infected using rat blood, consistent with results reported previously [23]. Transmission by partially blocked fleas was surprisingly inefficient, comparable to early-phase transmission, which may be because the transmission mechanisms are more or less the same (transient or partial impedance of blood feeding), although partial blockage, unlike early-phase, develops later and is biofilm-dependent [14, 16, 18]. However, no effect of blood source on transmission by partially blocked fleas was evident. Transmission by completely blocked fleas was the most efficient of the three stages, both in terms of transmission rate and the number of CFUs transmitted per flea (Fig 3). At this stage also, no difference was apparent between blocked fleas infected using mouse or rat blood. After their 1-h feeding attempts, 50 to 67% of individual blocked fleas had transmitted, and ~10% of these transmitted >1,000 CFUs, with >10,000 CFU recovered in three instances. On average, the number of CFU transmitted by a blocked *O. montana* flea is greater than the number transmitted by a blocked *Xenopsylla cheopis* flea [23, 29].

A second aspect of this study was to use the experimentally derived transmission efficiency data to parameterize an SEIR model of plague dynamics that we developed. Here the main goal was to compare the relative importance and contribution of the early-phase and proventricular blockage-dependent transmission modes in determining epizootic outbreaks. The model outputs indicated that blockage-dependent transmission was most important in producing epizootics, regardless of host blood source. Our modeling indicated that early-phase transmission alone resulted in significantly less mortality, and a large effect of host blood source was evident. Early-phase fleas infected using rat blood could drive an epizootic, but only in the most susceptible host population (LD = 1 CFU). Early-phase transmission by fleas infected using mouse blood was insufficient by itself to produce epizootic conditions in any host population.

Model parameter values were estimated on an individual flea bite basis. However, a feature of flea-borne transmission in nature is that several infected fleas feeding simultaneously on a single host in the early-phase window, or a blocked flea making repeated feeding attempts in succession can produce an augmented, cumulative transmission efficiency. For example, a blocked flea will bite continuously and persistently in the few days before it dies from starvation; and blocked flea transmission efficiency estimates based on single, short term exposure trials are acknowledged to be underestimates [8, 28]. The probability of a blocked flea cumulatively transmitting significant numbers of CFUs in repeated bites likely approaches 100%. In contrast, early-phase transmission is primarily limited to the first feeding after infection, with reduced transmission during later feeds unless reinfected [52]. Therefore, in a second version of the model we incorporated a specific scenario for effective efficiency of cumulative early-phase transmission (simultaneous bites by 10 fleas modeled) and also of blocked-flea transmission (conservatively, 2 consecutive bites by a single blocked flea modeled). In this version of the model, early-phase transmission by fleas infected using mouse blood was sufficient to drive

an epizootic, but only in the most susceptible population ($LD = 1$ CFU). Early-phase transmission by fleas infected using rat blood was sufficient to drive an epizootic in hosts in which the lethal dose was 10 CFU or less. In either version of the model, however, early-phase transmission was incapable of driving an epizootic in a more resistant population ($LD = 100$ CFU), which is more representative of most wild rodents. Blockage-dependent transmission was essential for epizootic scenarios in those populations. Notably, predicted mortality increased when early-phase transmission was set to zero, whereas the number of recovered hosts was higher when both transmission modes were operative. This suggests that early-phase transmission would act to dampen outbreaks in more resistant populations because the sublethal doses usually delivered lead to recovery and immunity rather than disease. In a previous experiment in which laboratory mice were fed on by 3 to 12 infected *O. montana* early-phase fleas, the majority of transmissions were detected by seroconversion only [27]. Thus, blocked flea transmission is likely the driving force behind epizootics in moderately resistant populations. Based on some prior reports that *O. montana* rarely becomes blocked, this mode of transmission was discounted *a priori* in an earlier model of plague dynamics as vectored by this flea [54]. Our results show that this needed to be reevaluated, because in this and a previous study [23] we show that blockage-dependent transmission by *O. montana* is more efficient than early-phase transmission. In accordance, another study reported that *O. montana* transmits at higher efficiency 7 to 21 days after infection than in the early phase [25].

The higher vector competence of blocked *O. montana* fleas was also reflected in the estimated flea burden required to drive an epizootic, which was lower than for early-phase transmission, particularly in a more resistant host population (Fig 7B). The value we estimated for early-phase transmission by *O. montana* infected using rat blood and a susceptible population was similar to a previous estimate for *X. cheopis* that used the same infection scenario [40]. That study came to an opposite conclusion than we did for blocked flea transmission, maintaining that the flea burden would have to be higher than for early-phase transmission. However, the parameter values for blocked flea transmission were based on a single separate study in which the fleas were infected by feeding on mice with varying levels of bacteremia, such that their blockage rate was only 7% [33]. When *X. cheopis* feed on highly bacteremic blood, as used for the early-phase experiments, however, several studies have shown that the blockage rate is 35 to 40% or higher [18, 23, 24, 28, 29, 55–58].

Early-phase transmission, although the first mode of transmission to be described, received relatively little attention after the blockage-dependent transmission mode was discovered. Some, however, proposed that early-phase transmission could add momentum to an epizootic when the plague incidence was high enough to reduce a population size, leading to increased flea burdens on remaining hosts [28, 59]. According to this scenario, the large number of questing fleas that had last fed on a host that died from plague septicemia could transmit when they next fed a new host in a phenomenon called mass transmission (the original name for early-phase transmission, reflecting the fact that many fleas feeding simultaneously are required for a high probability of transmission). Our results also indicate that a high flea burden is required for productive early-phase transmission. Transmission by blocked and partially blocked fleas was still considered to be essential for stable ecological maintenance of flea-borne plague [28, 60]. This premise is supported by the fact that all *Y. pestis* strains maintain the genes required for flea biofilm formation, even though they are not required for virulence in mammals [24, 61, 62]. A previous theoretical modeling study also concluded that gaining the ability to block fleas was evolutionarily adaptive for *Y. pestis* because transmissibility is superior to that of unblocked (*e.g.* early-phase) fleas [63].

More recently, a series of studies concluded that early-phase transmission might be the principal driving force of epizootics, particularly in rodent-flea cycles in which the primary

flea vector reportedly develops proventricular blockage at low incidence (reviewed in [21]). These conclusions were based on transmission results with highly susceptible laboratory mice challenged by groups of ~10 fleas that had been infected 1 to 4 days earlier by feeding on highly bacteremic rat blood. Our simulations also indicate that early-phase transmission could be sufficient to drive an epizootic in those conditions. However, early-phase transmission varies with host blood—it is less efficient following infection with bacteremic mouse blood, (Fig 3; [16]), and may be important in driving epizootics only with hosts whose blood, like that of brown rats, induce PIER in fleas. Furthermore, most wild rodents are more resistant to *Y. pestis* than laboratory mice. For example, the LD₅₀ of *Y. pestis* for the ground squirrel hosts of *O. montana* is reportedly at least 250 to >1,000 CFU [64–66], although this value is likely to vary locally as well as within a given population. It is not known whether transmission efficiency in conjunction with ground squirrel blood would be more like mouse or rat blood, and it will be important to examine that in future studies. In either case, our results suggest a different ecological role for early-phase transmission within most rodent populations. By transmitting a sublethal dose, early transmission may serve to increase the number of resistant individuals in a population in which plague is introduced, in a sense vaccinating them against an otherwise lethal challenge by the later, more efficient transmission by blocked fleas.

Long-term focal persistence of plague requires stable host-pathogen dynamics to maintain an enzootic state. A pathogen as virulent as *Y. pestis*, which depends on producing a lethal, high-density bacteremia in the host to infect its flea vectors would seemingly be at risk of burning through host populations too quickly for stable ecological maintenance. This epizootic scenario can occur in the most susceptible hosts. However, our models suggest that exposure of most wild rodents to sublethal, immunizing doses of *Y. pestis* transmitted during the early phase may ameliorate rapid epizootic spread by reducing the number of susceptible individuals in the population. The coexistence of resistant and susceptible hosts in a population has long been thought to be a factor in the enzootic persistence of plague despite its high virulence [11]. Thus, in many situations early-phase transmission may be more important in maintaining the enzootic state than in driving an epizootic. The effect of early-phase transmission in generating partially immune populations would also increase the time span of enzootic prevalence, during which the movement of individuals could spread the disease to new populations in new areas [67].

Summary

Transmission efficiency of individual *O. montana* fleas was measured over a four-week period after they had fed on mouse or rat blood containing 5 to 8 x 10⁸ *Y. pestis*/ml. Both early-phase and proventricular blockage modes of transmission were monitored for cohorts of fleas following a single infectious blood meal. The results indicate that: 1) blockage-dependent transmission is much more efficient than early-phase transmission, both in terms of the probability of transmission and the number of CFUs transmitted. The recurrent biting behavior of blocked fleas is a significant force multiplier of transmission [68], as a single blocked flea can cumulatively transmit thousands of CFUs before it dies of starvation. 2) Host blood source strongly affects early-phase transmission efficiency, but not blockage-dependent transmission efficiency. However, host blood source can influence the infection rate and therefore the subsequent blockage rate. 3) Early-phase transmission could drive an epizootic only in naïve, very susceptible host populations and when the flea burden is high. 4) The low CFU numbers typically transmitted in the early phase may “immunize” many individuals of more resistant host species, acting to limit epizootic spread and promote an enzootic state.

Materials and methods

Ethics statement

Fleas were from colonies maintained according to a protocol approved by the Institutional Animal Care and Use Committee of the Rocky Mountain Laboratories (RML) [23]. Animal work adhered to the institution's guidelines for animal use, the guidelines and principles in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals and was conducted by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care (AAA-LAC) International accredited facility.

Flea infections

O. montana fleas were from a laboratory colony originally established at the CDC, Fort Collins [69] and maintained at RML since 2011. Groups of ~300 *O. montana* that had not fed for 5 days were infected by allowing them to feed on 5 ml of defibrinated mouse or rat blood containing 5 to 8×10^8 CFU/ml of *Y. pestis* KIM6+ with pAGFP1 (Clontech/Takara Bio) a plasmid that encodes the green fluorescent protein and carbenicillin-resistance or with KIM6+ $\Delta hmsH$ (pAcGFP1) through a Parafilm M membrane stretched across an artificial feeding device, following a previously established standard protocol [23, 70, 71]. The pAcGFP1 plasmid is stably maintained by *Y. pestis* in infected fleas for at least 31 days in the absence of antibiotic pressure. After a 1-h feeding period, fleas were individually examined microscopically, and those that took an infectious blood meal (denoted by the presence of fresh red blood in the midgut) were collected; 20 of them were placed at -80°C for later determination of the initial infectious dose and the rest were placed in capsules containing a layer of sawdust and maintained at 21°C , 75% relative humidity [23].

Evaluation of flea infection, mortality, blockage, and transmission rates after infection

To assess early-phase transmission, 12–48 individual fleas (equal numbers of males and females) were allowed to feed for 1 h on ~300 μl of sterile defibrinated mouse or rat blood (corresponding to the infectious blood meal source), using miniaturized versions of the artificial feeding device, on days 2 to 4 after infection [71]. Each flea was then examined for evidence of feeding and for partial or complete proventricular blockage [24]. Partial blockage is diagnosed by the presence of fresh blood in the esophagus and midgut and complete blockage by the presence of fresh blood only in the esophagus, usually pooled just anterior to the proventriculus [71]. The blood in the individual reservoirs was removed and spread onto blood agar plates containing 100 $\mu\text{g/ml}$ carbenicillin. CFUs were counted after 48 h at 28°C to determine the number of bacteria transmitted. Fleas that fed were individually frozen at -80°C for subsequent bacterial load determinations.

All other fleas that took an infectious blood meal were provided maintenance feeds twice weekly (Monday and Thursday) beginning 2 to 4 days after infection for one month on a neonatal mouse or on sterile defibrinated rat blood in the same feeding system used to infect them. After the 1-h feeding period fleas were individually examined and the feeding rate and the incidence of partial or complete proventricular blockage was recorded. Flea mortality was also recorded throughout the experiment, and samples of 20 fleas were removed at 7, 14, and 30 days after infection and frozen for bacterial load determination. Fleas showing signs of partial or full blockage were removed from the group and stored separately. These partially and completely blocked fleas were permitted to feed individually on a miniaturized artificial feeder

every 1 to 3 days, and evidence of feeding, proventricular blockage status, and mortality were determined each time. Blood was collected from the reservoirs corresponding to those fleas that showed evidence of feeding or attempted feeding and plated to determine the number of CFUs transmitted. Transmissions by the same partially or completely blocked flea on multiple days were each considered as separate transmission events. Between-group differences in transmission probability and number of *Y. pestis* CFU transmitted were analyzed by chi-square and Mann-Whitney test, respectively (GraphPad Prism 9 software).

To determine infection rates and the average bacterial load per infected flea, samples of 20 fleas that had been collected and stored at -80°C at different times after the infectious blood meal were thawed, surface-sterilized, individually triturated in PBS, and dilutions plated in BHI soft agar overlays for CFU counts (lower limit of detection = 40 CFU/flea) as previously described [70, 71].

Deterministic host-vector model

We developed a continuous time, deterministic model formulated to reflect the dynamics of infection in the vector population as they relate to progression of disease in reservoir hosts (Fig 5). The model is based on Bailey's single host-single vector and Ross-Macdonald models [72, 73] and uses ordinary differential equations to describe the flow of individual vertebrate hosts and vectors through different infection categories (S1 Text). Our model is focused on understanding the relative contribution of the three transmission-competent states of infection in fleas to the dynamics of infection in a host population. Thus, we did not include a birth rate for either flea or rodent compartments.

The flea vector compartment follows a susceptible-infected (SI) pattern with the infected state divided into three successive transmission-competent stages: early-phase (I_{ep} ; here defined as the first four days after the infectious blood meal), partially blocked (I_{pb}), and completely blocked (I_b) [14, 23]. Additionally, we account for the subset of fleas that clear themselves of infection but that may become reinfected. However, there is no final recovery state because the endstage of biofilm development in the flea (complete blockage) is invariably fatal.

Hosts follow a susceptible-exposed-infected-recovered (SEIR) pattern of disease; however, the exposed-recovered progression is disjointed from the development of overt disease. When the number of CFUs transmitted is less than a lethal dose (LD), we expect that a susceptible host will resolve the infection and recover without ever developing the terminal, high-density bacteremia ($>10^7$ *Y. pestis*/ml of peripheral blood) that is required to reliably infect fleas that feed on it [29, 33, 43]. Thus, transmission by fleas in any of the three transmission-competent stages (I_{ep} , I_{pb} , I_b) to a susceptible host can lead to either exposed but never infectious stages (E, R), or to an infectious stage (I) characterized by a fatal bacteremia. We also included a latent stage (L) prior to the infectious stage to account for the time to develop high bacteremia after transmission.

Transmission parameter values and conditions for model simulations

Model simulations were performed using the programming language R [74] and were based on parameters generated from experimental results reported here or derived from literature sources. To simulate the cumulative transmission by 10 infected fleas feeding simultaneously in an early-phase challenge, the probability of transmission was calculated as $1 - (1 - p_{ep})^{10}$ and the probability of transmitting a lethal dose was calculated as $1 - (1 - t_{ep})^{10}$, with p_{ep} and t_{ep} being the probabilities for a single flea bite (Table 2). It was further assumed that an individual blocked flea would (conservatively) make at least two successive feeding attempts, and the p_b and t_b values were similarly adjusted to $1 - (1 - p_b)^2$ and $1 - (1 - t_b)^2$.

Simulation conditions were set for 100 days at 0.3-day time steps to capture acute infection dynamics. The flea population is represented by *O. montana*, and host populations with different susceptibilities ($LD_{100} = 1, 10$ or $100 Y. pestis$ CFUs) were considered. Model populations consisted of 10 hosts (1 bacteremic and 9 uninfected) and 50 uninfected fleas. When infected and dead hosts exceeded 50%, we considered the infection scenario an epizootic. Conversely, when more hosts survived than died from infection, we classified the infection as enzootic. See [S1 Text](#) for details of the model and R code.

To understand the role of the different modes of flea-borne transmission of *Y. pestis* in maintaining enzootic levels of the pathogen versus stimulating epizootic bursts, we evaluated all flea transmission states together and then systematically compared the individual capacity of early-phase transmission to transmission by blocked/partially blocked fleas by artificially setting one or the other transmission probabilities to zero.

Estimation of flea density required for enzootic and epizootic transmission

We formulated an expression for R_0 from our model using the next-generation matrix method [42]. See [S1 Text](#) for details and R code. Based on our parameter estimates (Tables 2 and 3, and [Fig 3](#)), the number of fleas per host (m) that would be required for an enzootic ($R_0 = 1$) or epizootic ($R_0 \geq 2$) was calculated.

Supporting information

S1 Text. The deterministic host-vector model; calculating R_0 ; and model code.
(PDF)

S1 Fig. Model output of the dynamics of plague in host populations with different levels of susceptibility.
(PDF)

S2 Fig. Model output of the dynamics of plague in host populations with different levels of susceptibility using the modified parameters to account for cumulative transmission by simultaneous flea bites.
(PDF)

S3 Fig. Model output of the dynamics of flea infection, blockage, and mortality in scenarios with different levels of host susceptibility.
(PDF)

S4 Fig. Model output of the dynamics of flea infection, blockage, and mortality in scenarios with different levels of host susceptibility using the modified parameters to account for cumulative transmission by simultaneous flea bites.
(PDF)

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