nature portfolio

Peer Review File

Transovarial transmission of Yersinia pestis in its flea vector, Xenopsylla cheopis

Reviewer #1 (Remarks to the Author):

Reviewer comments: NCOMMS-23-50286-T 'Transovarial transmission of Yersinia pestis in fleas'

Pauling et al describe a study in which they evaluated if Xenopsylla cheopis fleas harbor persistent small numbers of plague bacteria across a generation which would account for transovarial maintenance of plague bacteria in fleas. By analyzing infected fleas and their progeny under their specific experimental conditions, their data demonstrated presence of low numbers of Y. pestis in reproductive tissue, eggs and larvae from infected fleas.

While these observations may be relevant a major concern is that many aspects of the experimental design do not unequivocally preclude alternative explanations for the observations and question the evidence for transovarial transmission. A list of these is given below:

-The experimental design fails to account for potential contamination specifically in the protocols utilized for ascertaining bacterial colonization in eggs and larvae from infected fleas. A critical disinfecting step to eliminate contamination from the outer surface of eggs and larvae was not present. While authors describe a method of separation involving sifting to avoid contact with flea feces, the procedure's efficacy remains questionable.

Flea feces are heterogeneously expelled and can be distributed in the bedding by fleas moving around freely, presenting a significant risk of contamination of eggs and larvae. Besides larvae naturally feed on adult flea feces and crawl in the bedding. Feeding infected fleas on uninfected blood overlooks the possibility of bacteria being present in the feces excreted post feeding. Application of sterile water washes and removal to sterile medium is insufficiently rigorous to ensure surface sterilization of eggs and larvae. Consequently, any detection of Y. pestis could be attributed to fecal contamination rather than genuine colonization. Hence, all experiments with eggs and larvae could possibly have yielded positive results for presence of Y. pestis due to contamination from feces. For a robust validation of their findings, authors should incorporate a control wherein eggs and larvae from fleas fed on sterile blood are exposed to bedding from infected fleas. Subsequent identical processing of these samples would provide a comparative measure to ascertain the presence of Y. pestis, thus reinforcing the integrity of the experimental design and conclusively eliminating contamination as a variable.

-Any bacteria present in the F1 progeny would have been acquired through carryover of the contaminated organism and may account for the unusually low numbers detected in these adult fleas. Fleas are generally colonized at high numbers or not at all as seen in the literature primarily from the group of Joe Hinnebusch. Considering previous literature, a discussion of how low numbers of Y. pestis persist in the F1 fleas is warranted here. Were F1 progeny fed? Blood-feeding at low infectious dose leads to clearing (https://doi.org/10.1086/429931) by digestive processes which may eliminate these small numbers of surviving plague bacteria in the F1 fleas. How such a scenario will factor into maintenance of Y. pestis should be discussed. Better yet, an experiment in which the infected F1 progeny are fed and then bacterial burdens determined is needed.

-The resolution of the confocal images does not allow for appreciation of whether there are plague bacteria colonizing the flea midgut (Figs 1-5). Autofluorescence of the flea gut is a known issue with flea imaging. (DOI: 10.1128/aem.02091-22). How was autofluorescence addressed in these images and how were images collected and analyzed and 3D Z-stacks generated. Please provide details about the confocal imaging system used. Here are a few examples demonstrating high resolution imaging of fluorescing Y. pestis in the flea gut (see https://doi.org/10.1371/journal.ppat.1008440; 10.1128/aem.02091-22,

10.1371/journal.ppat.1009995, 10.1371/journal.ppat.1009995; https://doi.org/10.1128/jb.02000-12).
-A related concern is that microscopy serves only as qualitative evidence for the presence of Y. pestis in flea reproductive issue. All arthropods are known to harbor their own set of microbiota including fleas (https://doi.org/10.1038/ismej.2012.95, https://doi.org/10.1371/journal.pone.0141057) so verification that the bacteria visualized in reproductive tissue is Y. pestis is required. Use of an anti-Yersinia antibody (see https://doi.org/10.1111/j.1462-5822.2007.00986.x) or in situ hybridization assays (https://doi.org/10.1371/journal.pntd.0008688), or perhaps even immunogold-labeling in the case of electron microscopy, to unequivocally validate the presence of Y. pestis in flea reproductive tissue should be undertaken. Again, the argument here would be that currently microscopy serves circumstantial evidence and not an actual verification that bacteria visualized in the flea reproductive tissue are Y. pestis.
-It has been established that of Y. pestis through its flea vector predisposes the bacterium to an environment that is conducive to genetic exchange with the resident microflora of the flea digestive tract (ref doi: 10.1046/j.1365-2958.2002.03159.x.) Given this background it would be prudent to investigate the potential for plasmid transfer among gut microbes as a facilitative process for maintaining the TD tomato fluorescence, and possibly Y. pestis extrachromosomal plasmids, in eggs and larvae. Therefore, it remains a critical validation step in the detection of Y. pestis in eggs and larvae, to confirm the presence of Y. pestis by detecting a chromosomal genes and not just plasmid-encoded genes in PCR assays. This would serve as unequivocal identification of the bacterium irrespective of plasmid transfer dynamics.
-What was the larval bedding composed off as it is known that flea larvae feed on adult feces. For the larval forms to undergo development they would require optimally nutritious food.
-The Figshare link appears broken on this reviewer's computers so access to the files was not achievable.
-The Introduction does not cover all relevant literature. This is primarily important for Lines 49-51, 66-68 that fails to acknowledge reference 25 of the manuscript which presents a refined model for Y. pestis blockage-mediated transmission by experimentally demonstrating that plague bacteria colonize the foregut first, and that the midgut is a secondary site of colonization.
Ln 48 mentions that the early phase transmission mechanism is 'poorly understood mechanism'. Recent studies (https://doi.org/10.1371/journal.ppat.1006859) have provided more understanding of this mechanism through describing the phenomenon of post esophageal reflux (PIER) which should be acknowledged in the introduction.
-How was the experiment to determine RFU in the alimentary canal of fleas performed with box plots representing interquartile range. There appears to be no control of Y. pestis without a fluorescent marker included which would be a true control for background fluorescence? In fact, such a control should have been included in all experiment as it is an appropriate negative control for fluorescence experiments but should have cfu burdens and microscopy outcomes that mirror findings shown for experiments in which the fluorescently marked Y. pestis strain was used.
-Proper statistical analysis should be performed for Figs 1E and F and elsewhere when applicable. The sample numbers in Fig 5 are too small to allow for rigorous analysis. Pooling samples doesn't allow assessment of the proportion of the samples that were positive. This would be relevant to understand the extent of transovarial transmission and if it is a rare or common event.
-The Discussion section is scant and doesn't cover the breadth of literature relevant for

transovarial transmission in vector-borne disease, beyond flea-borne diseases. A more in depth-analysis and comparing findings in other vector-pathogen interactions with findings from this manuscript is needed. Moreover, that this bacterium is primarily found extracellularly in the gut lumen, and the lack of evidence for presence of Y. pestis in the hemocoel warrants a deeper explanation for how the bacteria would have reached the reproductive tissue.
-A model or proposal for how Y. pestis re-enters the sylvatic plague cycle is relevant in the discussion. Also, in consideration of the refined mechanism of transmission offered by reference 25, can the authors address how bacteria from the midgut in the F1 progeny transit to the foregut to be transmitted?

Reviewer #2 (Remarks to the Author):

“Transovarial transmission of Yersinia pestis in its flea vector, Xenopsylla cheopis" for Nature Communications.
The authors used different assessment methods to demonstrate that adult Xenopsylla cheopis fleas infected with the plague bacterium Yersinia pestis can transmit the infection to all the different developmental stages of their offspring.
Plague disease, in humans or in animals has been characterized by the alternance of long periods of quiescence and outbreaks. Several hypotheses have been advanced, mainly focused on the existence of enzootic and epizootic cycles, the role of hibernating mammal reservoirs, and the persistence in the soil or in unicellular microorganisms living in damp soils. The persistence of the bacterium in adult fleas from one season to another under hibernation conditions has been described, as well as the potential for flea larvae feeding on infected adult flea dejection to keep the bacterium until the adult life stage.
The authors demonstrated that Y. pestis can be transmitted vertically among fleas and this discovery has a capital impact on the understanding of the flea-borne transmission of plague and the persistence of the bacterium in the environment, especially during interepizootic period. This work is ultimately changing what is known about flea-Y. pestis interaction.
Since the work of Verjbitski et al. in 1908 which described in detail plague transmission by insects, through the iconic description of the flea blockage by Martin and Bacot in 1914, the presence of plague bacilli in the flea digestive tract and dejection has been well-accepted, and considered as a dogma. The demonstration of the blockage-independent transmission or early-phase transmission dismissed the possibility of a mechanical transmission where the plague bacterium would persist on the mouthparts, the infective bacterium is confined to the midgut solely. The body of the published research on Y. pestis persistence, survival, and transmission by the flea relies largely on the development of a biofilm, a prerequisite for Y. pestis survival and growth in its invertebrate host. Biofilm formation is an exclusive Y. pestis “life stage” that is only possible in the flea gut, and thus to survive, unlike arbovirus which can colonize other tissue, Y. pestis is confined in the flea gut. Therefore, the present research is introducing a totally new and unprecedented concept regarding the flea-borne transmission of Y. pestis.

The authors used various and complementary methods to support their findings. They used standardized and published methods for flea infection and transmission tests. Through careful consideration, the work supports the conclusions, although a more consequent sample size may help to reinforce the claims. I do understand that getting enough infected and alive fleas through the experiments may be a challenge.

Comments and questions for the authors
Line 61. “This observation suggests that increased population of rats should increase the likelihood of epizootic plague”. I do not agree with the statements because I feel like it is a misinterpretation or rather a shortcut of the original finding of the paper cited by the authors. Please consider the role of genes like ymt and its kinetics with host red blood cell composition (Bland et al., 2021. PloS Pathogens), which is more likely to demonstrate Y. pestis adaptation to its rodent hosts rather than having a direct consequence on the occurrence of epizootics.

Please justify the use of tdTomato instead of another fluorescent reporter. Do the authors confirm that similar results may be obtained for example with GFP?

Line 86: Please add a percentage of fleas with bright fluorescence.

Extended data Figure 1. Please show the Malpighian tubules in non-infected control fleas. Malpighian tubules are known to express autofluorescence like the pupae.

Line 118 to 122. Authors should discuss more about the contribution of infected blood meal or infected female and/ or male reproductive organs to the obtention of infected eggs. Which one would contribute more to infecting the egg? Maybe a way to test is to get eggs from an infected male and naive female or a naïve male and infected female.

Line 133. What is the proportion of flea larvae hatching from infected eggs? Is the hatching rate and larvae survival impacted by the infection? What is the proportion of infected larvae hatching from infected eggs?

For the transmission and infection experiments using an artificial feeding system, several host bloods were used. Is there any reason motivating the blood choice and what would be the impact of the blood sources on the issues of the experiments?

To optimize the number of feeding fleas, they were starved for a few days prior to the infection. In nature, fleas may have unrestricted access to host blood, so do you think that the amount of ingested infected blood may influence the recovery of bacteria from the different tissues and from the immature stages?

In the discussion, the authors should give more explanation on how Y. pestis migrates from the midgut to the other body parts. How can they confirm that Y. pestis is absent from the gut epithelial cells and the hemocoel?

Authors demonstrated that infected adults could transmit vertically to the egg, and subsequently to the larvae, pupae, and F1 generation. They also demonstrated that bacteria isolated from these stages remained viable and can infect a naïve adult flea with artificial feeding infection. For all results, authors should consider adding proportion and number of samples.

Line 199. Since the authors did not conduct any transmission test on potentially infected F1 adults to confirm that they were able to transmit enough bacterium in sterile blood, they should be more careful in concluding that “transovarial transmission could sustain low levels of Y.

pestis in rodent community". A flea infection in the gut does not mean that there would be a transmission. It would have been interesting to follow up on the infection in the F1 generation that was infected since they were eggs and accounted for a clearance rate through time.

Reviewer #3 (Remarks to the Author):

Transovarial transmission of Yersinia pestis in fleas
Pauling et al.

This very interesting paper describes evidence for vertical transmission of Y. pestis in its arthropod vector, which has never been documented before and is potentially of great significance both widely and to the field. Most compellingly, Y. pestis could be recovered from adult F1 progeny of infected fleas. Evidence for Y. pestis infection of ovarioles and testes of orally-infected fleas, ova, and subsequent larval and pupal life stages is also presented.

The results are novel and have obvious potential for adding another factor to the complex and incompletely understood dynamics of plague ecology. However, it would be premature to assign a significant role of vertical transmission to plague ecology based on the present results. Only small numbers of Y. pestis were detected in F1 fleas and it would be necessary to further follow a sample of F1 fleas to show that Y. pestis could go on to produce a transmissible infection and be vertically transmitted to the F3 generation; as well as an estimate of the vertical transmission rate. These caveats should at least be discussed to prevent readers' overinterpretation of the paper.

The report of significant infection of ovarioles and testes within 3-5 days after an infectious blood meal (Fig. 2) is highly striking and unexpected, since it would seemingly require Y. pestis to pass through the midgut epithelium, transit the hemocoel, invade the reproductive organs and multiply there despite no evidence over decades that Y. pestis infects beyond the digestive tract or even associates much with the midgut epithelium. As such, I think the burden of proof should be higher than showing amorphous patches of faint fluorescent signal within the reproductive tissues. Electron microscopy images (Fig. 6) are non-specific. Fleas are known to maintain a microbiome that includes intracellular bacteria that are presumably transovarially passaged. The bacteria found by TEM could be these; in fact, their morphology does not look like Y. pestis to me. The "safety pin" appearance referred to in the figure legend and line 166 is not a morphology but a bipolar staining characteristic and it is not evident here. Immunohistochemistry staining on sectioned tissues using anti-Y. pestis antibody should be performed instead as well as culturing viable bacteria from these tissues. The statement on line 171 that the TEM results are "confirmatory" of Y. pestis infection of ovarioles and testes is an overinterpretation.

A possible alternative route to the conclusion that Y. pestis escapes from the midgut (line 176) is based on the fact that infected fleas excrete large numbers of Y. pestis in their feces, which are viscous and tarry and can stick to the rear end of the flea. The opening for the male genital apparatus is directly below the anus, and it is possible that infected fecal material is picked up and transferred to the female genital tract during the complicated, prolonged rear-end to rear-end mating process.

Specific questions and comments:

1. More details on the confocal microscopy could be provided, such as the wavelength of the laser filter set used (were they highly specific for the Tomato protein?) and the magnification. In general, the fluorescent signals are not very bright and rather difficult to make out in all the figures. Also, it would have been more convincing if images were collected using a 60X objective lens that might indicate bacterial morphology rather than amorphous patches of signal.

2. The correlation between RFU by confocal and CFU by viable plating does not seem very high to me (Fig. 1E,F and Fig. 3K, 4G and Fig. 5A. If that impression is correct, is there a reason for it? Is it a reflection of high variability in small sample sizes?

3. What was the reason for using prairie dog blood and pig blood in some experiments instead of the biologically relevant rat blood for all the experiments? This introduces a confounding variable because host species blood can influence infection in the flea. What was the source of the infectious blood meals for the experiments described starting at line 107 in which eggs, larvae, pupae and F1 adults were collected? Also, what was the source of the maintenance blood meals for infected fleas kept longer than 7 days after infection- the same blood source they were infected with?

4. Is it possible to determine whether the fluorescent signals in larvae (Fig. 4) were intra- or extra-intestinal? This is pertinent because larvae feed on adult feces, which if infected could lead to transstadial infection. A previous examination of this possibility reported that Y. pestis ingested by flea larvae did not survive more than 48 h (D.H. Molyneux 1969 Investigations into the possibility of intervector transmission of Pasteurella pestis. Ann. Trop Med Parasitol 63:403-407). This relevant study should be mentioned and discussed.

5. The number of samples examined and the number of those with positive results should be stated for all the experiments. I couldn't find this information for some of the experiments and I suspect the sample sizes were low.

6. The Introduction contains some incorrect or unclear statements:

line 58- It is not correct to say that blockage in O. montana is "very infrequent" as it actually blocks readily (PLoS NTD 11:e0005276; 2017); although it is true that blockage in some fleas, such as cat fleas and the human flea is very infrequent compared to rat fleas.

Lines 59-64 state that host blood source affects proventricular blockage but not early-phase transmission. In fact the opposite has been reported. The early (pre-blockage) colonization of the proventriculus and subsesquent early-phase transmission is significantly higher with rat vs mouse blood (ref 22) but proventricular blockage rates are equivalent (ref 24).

Line 67- As the transmitted bacteria usually derive from the foregut (proventriculus), it would be more accurate to say that transmission occurs from the digestive tract rather than the midgut.

Line 61- The reasoning behind the statement beginning with "This observation…" is unclear to me, please explain and provide supporting references.

Line 71-72. This statement is also unclear as several modeling studies are found in the literature. Please explain.

7. What was the source of the Tomato gene- a high copy number plasmid?

8. It would be advisable to surface-sterile samples before triturating and plating; especially the eggs, which are coated with a sticky substance and could pick up contaminated fecal material during passage not easily removed by PBS washes.

Response to the Reviewer Critiques

General response:

We appreciate the positive comments from all three reviewers, as well as their expressed concern that additional experimentation was needed in order to achieve more *unequivocal* evidence that justifies a shift in paradigm. In the revised submission, we have included additional experimental support as detailed below to address the major concerns. Notably, a new figure 1 has been included that addresses the use of multiple host blood sources for infections in the artificial feeder. We have added a new experiment in figure 5 that demonstrates by immunohistochemistry that *Y. pestis* is present in eggs and ovariole tissue. We have added a new experiment in figure 8 that shows whole genome sequencing results from one of the egg isolates that demonstrates unequivocal identification of transovarially transmitted *Y. pestis*. We have also added additional TEM images from male and female infected fleas. We have highlighted the sections of the revised submission that contain new data.

Extensive additional modifications to the manuscript were made to clarify methods, background information, group sizes, and data interpretation. Below we present specific point-by-point responses to the thoughtful comments of the reviewers. We believe the new data and modifications made to the text strengthen the conclusions and appreciate the helpful suggestions made by each of the Reviewers.

Reviewer #1:

Reviewer comments: NCOMMS-23-50286-T 'Transovarial transmission of Yersinia pestis in fleas'
Pauling et al describe a study in which they evaluated if Xenopsylla cheopis fleas harbor persistent small numbers of plague bacteria across a generation which would account for transovarial maintenance of plague bacteria in fleas. By analyzing infected fleas and their progeny under their specific experimental conditions, their data demonstrated presence of low numbers of Y. pestis in reproductive tissue, eggs and larvae from infected fleas.
While these observations may be relevant a major concern is that many aspects of the experimental design do not unequivocally preclude alternative explanations for the observations and question the evidence for transovarial transmission. A list of these is given below:
-The experimental design fails to account for potential contamination specifically in the protocols utilized for ascertaining bacterial colonization in eggs and larvae from infected fleas. A critical disinfecting step to eliminate contamination from the outer surface of eggs and larvae was not present. While authors describe a method of separation involving sifting to avoid contact with flea feces, the procedure's efficacy remains questionable.
Flea feces are heterogeneously expelled and can be distributed in the bedding by fleas moving around freely, presenting a significant risk of contamination of eggs and larvae. Besides larvae naturally feed on adult flea feces and crawl in the bedding. Feeding infected fleas on uninfected blood overlooks the possibility of bacteria being present in the feces excreted post feeding. Application of sterile water washes and removal to sterile medium is insufficiently rigorous to ensure surface sterilization of eggs and larvae. Consequently, any detection of Y. pestis could be attributed to fecal contamination rather than genuine

colonization. Hence, all experiments with eggs and larvae could possibly have yielded positive results for presence of Y. pestis due to contamination from feces. For a robust validation of their findings, authors should incorporate a control wherein eggs and larvae from fleas fed on sterile blood are exposed to bedding from infected fleas. Subsequent identical processing of these samples would provide a comparative measure to ascertain the presence of Y. pestis, thus reinforcing the integrity of the experimental design and conclusively eliminating contamination as a variable.

Response: We appreciate this concern, and in the new immunohistochemistry experiment shown in figure 5, we included 2 washes with 70% ethanol in the processing protocol. In addition, we have addressed this concern by clarifying the description of the methodology and results. We wish to emphasize that in the original manuscript, we employed selective and non-selective media for recovering bacteria and only a single microbial species has been cultured on agar plates regardless of growth in the presence of antibiotic selection, suggesting the eggs and larvae were sterile. We have also revised the confocal images, now showing higher magnification with the ability to discern single rod shaped bacteria. We have also presented many of the confocal images in red rather than greyscale, allowing easier visualization of rod-shaped bacteria with dimensions consistent with the published size of *Y. pestis* in the reproductive tissue. We also wish to emphasize that the cross sections of the 3D renditions of reproductive tissue and eggs demonstrate rod-shaped bacteria in the interior not on the exterior surface. We hope these clarifications and the additional data satisfy the reviewer's concern about surface contamination as a variable.

-Any bacteria present in the F1 progeny would have been acquired through carryover of the contaminated organism and may account for the unusually low numbers detected in these adult fleas. Fleas are generally colonized at high numbers or not at all as seen in the literature primarily from the group of Joe Hinnebusch. Considering previous literature, a discussion of how low numbers of Y. pestis persist in the F1 fleas is warranted here. Were F1 progeny fed? Blood-feeding at low infectious dose leads to clearing (https://doi.org/10.1086/429931) by digestive processes which may eliminate these small numbers of surviving plague bacteria in the F1 fleas. How such a scenario will factor into maintenance of Y. pestis should be discussed. Better yet, an experiment in which the infected F1 progeny are fed and then bacterial burdens determined is needed.

Response: We respectfully disagree with some aspects of this statement, as it invokes more information than is available. However we believe we understand the Reviewer's point that compared to the primary infection, there is likely more than 3-$\log_{10}$ lower bacterial titer in the midguts of newly emerged F1 infected adults. We anticipate that *Y. pestis* would be able to grow in the midgut upon blood feeding and look forward to examining this question in future work. In this work and in the revised submission, we did not conduct blood feeding of F1 progeny and agree with the unknowns pointed out by all of the reviewers associated with the F1 adult infection. However, we suggest that to address the life cycle of *Y. pestis* in F1 adults is complex, requires high numbers of infected F1 adults and multiple experimental assays, and is therefore outside the scope of this submission. We have adjusted some text in the Discussion section of the revised manuscript to include the critical unknowns about the F1 adult infected fleas (lines 290-299).

-The resolution of the confocal images does not allow for appreciation of whether there are plague bacteria colonizing the flea midgut (Figs 1-5). Autofluorescence of the flea gut is a known issue with flea imaging. (DOI: 10.1128/aem.02091-22). How was autofluorescence addressed in these images and how were images collected and analyzed and 3D Z-stacks generated. Please provide details about the confocal imaging system used. Here are a few examples demonstrating high resolution imaging of fluorescing Y. pestis in the flea gut (see https://doi.org/10.1371/journal.ppat.1008440; 10.1128/aem.02091-22, 10.1371/journal.ppat.1009995, 10.1371/journal.ppat.1009995; https://doi.org/10.1128/jb.02000-12).

Response: We appreciate the concerns associated with resolution and autofluorescence when examining *Y. pestis* in the flea, egg, larva, and pupa by fluorescence microscopy as expressed by the Reviewer, and regret that we did not include sufficient detail in the methods in the original manuscript to mitigate this concern. The use of the brightly fluorescing tdTomato and Texas Red (new figure 5) works in a range of the spectrum where autofluorescence of fleas and tissues is low. One exception was the pupae, which had much higher autofluorescence in this range. In the revised submission, we have clarified controls and negative samples to illustrate that the positive signals were substantially brighter than the small amount of autofluorescence.

-A related concern is that microscopy serves only as qualitative evidence for the presence of Y. pestis in flea reproductive issue. All arthropods are known to harbor their own set of microbiota including fleas (https://doi.org/10.1038/ismej.2012.95, https://doi.org/10.1371/journal.pone.0141057) so verification that the bacteria visualized in reproductive tissue is Y. pestis is required. Use of an anti-Yersinia antibody (see https://doi.org/10.1111/j.1462-5822.2007.00986.x) or in situ hybridization assays (https://doi.org/10.1371/journal.pntd.0008688), or perhaps even immunogold-labeling in the case of electron microscopy, to unequivocally validate the presence of Y. pestis in flea reproductive tissue should be undertaken. Again, the argument here would be that currently microscopy serves circumstantial evidence and not an actual verification that bacteria visualized in the flea reproductive tissue are Y. pestis.

Response: Thank you for the suggestion. We have added additional samples from the TEM experiments in the revised submission and have added detail to the description of these data in order to more conclusively demonstrate bacteria outside the midgut. In addition, in new experiments now included in the revised submission, we carried out immunohistochemistry on tissues collected from fleas infected with the non-recombinant *Y. pestis* strain using a validated and highly specific monoclonal antibody to *Y. pestis* and we have included appropriate control samples from fleas that were not infected. We elected not to pursue immunogold labeling due to time and cost restrictions, however we note that the new immunohistochemistry shown in Figure 5, confocal microscopy on tdTomato-expressing *Y. pestis* in figure 3, and the TEM results in figure 6 are all in agreement with the size, morphology and localization of *Y. pestis* in the reproductive tissues. Therefore the evidence for midgut escape is very strong, and justifies a shift in paradigm.

-It has been established that of Y. pestis through its flea vector predisposes the bacterium to

an environment that is conducive to genetic exchange with the resident microflora of the flea digestive tract (ref doi: 10.1046/j.1365-2958.2002.03159.x.) Given this background it would be prudent to investigate the potential for plasmid transfer among gut microbes as a facilitative process for maintaining the TD tomato fluorescence, and possibly Y. pestis extrachromosomal plasmids, in eggs and larvae. Therefore, it remains a critical validation step in the detection of Y. pestis in eggs and larvae, to confirm the presence of Y. pestis by detecting a chromosomal genes and not just plasmid-encoded genes in PCR assays. This would serve as unequivocal identification of the bacterium irrespective of plasmid transfer dynamics.

Response: We agree and have already begun to address this issue by immunohistochemistry as detailed above. In addition, we have initiated whole genome sequencing of bacteria harvested from eggs, larvae and pupae with the broader goal to understand evolutionary pressures of transovarial transmission. We have begun sequencing 8 isolates and the parent strain, including egg, larval and pupal isolated strains, all of which indicate strong alignment to published *Y. pestis* genome sequence. In this revised submission figure 8, we have included sequence data from one of these egg-isolates as additional evidence that the recovered bacteria are *Y. pestis*.

-What was the larval bedding composed off as it is known that flea larvae feed on adult feces. For the larval forms to undergo development they would require optimally nutritious food.

Response: The formula used for larval food has been published and is included in the revised Methods. We had minimal problems rearing eggs to adults in our system (lines 450-451).

-The Figshare link appears broken on this reviewer's computers so access to the files was not achievable.

Response: We apologize for this issue and have created a new Figshare link that is functional.

-The Introduction does not cover all relevant literature. This is primarily important for Lines 49-51, 66-68 that fails to acknowledge reference 25 of the manuscript which presents a refined model for Y. pestis blockage-mediated transmission by experimentally demonstrating that plague bacteria colonize the foregut first, and that the midgut is a secondary site of colonization.

Response: Thank you for the suggestion. This model is now discussed as suggested (lines 52-54).

Ln 48 mentions that the early phase transmission mechanism is 'poorly understood mechanism'. Recent studies (https://doi.org/10.1371/journal.ppat.1006859) have provided more understanding of this mechanism through describing the phenomenon of post esophageal reflux (PIER) which should be acknowledged in the introduction.

Response: We have adjusted this wording as suggested (lines 36-37).

-How was the experiment to determine RFU in the alimentary canal of fleas performed with box plots representing interquartile range. There appears to be no control of Y. pestis

without a fluorescent marker included which would be a true control for background fluorescence? In fact, such a control should have been included in all experiment as it is an appropriate negative control for fluorescence experiments but should have cfu burdens and microscopy outcomes that mirror findings shown for experiments in which the fluorescently marked Y. pestis strain was used.

Response: We apologize for not including a clear description of RFU determination. This has been corrected. However, we do not understand the suggestion that *Y. pestis* with no fluorescence is needed to assure the reader that *Y. pestis* is fluorescing red due to tdTomato and not autofluorescence, because either way, the fluorescent signal is from *Y. pestis* and the data interpretation is unchanged. Nevertheless, we can assure the reviewer that *Y. pestis* without tdTomato exhibits minimal autofluorescence. We have improved the presentation of the confocal microscopy data and hope these revisions satisfy the Reviewer's concern.

-Proper statistical analysis should be performed for Figs 1E and F and elsewhere when applicable. The sample numbers in Fig 5 are too small to allow for rigorous analysis. Pooling samples doesn't allow assessment of the proportion of the samples that were positive. This would be relevant to understand the extent of transovarial transmission and if it is a rare or common event.

Response: Statistical analysis has been included in Figs 1E and F (now 2E and F) as suggested. While we appreciate the limitations of using pools of 3 adult midguts (which was done to facilitate plating of replicates), we do not think this process negates the importance of the data because the pool size is low. Furthermore, in some cases (see for example figure 8, previously figure 5 of the original manuscript), we were limited in sample number of the different life stages and chose to include lower numbers in order to expand the rigor of each life stage by conducting microscopy and CFU determination. Overall the numbers of fleas that have been examined for transovarial transmission is quite high: by microscopy (figure 4) we analyzed 14 eggs and by plating (figure 8) we analyzed 32 eggs (information previously omitted, now included in figure 8 legend), totaling 46 eggs, with 53% positive for *Y. pestis*. Third, by microscopy (figure 3, Extended Data 1-2), we have also analyzed the reproductive tissues of 10 females and 9 males. Finally, using a non-recombinant *Y. pestis* strain, in the new figure 5, we present microscopy data collected from females and eggs using immunohistochemistry with specific antibody against *Y. pestis* whose specificity has been validated. Therefore with regard to the transovarial transmission studies, they are high-powered and, even though we are just beginning to understand this process, it is reasonable to estimate that the frequency of *transovarial* transmission is approximately 50%.

Transstadial transmission between life stages seems likely to be even more efficient, although we concur with the reviewers that our power for pupae and F1 adults is too small to conclude. Nevertheless, if we only looked at CFU, there were 4 individual larvae, 2 individual pupae, and 3 (2 of which were pooled) F1 adult fleas: 5 of 6 larvae/pupae were positive and we identified *Y. pestis* in the pooled F1 adult sample as well as in the individual flea. Given that the later life stages must also have been infected eggs, the evidence for live bacteria successfully undergoing transstadial transmission from eggs to larvae has n=9, with at least 7 positive samples. Representative isolated colonies from each sample were verified, minimally, by PCR, and

several by whole genome sequencing. We have revised some text to better discuss these topics and hope these revisions satisfy the Reviewer's concern.

-The Discussion section is scant and doesn't cover the breadth of literature relevant for transovarial transmission in vector-borne disease, beyond flea-borne diseases. A more in depth-analysis and comparing findings in other vector-pathogen interactions with findings from this manuscript is needed. Moreover, that this bacterium is primarily found extracellularly in the gut lumen, and the lack of evidence for presence of Y. pestis in the hemocoel warrants a deeper explanation for how the bacteria would have reached the reproductive tissue.

Response: Thank you for the suggestion to add depth to the discussion; we have included this direction in the revised manuscript. We agree with the perspective that invasion of the epithelium is the most likely route of midgut escape, as *Y. pestis* have invasive properties even when grown in fleas, however this could be due to intracellular invasion followed by systemic circulation through hemocoel, exit through Malpighian tubules, a cytotoxic effect on epithelial cells, or may even relate to physiological changes that occur during feeding. Investigation into the mechanism of escape will be pursued as an independent study (lines 280-286).

-A model or proposal for how Y. pestis re-enters the sylvatic plague cycle is relevant in the discussion. Also, in consideration of the refined mechanism of transmission offered by reference 25, can the authors address how bacteria from the midgut in the F1 progeny transit to the foregut to be transmitted?

Response: We have addressed this point in the discussion as suggested (lines 290-296).

Reviewer #2 (Remarks to the Author):

"Transovarial transmission of Yersinia pestis in its flea vector, Xenopsylla cheopis" for Nature Communications.
The authors used different assessment methods to demonstrate that adult Xenopsylla cheopis fleas infected with the plague bacterium Yersinia pestis can transmit the infection to all the different developmental stages of their offspring.
Plague disease, in humans or in animals has been characterized by the alternance of long periods of quiescence and outbreaks. Several hypotheses have been advanced, mainly focused on the existence of enzootic and epizootic cycles, the role of hibernating mammal reservoirs, and the persistence in the soil or in unicellular microorganisms living in damp soils. The persistence of the bacterium in adult fleas from one season to another under hibernation conditions has been described, as well as the potential for flea larvae feeding on infected adult flea dejection to keep the bacterium until the adult life stage.
The authors demonstrated that Y. pestis can be transmitted vertically among fleas and this discovery has a capital impact on the understanding of the flea-borne transmission of plague and the persistence of the bacterium in the environment, especially during interepizootic period. This work is ultimately changing what is known about flea-Y. pestis interaction.
Since the work of Verjbitski et al. in 1908 which described in detail plague transmission by

insects, through the iconic description of the flea blockage by Martin and Bacot in 1914, the presence of plague bacilli in the flea digestive tract and dejection has been well-accepted, and considered as a dogma. The demonstration of the blockage-independent transmission or early-phase transmission dismissed the possibility of a mechanical transmission where the plague bacterium would persist on the mouthparts, the infective bacterium is confined to the midgut solely. The body of the published research on Y. pestis persistence, survival, and transmission by the flea relies largely on the development of a biofilm, a prerequisite for Y. pestis survival and growth in its invertebrate host. Biofilm formation is an exclusive Y. pestis "life stage" that is only possible in the flea gut, and thus to survive, unlike arbovirus which can colonize other tissue, Y. pestis is confined in the flea gut. Therefore, the present research is introducing a totally new and unprecedented concept regarding the flea-borne transmission of Y. pestis.

Response: We appreciate these positive comments on the potential impact of the work.

> *The authors used various and complementary methods to support their findings. They used standardized and published methods for flea infection and transmission tests. Through careful consideration, the work supports the conclusions, although a more consequent sample size may help to reinforce the claims. I do understand that getting enough infected and alive fleas through the experiments may be a challenge.*

Response: We appreciate this concern and agree with the Reviewer that the previous submission left open the impression of small sample sizes. In the revised submission, and in our response to Reviewer 1, we have added new data and outlined the power of the study to illustrate high rigor.

> *Comments and questions for the authors*
> *Line 61. "This observation suggests that increased population of rats should increase the likelihood of epizootic plague". I do not agree with the statements because I feel like it is a misinterpretation or rather a shortcut of the original finding of the paper cited by the authors. Please consider the role of genes like ymt and its kinetics with host red blood cell composition (Bland et al., 2021. PloS Pathogens), which is more likely to demonstrate Y. pestis adaptation to its rodent hosts rather than having a direct consequence on the occurrence of epizootics.*

Response: We agree and have adjusted the discussion of this section (lines 57-61).

> *Please justify the use of tdTomato instead of another fluorescent reporter. Do the authors confirm that similar results may be obtained for example with GFP?*

Response: We have had great results with the red end of the spectrum and have been working with this tdTomato construct since 2011. Occasionally we use GFP in *Y. pestis* but not extensively in our flea research. There is very low autofluorescence in adult fleas (as well as eggs and larvae) with excitation at 554nm, emission at 581nm (tdTomato) or 596nm excitation, 615nm emission (Texas red). We did not have the same good fortune with GFP in the expression plasmid, where we had a much greater degree of autofluorescence with excitation at 405nm compared to tdTomato. We have seen plenty of negative samples of infected midguts and eggs, and do not believe autofluorescence can be attributed to the positive results we are reporting. In

the revised submission, we have paid more attention to the midgut life cycle by adding new confocal data in Figure 1, which is a high-powered microscopy study that includes samples were fluorescent signal was minimal or absent.

Line 86: Please add a percentage of fleas with bright fluorescence.

Response: We have added this information and expanded the data presented on the experimental model. This is presented as Figure 1 in the revised submission.

Extended data Figure 1. Please show the Malpighian tubules in non-infected control fleas. Malpighian tubules are known to express autofluorescence like the pupae.

Response: We have added images the Malpighian tubules from non-infected control fleas.

Line 118 to 122. Authors should discuss more about the contribution of infected blood meal or infected female and/ or male reproductive organs to the obtention of infected eggs. Which one would contribute more to infecting the egg? Maybe a way to test is to get eggs from an infected male and naive female or a naïve male and infected female.

Response: We thank the reviewer for raising this very interesting point. On close examination, we think it is likely that the female is contributing more to infecting the egg because it appears the oocytes become infected, whereas in males, we think the infection is in the interstitial tissue or seminiferous tubules but not the spermatocytes themselves. We have modified the text to include a clear discussion of this and will pursue experimental validation in the future (see discussion of TEM data, lines 181-198).

Line 133. What is the proportion of flea larvae hatching from infected eggs? Is the hatching rate and larvae survival impacted by the infection? What is the proportion of infected larvae hatching from infected eggs?

Response: The hatching of flea larvae from infected eggs was efficient. In the revised manuscript, we have added detail on rearing the infected eggs in the Methods section (lines 451-453).

For the transmission and infection experiments using an artificial feeding system, several host bloods were used. Is there any reason motivating the blood choice and what would be the impact of the blood sources on the issues of the experiments?

Response: We appreciate this comment and potential concern. In the revised submission, we address this concern with new data presented in Figure 1 where we illustrate the model and differences observed in the foregut and midgut when different host species blood meals are used for the infection. While there are differences in these compartments related to the aggregation and growth of *Y. pestis*, it appears that the aggregation of bacteria in the foregut/midgut infection may not directly impact transovarial transmission as we have observed it at all times post-infection and with each of the four host species we use for flea infections. In addition, we include a brief discussion of this topic in the revised text (lines 99-101, 119-121, 141-144).

To optimize the number of feeding fleas, they were starved for a few days prior to the infection. In nature, fleas may have unrestricted access to host blood, so do you think that the amount of ingested infected blood may influence the recovery of bacteria from the different tissues and from the immature stages?

Response: This is another interesting point, given that fleas are typically, though not exclusively, on host. We anticipate that blood feeding provides opportunity for midgut escape, and therefore would expect that on-host fleas efficiently transfer *Y. pestis* to progeny eggs. We plan to address this directly in future experiments. In the revised discussion, we have raised this issue as an open question that could impact the sylvatic cycle (lines 293-301).

In the discussion, the authors should give more explanation on how Y. pestis migrates from the midgut to the other body parts. How can they confirm that Y. pestis is absent from the gut epithelial cells and the hemocoel?

Response: We agree with the Reviewer that we cannot rule out invasion of the epithelium and temporary residence in hemocoel. We have adjusted the discussion as suggested (lines 280-282).

Authors demonstrated that infected adults could transmit vertically to the egg, and subsequently to the larvae, pupae, and F1 generation. They also demonstrated that bacteria isolated from these stages remained viable and can infect a naïve adult flea with artificial feeding infection. For all results, authors should consider adding proportion and number of samples.

Response: Thank you for this suggestion. This information has been included in the figure legend of revised figure 8 as suggested.

Line 199. Since the authors did not conduct any transmission test on potentially infected F1 adults to confirm that they were able to transmit enough bacterium in sterile blood, they should be more careful in concluding that "transovarial transmission could sustain low levels of Y. pestis in rodent community". A flea infection in the gut does not mean that there would be a transmission. It would have been interesting to follow up on the infection in the F1 generation that was infected since they were eggs and accounted for a clearance rate through time.

Response: We agree and have adjusted the wording in several sections as suggested.

Reviewer #3 (Remarks to the Author):

The results are novel and have obvious potential for adding another factor to the complex and incompletely understood dynamics of plague ecology. However, it would be premature to assign a significant role of vertical transmission to plague ecology based on the present results. Only small numbers of Y. pestis were detected in F1 fleas and it would be necessary to further follow a sample of F1 fleas to show that Y. pestis could go on to produce a

transmissible infection and be vertically transmitted to the F3 generation; as well as an estimate of the vertical transmission rate. These caveats should at least be discussed to prevent readers' overinterpretation of the paper.

Response: Thank you for the positive comments. As suggested, we have adjusted our discussion of plague ecology to reduce the potential for overinterpretation (lines 291-301).

The report of significant infection of ovarioles and testes within 3-5 days after an infectious blood meal (Fig. 2) is highly striking and unexpected, since it would seemingly require Y. pestis to pass through the midgut epithelium, transit the hemocoel, invade the reproductive organs and multiply there despite no evidence over decades that Y. pestis infects beyond the digestive tract or even associates much with the midgut epithelium. As such, I think the burden of proof should be higher than showing amorphous patches of faint fluorescent signal within the reproductive tissues. Electron microscopy images (Fig. 6) are non-specific. Fleas are known to maintain a microbiome that includes intracellular bacteria that are presumably transovarially passaged. The bacteria found by TEM could be these; in fact, their morphology does not look like Y. pestis to me. The "safety pin" appearance referred to in the figure legend and line 166 is not a morphology but a bipolar staining characteristic and it is not evident here. Immunohistochemistry staining on sectioned tissues using anti-Y. pestis antibody should be performed instead as well as culturing viable bacteria from these tissues. The statement on line 171 that the TEM results are "confirmatory" of Y. pestis infection of ovarioles and testes is an overinterpretation.

Response: We have adjusted the text, improved magnification of the tdTomato microscopy, and also added new data with immunohistochemistry to address this concern. We note that in all assays, whether recombinant or non-recombinant *Y. pestis* strain labeled by anti-*Yersinia* monoclonal antibody or with expression of tdTomato or by TEM, we have identified rod-shaped bacteria that are approximately 1μm in length. In addition, we agree with the reviewer about the likely existence of a microbiome in the midgut and reproductive tissue as we have observed other bacteria by TEM. However the infected adult ovarioles and testes are uniquely infected by rod shaped bacteria and there is evidence in the testes of minor pathology caused by the infection due to the enlarged basement membrane which was only seen in *Y. pestis*-infected fleas. Additional TEM images from male and female infected fleas have been included in the revised submission and we hope the reviewer is assured that we did not overinterpret the TEM data.

A possible alternative route to the conclusion that Y. pestis escapes from the midgut (line 176) is based on the fact that infected fleas excrete large numbers of Y. pestis in their feces, which are viscous and tarry and can stick to the rear end of the flea. The opening for the male genital apparatus is directly below the anus, and it is possible that infected fecal material is picked up and transferred to the female genital tract during the complicated, prolonged rear-end to rear-end mating process.

Response: Thank you for raising this interesting point. We do not have good information on how long *Y. pestis* is shed in the feces after infection, but we have observed *Y. pestis* outside the midgut compartment 15 weeks after the adult flea was infected (shown in Extended Data Figure 2). The type of transmission referred by the Reviewer seems unlikely given that we have also

observed *Y. pestis* in testes, Malpighian tubules and in the spermatheca, with 3D cross-sections illustrating rod shaped, 1µm length bacteria in the interior of these tissues.

Specific questions and comments:

1. More details on the confocal microscopy could be provided, such as the wavelength of the laser filter set used (were they highly specific for the Tomato protein?) and the magnification. In general, the fluorescent signals are not very bright and rather difficult to make out in all the figures. Also, it would have been more convincing if images were collected using a 60X objective lens that might indicate bacterial morphology rather than amorphous patches of signal.

Response: This information has been added to the methods, and new immunohistochemistry data have been included as Figure 5. Appropriate magnification was used to visualize the length of the cell, which agrees with the expected morphology and size of *Y. pestis* (lines 455-464).

2. The correlation between RFU by confocal and CFU by viable plating does not seem very high to me (Fig. 1E,F and Fig. 3K, 4G and Fig. 5A. If that impression is correct, is there a reason for it? Is it a reflection of high variability in small sample sizes?

Response: We agree with this observation that correlation between RFU and CFU is not apparent as anticipated, and think it is important to point out that the RFU data points represent individual fleas, whereas the CFU data points are pools of 3 midguts. The pooling would mask low level colonization in favor of high titer midguts if both were present in the pool. To improve rigor in our model system, we have added microscopy data, presented in figure 1, from 243 fleas, fed with each of the host species used in the remaining paper, and we believe our results for bacterial aggregation in the early phase after flea infection are in-agreement with published data.

3. What was the reason for using prairie dog blood and pig blood in some experiments instead of the biologically relevant rat blood for all the experiments? This introduces a confounding variable because host species blood can influence infection in the flea. What was the source of the infectious blood meals for the experiments described starting at line 107 in which eggs, larvae, pupae and F1 adults were collected? Also, what was the source of the maintenance blood meals for infected fleas kept longer than 7 days after infection- the same blood source they were infected with?

Response: This concern was also expressed by Reviewer 2. The driving force behind the different blood sources was to investigate how this might impact the early phase of the *Y. pestis* life cycle and midgut escape. We also concur that the pig and prairie dog blood feeding of *X. cheopis* represents new information that should be included in this study. Accordingly we have added this data as Figure 1 in the revised manuscript.

4. Is it possible to determine whether the fluorescent signals in larvae (Fig. 4) were intra- or extra-intestinal? This is pertinent because larvae feed on adult feces, which if infected could lead to transstadial infection. A previous examination of this possibility reported that Y. pestis ingested by flea larvae did not survive more than 48 h (D.H. Molyneux 1969

Investigations into the possibility of intervector transmission of Pasteurella pestis. Ann. Trop Med Parasitol 63:403-407). This relevant study should be mentioned and discussed.

Response: Thank you for the suggestion and interesting reference, we agree that this is an important issue. In the Molyneux paper, larvae were infected by feeding on liquid or dried blood, with no detection of *Y. pestis* after 24-48 hours, suggesting the bacteria cannot survive digestion in larvae. Interestingly, we have on numerous occasions studied *D. melanogaster* larvae infected with *Y. pestis* during feeding on agar plates wherein bacterial survival and growth occurs over several days thereby indicating *Y. pestis* can survive digestion (Earl, et al, 2015, *PLoS One*). These observations seem to contradict one another and we look forward to investigating this further. Nevertheless, assuming the Molyneux results are confirmed, it is probable that the disparate observations can be reconciled by invoking extra-intestinal localization of *Y. pestis* in larvae where it may survive better. We have adjusted the discussion to include the Molyneux paper as suggested, but do not believe this paper rules out transstadial transmission (lines 264-277).

5. The number of samples examined and the number of those with positive results should be stated for all the experiments. I couldn't find this information for some of the experiments and I suspect the sample sizes were low.

Response: We regret the omission and have included this information in all of the figure legends of the revised manuscript.

6. The Introduction contains some incorrect or unclear statements:

line 58- It is not correct to say that blockage in O. montana is "very infrequent" as it actually blocks readily (PLoS NTD 11:e0005276; 2017); although it is true that blockage in some fleas, such as cat fleas and the human flea is very infrequent compared to rat fleas.

Response: We agree and have clarified this section, as we were referring to the early surveillance study where blockage of *O. montana* (then *Diamanus montanus*) was 3% (lines 49-52).

Lines 59-64 state that host blood source affects proventricular blockage but not early-phase transmission. In fact the opposite has been reported. The early (pre-blockage) colonization of the proventriculus and subsesquent early-phase transmission is significantly higher with rat vs mouse blood (ref 22) but proventricular blockage rates are equivalent (ref 24).

Response: We have clarified this point as suggested (lines 54-56).

Line 67- As the transmitted bacteria usually derive from the foregut (proventriculus), it would be more accurate to say that transmission occurs from the digestive tract rather than the midgut.

Response: This wording has been changed throughout the introduction as suggested.

Line 61- The reasoning behind the statement beginning with "This observation…" is unclear

to me, please explain and provide supporting references.

Response: We have clarified this point and provided references as suggested (lines 58-64).

Line 71-72. This statement is also unclear as several modeling studies are found in the literature. Please explain.

Response: We have clarified this point as suggested (lines 67-70).

7. What was the source of the Tomato gene- a high copy number plasmid?

Response: The reference cited shows this strain as recombinant with a high copy plasmid. To make sure this is clear to the reader, we have added this descriptor to the methods (lines 78-80, 425-426).

8. It would be advisable to surface-sterile samples before triturating and plating; especially the eggs, which are coated with a sticky substance and could pick up contaminated fecal material during passage not easily removed by PBS washes.

Response: We appreciate the concerns over surface contamination of eggs. As detailed above, nearly 50% of the eggs (15 of 32) were negative for *Y. pestis* by plating either on selective or non-selective media. This result indicates a strong probability that the processing methods successfully removed any contaminating material. In addition, the 3D cross sections of confocal microscopy and the TEM support an interior, not exterior, localization.

Reviewer #1 (Remarks to the Author):

In the rebuttal, many of the reviewers' comments have been addressed. Addition of more TEM images of bacteria is helpful. But there remain concerns. A major concern is that the burden of proof remains as the immunohistochemistry leaves an opening for alternate interpretation of the findings based on the study design. Image quality is still not optimal, showing mainly patches of color that supposedly depict Y. pestis. Fig 3E and 3F and perhaps Fig 4F, 4H and 4I are an improvement and the change to represent the images in the red channel has been helpful. Because this study will highlight a significant new paradigm associated with the understanding of how Y. pestis persists in the environment during the interepizootic cycle, it requires rigorously acquired evidence of these claims.

-In reference to Fig 5 it remains unclear as to the verification of Y. pestis in the ovariole tissue because dissected, instead of tissue sections was used. (Comments by Reviewer three, mentioned use of sectioned tissue and I suggest referring to https://doi.org/10.1371/journal.pbio.3002625 for how to detect Y. pestis in flea tissue sections in a new paper that also presents a brand new paradigm for Y. pestis infection and transmission.). Cross contamination is a possibility when tissues are dissected from tiny organisms like fleas. In Ln 621 sterilizing fleas with water washes may not necessarily remove any bacteria stuck to the outer surface of the tissue. Sterilization is only relevant for viable bacterial counts in tissue. It's known that dissecting fleas often results in some leakage of bacteria from the gut to the exterior of the gut that can contaminate other tissue (see in https://doi.org/10.1371/journal.ppat.1008440 (Fig 2 and 8); https://doi.org/10.1371/journal.ppat.1009995 (Fig 1D, 3D); https://doi.org/10.1371/journal.ppat.1006859 (Fig 2 and 4)). Therefore, the reproductive tissue could have bacteria sticking to the outer surface. Besides, to me, Fig, 5D and 5F show clearly that the tissue is surrounded by large cells that look like fat body cells. And Fig H shows that the staining is associated mostly with these cells. The uninfected Control sample B doesn't appear to show the fat body like cells. Additionally, the staining conditions were optimized on bacteria growing in HIB in vitro (Ln 479) and does not account for non-specific staining of flea tissue (e.g., fat body cells). Overall, the image quality doesn't allow for conclusive identification of Y. pestis or the localization of the staining within the tissue. Optimally, ovarioles that were not subject to Triton X-100 permeabilization step could have helped to distinguish between surface associated and internalized Y. pestis.

-Ln 88 – please include reference/s that supports the statement 'consistent with published data'.

-Ln 185 – how is it known that the bacteria are Gram negative in the TEM images?

-Lns 183-198 For Fig 6 please label the cell types referred to in the text (e.g., follicle cells, seminiferous tubules, basement membrane enclosure, spermatocyte and so on. Please indicate the basement membrane in Fig 6G.

-Ln 565-568 Can the authors please define 'early' aggregation as they are evaluating fleas collected from Days 1 to 14 for this experiment as per Fig 1 legend? Does this refer to early phase transmission or other phenomena? Additionally, in the response to Reviewer three comments, the authors refer to 'aggregation in the early phase' which apparently encompasses days 1 to14 as per Fig 1 legend. Currently the literature indicates that Y. pestis colonizes the proventriculus within 24h

-Ln 116-118 Please provide the references for 'published reports' mentioned here that 'bacterial aggregation in the foregut occurs around Day 3 post-infection and then subsides until biofilm-mediated aggregation occurs' and please clarify what this statement means in light of current

literature on the subject. This comments also reflects on the title for Fig 2 'Aggregation of Y. pestis in X. cheopis peaks on day 3 post-infection.' Y. pestis aggregates in the proventriculus within 24h and bacteria are also noted to multiply in that time period (see Fig 1 D in https://doi.org/10.1371/journal.ppat.1006859 and see Fig 2 https://doi.org/10.1371/journal.ppat.1009995) and is the current paradigm for bacterial colonization of the proventriculus.
-On occasion the bacterial colonization extends into the midgut or bacteria are observed around the proventriculus stomodeal region. It is therefore important to include in the methods for Fig 2, how a predefined set of criteria were established for RFU data collection for the PV and midgut to improve objectivity in the analysis.
-Ln 621 – Please describe clearly what the control refers to in Ln 621. How many eggs were collected from uninfected controls?
-Extended Data Fig 2 requires a non-infected control image.
-Additionally, with regards to DNA sequence evidence of Y. pestis in the flea tissue, can the authors address information as to whether any Rickettsial DNA was found. For example, it is known that Rickettsia felis can survive in laboratory reared flea colonies, have been found associated with rat fleas naturally, and are able to be transovarial transmitted. Additionally, the bacteria is approx. 1.5 micron long similar to Y. pestis (see DOI: https://doi.org/10.4269/ajtmh.1990.43.400) so it would be great to eliminate Ricketssial species as a potential confounding variable in the flea colonies. After all, fewer control samples were used in studies. In fact, the data, and details about uninfected controls in terms of numbers is less than those for infected test samples. This is important especially because transovarial transmission is estimated to be at a 50% occurrence, so findings in uninfected controls may have been missed by not evaluating enough of such samples (e.g., Fig 7 have 8 infected and 3 uninfected samples).
-In relation to the comment above, please provide in the discussion information on localization of R. felis in the reproductive tissue and how these overlaps, or differs, with the localization of Y. pestis in the reproductive tissue presented here.

Reviewer #2 (Remarks to the Author):

I appreciate the effort made by the authors to address reviewers' comments, including mine. The manuscript has significantly improved in clarity, and the incorporation of new experiments has solidified the research hypothesis. I want to commend the authors for their dedication in executing each step of these experiments, which undoubtedly demanded a considerable amount of patience and expertise.

I have only minor comments and recommendations:

Please ensure that references are added for any mention of data from previous research, as noted in Lines 88, 117, and 255.
It would be beneficial to include a summary table detailing the number of samples processed for each experiment and the number of samples with positive outcomes. Using precise numbers instead of approximations would enhance clarity. For instance, in Figure 1, provide the exact number of midguts dissected per blood type and per day post-infection (dpi).

Reviewer #3 (Remarks to the Author):

Transovarial transmission of Yersinia pestis in its flea vector, Xenopsylla cheopis (revised) Pauling et al.

In this revision, the authors have addressed the reviews and added additional details and discussion. They have convincingly documented some very interesting observations, which I believe warrant acceptance for publication. This work should inspire future investigation into the role, if any, of the detected transovarial transmission in the ecological maintenance of plague. There remain a few issues that could be additionally clarified:

1. The second sentence of the Discussion mentions quantitative data, but what I was looking for were data that quantified the percentage of infected females that laid infected eggs, the percentage of positive larvae from infected eggs, the percentage of positive pupae from infected larvae (or infected eggs) and the percentage of F1 infected adults that arose from infected pupae, larvae, or eggs. In other words, some indication of the efficiency of transovarial and transstadial transmission. The new information in the figure legends does not adequately address this. I could find that about 50% of infected fleas had bacteria in the genital tract (Fig 3 legend). The same data should be provided for eggs laid by infected fleas (How many of the 14 eggs tested in Fig. 4 were positive; how many of the 25 eggs tested in Fig. 5 were positive). How many of the 5-10 fleas tested in Fig. 6 were positive. How many larvae were examined for Fig. 8 (the legend states 8 were positive, but out of how many examined?). How many pupae were examined for the extended data Fig. 3, and how many were positive? The new information in the Fig. 8 legend indicates that 2 of 3 F1 progeny were positive, but only one was examined for Fig. 8B,C? For these percentages, it would be best to rely on the direct microscopic evidence of infection, because the samples were not surface-sterilized (only washed) before plating for CFU counts.

2. The Abstract states that evidence was found that may account for sustained, low-level infection of a flea population via transovarial transmission and that the evidence implicates vertical transmission as a mechanism capable of sustaining Y. pestis in the environment for extended periods. I think these statements are overly strong and urge more caution because that was not examined in this study, and perhaps it would be better to say that the results suggest a potential role rather than implicate. Those reading only the Abstract may jump to the conclusion that this has been established and overinterpret the ecological significance of the present results. Ecological significance would only be concluded by examining how many of the

infected F1 progeny went on to develop a chronic, transmissible infection (following a cohort of F1 fleas to determine what percentage developed a heavy proventricular infection); and what percentage of F2 progeny of the original infected fleas were infected. The authors make that point in the last paragraph of the Discussion, stating that this was not examined. They state that it seems probable that the positive F1 fleas would maintain and transmit, but it remains to be seen if the transovarial transmission they document is just a curiosity or if it has real ecological significance. Since only newly-emerged F1 adults were examined and they contain only few bacteria (~170 CFU), my suspicion is that, although possible, continued infection would be rare and these fleas would eliminate the infection quickly once they were allowed to feed. I base this on a previous study (JID 2005 191:1907-1912) that showed that 94% of fleas that fed on blood with a low bacteremia level such that they ingested only about 1,000 Y. pestis CFU rapidly cleared the bacteria. Hopefully these issues will be addressed and resolved by this group or others before it becomes entrenched in the literature that transovarial transmission is ecologically significant in plague maintenance.

3. Other minor points of consideration:

-How were the pupa samples handled- dissected from their cocoons and PBS-washed? I did not find it in the methods.

-Direct spread from the digestive tract to the genital tract is strongly inferred as the mechanism, but no evidence for this is presented, and no bacteria were reportedly detected in midgut epithelia. Infected fecal contamination of the male aedeagus (the long elaborate penis apparatus) during the complex flea mating process could serve to introduce bacteria into both the male and female reproductive tract and is another plausible and simpler mechanism for what appears to be rapid and targeted spread to reproductive tissues. The mechanism of dissemination is a matter of speculation at this point.

Reviewer #1 (Remarks to the Author):

In the rebuttal, many of the reviewers' comments have been addressed. Addition of more TEM images of bacteria is helpful. But there remain concerns. A major concern is that the burden of proof remains as the immunohistochemistry leaves an opening for alternate interpretation of the findings based on the study design. Image quality is still not optimal, showing mainly patches of color that supposedly depict Y. pestis.

Response: We appreciate the positive comments. In the immunohistochemistry figure, prepared in response to the previous review, we worked with the experts in our Advanced Microscopy Core to image the tissues and have presented the highest quality that was possible. We have reviewed the fluorescent images in several recent *Y. pestis* papers, including those referred to by the Reviewer below. We believe our images are of comparable resolution and clarity, and the study design included appropriate controls capable of detecting autofluorescence.

Fig 3E and 3F and perhaps Fig 4F, 4H and 4I are an improvement and the change to represent the images in the red channel has been helpful. Because this study will highlight a significant new paradigm associated with the understanding of how Y. pestis persists in the environment during the interepizootic cycle, it requires rigorously acquired evidence of these claims.
-In reference to Fig 5 it remains unclear as to the verification of Y. pestis in the ovariole tissue because dissected, instead of tissue sections was used. (Comments by Reviewer three, mentioned use of sectioned tissue and I suggest referring to https://doi.org/10.1371/journal.pbio.3002625 for how to detect Y. pestis in flea tissue sections in a new paper that also presents a brand new paradigm for Y. pestis infection and transmission.). Cross contamination is a possibility when tissues are dissected from tiny organisms like fleas.

Response: Related to the previous comment, we appreciate this concern and the need to rule out surface localized bacteria. Figure 5 is an immunostain with a commercial monoclonal antibody, validated against 26 gram-negative bacterial species, therefore the signal we have reported is a specific signal. With regard to tissue sectioning of whole fleas, we have some experience with this and are familiar with the new paper in *PLoS Biology* which uses histochemistry in whole fleas. Although tissue sectioning represents another valid method of sample preparation, we have explored this technology in the past and found that the exoskeleton becomes brittle during the preparation of blocks. During sectioning, there is significant risk of fragmenting the tissues, resulting in data that cannot be interpreted. For these reasons, we developed the fixation method in dissected tissues.

Related to cross contamination, this experiment was conducted in response to the previous review and therefore we carried out 70% ethanol washes of the samples prior to imaging. As it is well known that *Y. pestis* are highly sensitive to ethanol even with low contact time, there can be no surface-located *Y. pestis* contamination in this experiment. We have added this detail to the figure legend to avoid confusion (line 659).

In Ln 621 sterilizing fleas with water washes may not necessarily remove any bacteria stuck to the outer surface of the tissue.

Response: There have been no observations that suggest our water washes were unsuccessful in removing bacteria: We observe only one species when we plate the washed eggs, and we have not observed tdTomato-expressing bacteria on the surface of the tissues. Nevertheless, the eggs and fleas used in the immunohistochemistry experiment were sterilized with ethanol as indicated in the methods which kills bacteria that may be on the outer surface. We have added this detail to the figure legend to avoid confusion (lines 659).

Sterilization is only relevant for viable bacterial counts in tissue. It's known that dissecting fleas often results in some leakage of bacteria from the gut to the exterior of the gut that can contaminate other tissue (see in https://doi.org/10.1371/journal.ppat.1008440 (Fig 2 and 8); https://doi.org/10.1371/journal.ppat.1009995 (Fig 1D, 3D); https://doi.org/10.1371/journal.ppat.1006859 (Fig 2 and 4)). Therefore, the reproductive tissue could have bacteria sticking to the outer surface.

Response: The cross sections and z-stacking for imaging shown in Figures 4F, 4I, and 5H demonstrate that the fluorescent signal in the reproductive tissue is not on the surface.

Besides, to me, Fig, 5D and 5F show clearly that the tissue is surrounded by large cells that look like fat body cells. And Fig H shows that the staining is associated mostly with these cells. The uninfected Control sample B doesn't appear to show the fat body like cells.

Response: We appreciate this concern and wish to emphasize that Figure 5G shows that the fluorescent signal is not at all limited to the cellular material that the reviewer refers to as fat body like cells. We have revised Figure 5F-H to include arrows that identify abundant bacteria in the tissue, not at all localized to the fat-body cells. In addition, we have added detail to Figure 5G showing the location of the cross-section (Figure 5 and lines 664-665).

Additionally, the staining conditions were optimized on bacteria growing in HIB in vitro (Ln 479) and does not account for non-specific staining of flea tissue (e.g., fat body cells). Overall, the image quality doesn't allow for conclusive identification of Y. pestis or the localization of the staining within the tissue. Optimally, ovarioles that were not subject to Triton X-100 permeabilization step could have helped to distinguish between surface associated and internalized Y. pestis.

Response: Confocal microscopy on tissues and cells is standard for demonstrating localization to the interior rather than surface. As stated above, we have revised figure 5F-H to illustrate more clearly the localization of the abundant signal observed within the infected ovarioles.

-Ln 88 – please include reference/s that supports the statement 'consistent with published data'.

Response: We have added the reference and modified the text to clarify the published data that is referred to (lines 90-95, references 19 and 28).

-Ln 185 – how is it known that the bacteria are Gram negative in the TEM images?

Response: Due to the high resolution of the TEM, the inner and outer membranes of the bacteria can be visualized. They are not gram-positive. We have made a minor modification to the Results to point this out (lines 191-192).

-Lns 183-198 For Fig 6 please label the cell types referred to in the text (e.g., follicle cells, seminiferous tubules, basement membrane enclosure, spermatocyte and so on. Please indicate the basement membrane in Fig 6G.

Response: We have added these labels as suggested (Figure 6 and lines 675-677).

-Ln 565-568 Can the authors please define 'early' aggregation as they are evaluating fleas collected from Days 1 to 14 for this experiment as per Fig 1 legend? Does this refer to early phase transmission or other phenomena? Additionally, in the response to Reviewer three comments, the authors refer to 'aggregation in the early phase' which apparently encompasses days 1 to14 as per Fig 1 legend. Currently the literature indicates that Y. pestis colonizes the proventriculus within 24h

Response: We agree with the Reviewer on these points and regret that the text was unclear. Proventricular *Y. pestis* has been published to occur within 24 hours when using an artificial feeder with rat blood, and more intense fluorescence was seen on day 3, giving the appearance of partial or full proventricular blockage. The results presented in figure 2 agree with the published data. Additionally, aggregation is a generic term that we used to distinguish dispersed bacteria from those that appear as an aggregate or colony. We have defined "early aggregation" in the revised text and also modified the wording to better describe the interpretation; in addition, we have modified the figure 1 title to clarify the interpretation of these data (lines 88-90; line 105; line 602, lines 607-608).

-Ln 116-118 Please provide the references for 'published reports' mentioned here that 'bacterial aggregation in the foregut occurs around Day 3 post-infection and then subsides until biofilm-mediated aggregation occurs' and please clarify what this statement means in light of current literature on the subject. This comments also reflects on the title for Fig 2 'Aggregation of Y. pestis in X. cheopis peaks on day 3 post-infection.' Y. pestis aggregates in the proventriculus within 24h and bacteria are also noted to multiply in that time period (see Fig 1 D in https://doi.org/10.1371/journal.ppat.1006859 and see Fig 2

https://doi.org/10.1371/journal.ppat.1009995) and is the current paradigm for bacterial colonization of the proventriculus.

Response: Thank you for providing this comment. We have made several modifications the text to improve our discussion of data interpretation. We did not measure biofilm-dependence or independence and were referring only to previously published transmission data. As this point is not necessary for data interpretation, as we removed reference to biofilm-dependence in this section of the revised manuscript (lines 105, lines 122-123, lines 619-620).

-On occasion the bacterial colonization extends into the midgut or bacteria are observed around the proventriculus stomodeal region. It is therefore important to include in the methods for Fig 2, how a predefined set of criteria were established for RFU data collection for the PV and midgut to improve objectivity in the analysis.

Response: We have added detail to the methods that clarifies the predefined criteria for RFU data collection (lines 508-510).

-Ln 621 – Please describe clearly what the control refers to in Ln 621. How many eggs were collected from uninfected controls?

Response: We have added this detail as suggested and the information has been included in the summary table. In Figure 5, we analyzed a total of 20 eggs from uninfected controls (n=4 control samples per trial). The uninfected control eggs were stained at the same time as the infected eggs. They do not stain with the anti-Yersinia antibody and have low autofluorescence. This information has been included in Extended Data Table 1 of the revised manuscript.

-Extended Data Fig 2 requires a non-infected control image.

Response: We did not include the non-infected control images for the data showing *Y. pestis* in reproductive tissues 15 weeks after infection, as this background fluorescence was documented in the negative samples represented in Figure 3 and Extended Data Figure 1.

-Additionally, with regards to DNA sequence evidence of Y. pestis in the flea tissue, can the authors address information as to whether any Rickettsial DNA was found. For example, it is known that Rickettsia felis can survive in laboratory reared flea colonies, have been found associated with rat fleas naturally, and are able to be transovarial transmitted. Additionally, the bacteria is approx. 1.5 micron long similar to Y. pestis (see DOI: https://doi.org/10.4269/ajtmh.1990.43.400) so it would be great to eliminate Ricketssial species as a potential confounding variable in the flea colonies.

Response: The DNA sequencing was conducted on bacteria harvested from a plate. As *Rickettsia* is an obligate intracellular bacterium, it would not be alive after plate isolation, is not expected to be present in the sequence, nor was it or any other bacteria observed to grow on the non-selective

agar plates used to isolate *Y. pestis* from eggs and other developmental stages of fleas. Likewise, the bacteria identified in the male fleas by TEM are extracellular and therefore cannot be *Rickettsia*. Nevertheless, we agree with the Reviewer that *Rickettsia* or other bacteria could be present in the midgut or reproductive tissues of *X. cheopis*. In preliminary experiments, by PCR, we have not been able to amplify *Rickettsia* 16S rRNA; however, deep sequencing picked up a low *Rickettsia*-specific signal in our laboratory *X. cheopis* flea colony that has not yet been validated. We are continuing to investigate this question, as the presence of *Rickettsia* could impact *Y. pestis* colonization of the reproductive tissues.

We have added labels to the TEM images to emphasize the structures and believe this helps to clarify the localization patterns. In addition, we have updated the Discussion section to provide clarity in the interpretation of the TEM data (Figure 6, lines 295-303).

After all, fewer control samples were used in studies. In fact, the data, and details about uninfected controls in terms of numbers is less than those for infected test samples. This is important especially because transovarial transmission is estimated to be at a 50% occurrence, so findings in uninfected controls may have been missed by not evaluating enough of such samples (e.g., Fig 7 have 8 infected and 3 uninfected samples).

Response: We appreciate this comment and at the suggestion of Reviewer 2, we have prepared a summary table in **Extended Data Table 1** to clearly represent the power of the study. There is no evidence that the larvae display significant autofluorescence, as we have examined 3 larvae that were reared from fleas that were not infected, with no detectable signal in the red/far red range of the excitation/emission spectra whereas autofluorescence (when it occurs) is readily detectable in every sample. Further, we report that nearly half of the infected larvae were negative, while 5 of 8 of the infected larvae had distinct fluorescent signal that appeared to be abundant and in a similar compartment. In addition, we carried out confocal examinations of larvae from 3 independent infections of the parent fleas, and each trial produced similar results. Additional power is added when we consider that live bacteria were recovered from a similar proportion of larvae (eg. Half of the group), and that the later stages (pupae and F1 adults) also carried *Y. pestis*. Therefore the results consistently support the conclusion that viable *Y. pestis* is transmitted transstadially between life stages of the developing flea.

-In relation to the comment above, please provide in the discussion information on localization of R. felis in the reproductive tissue and how these overlaps, or differs, with the localization of Y. pestis in the reproductive tissue presented here.

Response: The most significant difference is that *Rickettsia spp.* are obligate intracellular and cannot be found in extracellular spaces in the tissue. In the testes, *Y. pestis* appear to be extracellular in the seminiferous tubules. In addition, we observed intracellular *Y. pestis* in the oocytes and follicle cells of female fleas which are distinct from the published reports of *Rickettsia* (for example, *Y. pestis* are vacuolar, whereas *Rickettsia* escapes the vacuole). In addition, we reviewed the published data for *R. felis* and found distinct differences visualized by TEM and immunofluorescence microscopy. We have expanded our previous discussion of this

point in the Results and Discussion sections of the revised manuscript (lines 203-205, 289-293, lines 295-303).

Reviewer #2 (Remarks to the Author):

I appreciate the effort made by the authors to address reviewers' comments, including mine. The manuscript has significantly improved in clarity, and the incorporation of new experiments has solidified the research hypothesis. I want to commend the authors for their dedication in executing each step of these experiments, which undoubtedly demanded a considerable amount of patience and expertise.

I have only minor comments and recommendations:

Please ensure that references are added for any mention of data from previous research, as noted in Lines 88, 117, and 255.

Response: Thank you for your comments and suggestions. We have added references for the statements made in lines 88 and 255, as these statements referred to data that was discussed in the introduction. For line 117 (referring to published data), we removed the reference to published data, as it was redundant to the discussion presented earlier in the paragraph and did not provide additional information (lines 90-95, references 19 and 28; lines 264-267).

It would be beneficial to include a summary table detailing the number of samples processed for each experiment and the number of samples with positive outcomes. Using precise numbers instead of approximations would enhance clarity. For instance, in Figure 1, provide the exact number of midguts dissected per blood type and per day post-infection (dpi).

Response: Thank you for this suggestion. We have added a summary table with this detail (Extended Data Table 1).

Reviewer #3 (Remarks to the Author):

Transovarial transmission of Yersinia pestis in its flea vector, Xenopsylla cheopis (revised) Pauling et al.

In this revision, the authors have addressed the reviews and added additional details and discussion. They have convincingly documented some very interesting observations, which I believe warrant acceptance for publication. This work should inspire future investigation into the role, if any, of the detected transovarial transmission in the ecological maintenance of plague. There remain a few issues that could be additionally clarified:

1. The second sentence of the Discussion mentions quantitative data, but what I was looking for were data that quantified the percentage of infected females that laid infected eggs, the

percentage of positive larvae from infected eggs, the percentage of positive pupae from infected larvae (or infected eggs) and the percentage of F1 infected adults that arose from infected pupae, larvae, or eggs. In other words, some indication of the efficiency of transovarial and transstadial transmission. The new information in the figure legends does not adequately address this. I could find that about 50% of infected fleas had bacteria in the genital tract (Fig 3 legend). The same data should be provided for eggs laid by infected fleas (How many of the 14 eggs tested in Fig. 4 were positive; how many of the 25 eggs tested in Fig. 5 were positive). How many of the 5-10 fleas tested in Fig. 6 were positive. How many larvae were examined for Fig. 8 (the legend states 8 were positive, but out of how many examined?). How many pupae were examined for the extended data Fig. 3, and how many were positive? The new information in the Fig. 8 legend indicates that 2 of 3 F1 progeny were positive, but only one was examined for Fig. 8B,C? For these percentages, it would be best to rely on the direct microscopic evidence of infection, because the samples were not surface-sterilized (only washed) before plating for CFU counts.

Response: Thank you for your comments and suggestions. This information, also requested by Reviewer 2, has been added to the summary table for improved clarity (Extended Data Table 1).

2. The Abstract states that evidence was found that may account for sustained, low-level infection of a flea population via transovarial transmission and that the evidence implicates vertical transmission as a mechanism capable of sustaining Y. pestis in the environment for extended periods. I think these statements are overly strong and urge more caution because that was not examined in this study, and perhaps it would be better to say that the results suggest a potential role rather than implicate. Those reading only the Abstract may jump to the conclusion that this has been established and overinterpret the ecological significance of the present results. Ecological significance would only be concluded by examining how many of the infected F1 progeny went on to develop a chronic, transmissible infection (following a cohort of F1 fleas to determine what percentage developed a heavy proventricular infection); and what percentage of F2 progeny of the original infected fleas were infected. The authors make that point in the last paragraph of the Discussion, stating that this was not examined. They state that it seems probable that the positive F1 fleas would maintain and transmit, but it remains to be seen if the transovarial transmission they document is just a curiosity or if it has real ecological significance. Since only newly-emerged F1 adults were examined and they contain only few bacteria (~170 CFU), my suspicion is that, although possible, continued infection would be rare and these fleas would eliminate the infection quickly once they were allowed to feed. I base this on a previous study (JID 2005 191:1907-1912) that showed that 94% of fleas that fed on blood with a low bacteremia level such that they ingested only about 1,000 Y. pestis CFU rapidly cleared the bacteria. Hopefully these issues will be addressed and resolved by this group or others before it becomes entrenched in the literature that transovarial transmission is ecologically significant in plague maintenance.

Response: Thank you for this comment. We agree with these many unknowns and continue to address them in our research. We have revised the abstract and expanded the discussion as suggested (Lines 26, 27; lines 305-307, lines 316-317, 323-332).

3. Other minor points of consideration:

-How were the pupa samples handled- dissected from their cocoons and PBS-washed? I did not find it in the methods.

Response: Thank you for pointing this out. We have added this detail to the methods (lines 494-498).

-Direct spread from the digestive tract to the genital tract is strongly inferred as the mechanism, but no evidence for this is presented, and no bacteria were reportedly detected in midgut epithelia. Infected fecal contamination of the male aedeagus (the long elaborate penis apparatus) during the complex flea mating process could serve to introduce bacteria into both the male and female reproductive tract and is another plausible and simpler mechanism for what appears to be rapid and targeted spread to reproductive tissues. The mechanism of dissemination is a matter of speculation at this point.

Response: We appreciate this comment. Upon review of published data related to mechanisms of transovarial transmission of microbial pathogens, we found spread from the digestive tract was commonly reported. While a fecal-contaminated aedeagus theoretically could transfer *Y. pestis* to the ovarioles, it would not cause colonization of the seminiferous tubules in the males, nor the spermatheca in the females, or the Malpighian tubules. These observations support a more likely model of spread from the digestive tract. We have expanded our discussion of the localization of *Y. pestis* in the male and female reproductive tissue and have indicated that experimental validation of the model is needed (lines 289-314).

Reviewer #1 (Remarks to the Author):

I thank the reviewers for responses to the numerous questions and clarifications that were requested by reviewers. These appear to be intricate studies and the work is much appreciated. I believe this study now more thorough details experimental detail on the observation of the plague bacterium in reproductive tissues.

However, I do respectfully disagree with the explanation that ethanol washes are sufficient to remove bacteria stuck on the surface of interior tissues (e.g., ovarioles) dissected from fleas in the immunostaining experiment. While ethanol might kill bacteria, it doesn't necessarily remove bacterial material including bacterial proteins that the antibody recognizes. But OK I will let that go because I can imagine that immunostaining sections will require some time to optimize as alluded to by the authors.

Reviewer #2 (Remarks to the Author):

The research presented is groundbreaking and will significantly enhance our understanding of plague transmission and maintenance in the environment. I would like to extend my gratitude to the authors for thoroughly addressing all comments and providing new, insightful data. The addition of tables showing the number of experiments is particularly appreciated. Although the number of replicates is somewhat low, I believe that this study merits publication due to its substantial contributions to the field.
It is also interesting to note that vertical transmission is not influenced by the blood source. It would be beneficial if the authors could add a few words in the discussion to hypothesize why this might be the case.

Reviewer #3 (Remarks to the Author):

[No comments for author]

Response to the Reviewer Comments

Reviewer #1 (Remarks to the Author):

I thank the reviewers for responses to the numerous questions and clarifications that were requested by reviewers. These appear to be intricate studies and the work is much appreciated. I believe this study now more thorough details experimental detail on the observation of the plague bacterium in reproductive tissues.

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Response: We appreciate the Reviewer's positive comments, as well as the comment related to ethanol. We agree that ethanol would not remove biological material present on the bacteria, whether on the interior or exterior of the sample. Following ethanol, the eggs were also extensively washed with PBS. In addition, the 3D cross-sectional z-stacked images provide supporting data that the bacteria that are shown in the image are located in the interior of the tissue. As this information was already present in the text, no additional changes to the text have been made related to this issue.

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It is also interesting to note that vertical transmission is not influenced by the blood source. It would be beneficial if the authors could add a few words in the discussion to hypothesize why this might be the case.

Response: We appreciate the Reviewer's positive comments and helpful suggestions. We have revised the Discussion as suggested with a hypothesis for vertical transmission based on the observations of blood source and kinetics (lines 310-316).

Reviewer #3 (Remarks to the Author):

[No comments for author]