nature portfolio

Peer Review File

Trypanosoma congolense manipulates the chemosensory system and behavior of Glossina pallidipes to enhance its transmission

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study aims to gain knowledge on how T. congolense parasites may shape G. pallidipes sensory modalities as well as the mammalian host chemistry that enhance its transmission efficacy.

Major comments:
This is a very interesting research question but, in my view, the rationale of the study and its experimental design is not well explained and the experimental approach is patchy and lacks coherence. Moreover, some of the experimental set-ups lack clarity and seems to have flaws which questions the robustness of the generated data and related conclusions. Moreover, I have the impression that the authors systematically overinterpret the data/results not fully supported by a robust underlying data-set.

Line 120- 139. Glossina pallidipes interrupted feeding experiment: This experiment is highly unclear in its rationale, set-up and relevance in the context of this paper and how it links with the main hypothesis to be addressed. Reference is made to Muita et al., 2025 but not clear why; this reference is not taken up in the reference list. It is stated that the authors wanted to verify the maximum number of hosts that a single G. pallidipes fly can infect during an interrupted feeding cycle, mimicking host defense using a mouse model. What is meant here? Is this to verify whether mechanical transmission occurs when the fly feeding on a trypanosome-infected host is interrupted and jumps from one host to another. Please clarify. A single fly was allowed to feed on a maximum of five mice, consisting of one T. congolense-infected and four healthy mice restrained individually. Each fly was offered one minute/mouse: did the authors check whether the fly was actually probing during this short time period on each individual mouse? I guess a non-infected fly is used in this experiment. If not, and an infected fly is used, it is not clear from the Materials and methods how the authors selected for a metacyclic-infected G. pallidipes without dissecting it. This whole section needs to be rewritten to make the rationale and methodology more clear and how it fits within the paper.

Line 150-167: Headspace volatiles of T. congolense and uninfected mice. to enable us to efficiently conduct laboratory infections and feeding experiments. Not clear what the authors mean here. How will the headspace volatiles will help to efficiently conduct laboratory infections and feeding experiments? Please revise.
A single mouse per trial was placed inside a volatile collection glass jar during 12 hours to capture the volatiles. This seems to me very stressful for the mouse being isolated in a glass jar for 12 hours. Will this stressful situation not affect significantly the volatiles that are ‘released’ by the animals? It is not clear whether the control mice are truly controls that can be compared with: uninfected mice of the same age/sexe/ as the T. congolense-infected mice and that were maintained under similar conditions and where the volatile-capturing occurs at the same time/place as the infected mice. What is the observed variability in the volatile profiles within the ininfected and T. congolense-infected mice. Figure 1A en B only shows the profile of one individual each.

Line 170-190: Laboratory behavioral assays. First assay: small cage (10 cm × 10 cm × 15 cm) with a both sides a restrained mouse (infected versus non-infected). It is not clear from the description and data whether this experimental set-up was first validated for reproducibility and variability by using i) at both sides a non-infected mouse and ii) at both sides an infected mouse? In both case you would expect that there are no significant differences in the response index at both sides.

Related to this set-up, could an increased body temperature and/or CO2 production by the infected mouse (this animal has an increased respiration and body temperature due to severe parasite-induced sickness) result in more fly attraction compared to the uninfected mouse irrespective of other volatiles. I think the used experimental set-up cannot exclude for this possibility. For the urine testing, 20 G. pallidipes/ cage were used in a sex ratio 1:1. It is not clear why in this test both males and females were used whereas in the 'mouse' test nothing is mentioned about this. Several questions rises here: 1) why a different fly set-up in both experiments; 2) what is the rationale of having males and females in the same testing; why not testing them separately; I guess having them together in the same cage will induce some behavioural changes that are not linked to the urine component and could compromise the data outcome.

It is not clear from the methodogical section how metacyclic-infected flies were selected prior to the tsetse feeding experiments (for this you need live flies and not dissected ones). As the authors correctly describe in the introduction, G.pallidipes is a highly refractory tsetse fly vector and only very few flies will develop a final metacyclic infection in the mouthparts after an infective bloodmeal. So what is the achieved infection rate and how are metacyclic-infected flies selected without killing them. A T. congolense mouthpart infection does not automatically mean there are metacyclics present.

Line 191-222: Field trapping + dissection + tissue collection & storage in liquid nitrogen.? It is not clear from the text what the rationale /main objectives are for this section. Field flies are captured and dissected for scoring the trypanosome infection. Then, tissues for each different infection-status category were subjected to transcriptome analysis by RNA-seq.
Line 238-205: RNA extraction and sequencing. Total RNA was isolated from only one single female G. pallidipes head tissue. Are these females from the field-collected flies or are they experimentally infected flies? In case of the former, age is determined by the wing fray method so variability in fly age range from 32-50 days. And what about variability in nutritional status. Could these two variables have an impact on the transcriptome outcome which is not related to the trypanosome-infection status? It would be highly complementary to have well-controlled G.pallidipes flies in the lab conditions (with a different infection-status) to incude in this RNA-seq approach. Here, potential variables such as age and nutrionational status that could affect the transcriptome are un der control.
Line 336: Data analysis: Transcripts were considered differentially expressed between the comparison pairs if they had a log2 fold >1 and an adjusted p-value <0.05. In Fig. 3 (line 425) most of the differentially expressed genes have extremely high log2 Fold changes. Was there a threshold handled for minimum # of reads for a specific gene? Extreme log2fold changes are often observed for genes that are not or hardly expressed in one or both conditions (e.g. in both conditions there is a low number of gene reads/million total reads for that gene) resulting in unsignificant but high fold-changes if you compare them. E.g. calculating the fold change of a gene with 1 read/million versus 10 reads/million results in a high fold change although the gene can be considered not significantly expressed in both conditions. Therefore often a threshold of a gene read/million is handled of > 50 or even more (to be observed at least in one of the conditions that are compared) to calculate the log2fold changes of significantly expressed genes. Can the authors give more clarifications here for the observed very high log2 fold changes? These basic data are not shown.
For the RNA-seq. data: please show (in supplementary data-table + excel file) the full basic RNA-seq data set and differential expression analysis of the different tissues and fly groups

Minor comments:

Line 103-119: Not clear what the relevance this information is for the paper. It mainly describes how mice are infected with a cryostabilate of T. congolense to make new stabilates.

Line 140-149: Laboratory fly infection and feeding experiment. This paragraph describes how the flies were given a T. congolense infected bloodmeal (on infected mice) and were dissected to determine the infection status. It is not clear i) what the feeding experiment is referring to and ii) what the tsetse infection data after dissection are used for. Please revise.

Line 309. On feeding days, each infected fly infects up to 3 cattle and increases to 5 after 21 days post-infection to represent elevated aggressiveness. Is this evidence-based – on which data is this based? Please give a reference.

Fig.2 A. > variation in the GMF group? B. Please explain SRP_RNA in the legend

410-412: More genes are expressed within the vector when the trypanosome breaks midgut barriers to colonize the midgut, compared to the expression during its progression to the mouthpart metacyclic stage? What do the authors mean with 'breaking' the midgut barriers; what are these midgut barriers that a trypanosome has to 'break?

421-423: Most genes that were initially upregulated to promote T. congolense midgut establishment became downregulated in the GMF vs GOF comparison, suggesting a shift toward resource conservation that enables a more balanced co-existence between the parasite and the vector? shift to discussion; not clear on which basis this is stated?

Reviewer #2

(Remarks to the Author)
Dear Dr. Stortz,

The manuscript #COMMSBIO-25-10906-T entitled "Trypanosoma congolense manipulate the chemosensory 1 system and behavior of Glossina pallidipes to enhance their transmission" authored by Tawich and colleagues addresses an important question in vector neurobiology and ecology: Are parasites capable of modulating vector's nervous system and behavior in

order to improve transmission? The authors addressed this question using a series of experiments encompassing GC-MS, parasite detection, RNA-Seq, qRT-PCR, lab and field behavior, and mathematical model. In my evaluation, the experimental design and the results presented proved the main hypothesis of the manuscript: tryps manipulation of brain gene expression and host-seeking behavior. On the other hand, authors included experiments that are off-topic, diverting from the manuscript's main question. Besides that, there are concerns regarding data analysis and interpretation that need to be addressed by the authors before the manuscript be considered for publication in Commons Biology. Therefore, my recommendation is for Major Revision.

Major Concerns:

Lines 388-393: Mapping statistics and Fig 2b: This has not biological meaning for the manuscript's hypothesis. It should be transferred to the methods or authors can write a specific results' subsection pertaining to the sequencing results (#reads, mapping, filtering).
Lines 416-418: Authors need to state (1) What is the information that supports this statement? (2) Which genes are those? (3) Why they facilitate parasite progression?
Lines 419-424: Authors need to describe/mention in text (and/or figure 3) the putative functions of such genes. Access number only does not suffice. Tables comprehensively describing the expression levels and functions of these genes need to be provided
Figures 2 and 3: Tables comprehensively describing the expression levels and functions of these genes need to be provided.
Lines 458-463: Even though these genes were differentially regulated, this discussion about gene expression supporting parasite development is off-topic. Authors should focus on neuro-receptos/transmittors/modulators, as the hypothesis of the manuscript inquiries about such classes of genes.
Lines 502-502: I don't believe the qRT-PCR validates the RNA-Seq experiments as these used different experimental designs. The experimental set up as well as the comparisons in figure 6 are not the same as in Figure 5. Therefore, this is not a fair validation. RNA-Seq addressed the effects of parasite development and localization in head gene expression. qRT-PCR addressed the effects of parasite load in head gene expression. These independent experiments are not overlapping, answer different questions, and should be discussed as such independently.
In that regard, Lines 529-531: This explanation has no scientific basis - low-transcript abundance and normalization effects does not affect gene expression output.
Figure 6: Authors should revisit these results/raw data/analyses. It is unusual to see every single tested gene being up-regulated in high infection. The libraries showed a more variable pattern of genes being up or down-regulated. Authors should choose two unrelated genes (serina protease and aconitase, for instance) and run the qRT-PCRs as controls.
Lines 537-576: These phylogenetic analyses do not fit in this manuscript pertained to the demonstration that parasite infection affects sensory system-specific gene expression and host-seeking behavior. Authors should use this information in another manuscript dealing with evolution of such genes.
In that regard: Statement in lines 549-553 - I don't see the point of building phylogenetic trees to explain gene expression results.
Lines 590-593: A heatmap figure (or make a figure) with these RH gene results should be included.
Lines 597-599: Again, qRT-PCR and libraries experiments were not designed in the same way. Therefore, they should not be comparable. Authors should describe them as independent results for the questions they address based on specific experimental designs.
Lines 623-642: Experiment with engorgement and parasite transmission is off-topic in a manuscript designed to investigate vector neuromodulation by parasite. The experimental design does not encompass any comparative condition of differential feeding between infected and uninfected tsetse. This is another misplaced subsection that does not address the hypothesis of the manuscript. It should be removed.
Lines 650-653: Are these differences (fig 9A) statistically significant? If so statistical test and p-values should be indicated for each comparison. If not significant, it should also be stated.
Lines 662-663: Are these differences (fig 9B) statistically significant? If so statistical test and p-values should be indicated for each comparison. If not significant, it should also be stated.
Lines 665-672: All these results (trap catches) do not address the main hypothesis of the manuscript and should be removed (publish elsewhere).
Lines 768-772: I believe the authors implied that the tsetse HDAC can influence gene expression of Tryps. This is misleading and should be removed.
Lines 773-794: All this discussion has no place in the manuscript as it does not deal with neuromodulation of sensory systems by tryp infection. This should be removed, along with the correspondent result's subsection

Minor Concerns:

Lines 377-378: Sentence "After filtering at 0.5 count per library, 377 retained data were normalized by EdgeR within the iDEP platform" should be transferred to Methods section.
Lines 404-405: Authors need to mention that the differentially expressed genes of tsetse's heads are on display.
Lines 445-448: Reference #53 is related to midgut expressed genes. This is not a good comparison with head/brain gene expression. Author could compare results with other brain transcriptomes of vectors infected with pathogens, for instance.
Lines 451-452: Reference supporting statement needs to be included.

Reviewer #3

(Remarks to the Author)
This is a well-written manuscript that investigates how Trypanosoma congolense infection modulates Glossina pallidipes chemosensory system and promotes transmission. The strength of the study is that it is multidisciplinary as it integrates transcriptomics, metabolomics, behavioral assays and modeling. The topic is critical as it addresses animal trypanosomiasis in terms of vector biology, parasitology and disease control in sub-Saharan Africa. While the study is novel, innovative and has strong potential of application, the following revisions are proposed and require attention before publication.

1. The rationale as presented in the abstract is clear with the aim emphasizing on the multidisciplinary approach. The result section of the abstract didn't indicate the quantitative data.
2. The authors presented a strong background on vector-parasite ecology which provided the basis and justification of the study. The hypothesis is specified and the investigation carried out clearly stated
3. The ethical approvals are clearly documented followed by a detailed molecular and behavioral protocols. Format for reference citation in line 131 should be changed
4. The period (months and year) that the field trapping of tsetse flies was done in Kwale County should be specified
5. The sample size for the behavioral assays was limited as 20 replicated is too low and may be under powered for ecological inference and weaken confidence in conclusions
6. There is no evidence of using synthetic compounds to validate the behavioral assays. The authors to clarify why this wasn't done
7. The statistical results should state the False Discovery Rate (FDR) thresholds
8. The RNA-seq and RT-qPCR results are inconsistent as some OR genes show opposite regulation between methods
9. The conclusions on olfaction enhanced by parasite infection, energy metabolism changes linked to parasite development require functional validation and not rely exclusively on transcriptomic

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Although the authors made efforts to improve the manuscript based on the comments of the reviewers, there are still major flaws in the manuscript. Moreover, quite some relevant comments of the different reviewers are only somehow addressed in the rebuttal letter but did not result in improvement/substantial changes in the manuscript. This give me the impression that some highly relevant comments of reviewers are ignored for improving the manuscript.
The whole experimental section on tsetse fly interrupted feeding and trypanosome transmission remains highly unclear in its rationale and set-up. The explanation that the authors give for linking with the main hypothesis of this paper (i.e. olfactory modulation by the parasite infection in the host) is not relevant/satisfactory and is missing a sound scientific logic. Moreover, the experimental set-up remains scientifically unsound. In my view this whole section on fly infection and interrupted feeding should be removed as it makes no sense. It has already been demonstrated in the past that T. congolense infected flies have difficulties to feed because of a mechanical obstruction of the ingested blood flow by the attached parasite rosettes to the chitinous lining of the mouthparts. As tsetse flies are easily disturbed, the probability that a T.congolense-infected fly is interrupted and continue its feeding on another host is increased. This behavioural change by the parasite infection has nothing to do with olfactory modulation. So the infected fly/interrupted feeding experiment included in this manuscript will not allow to make any interpretation/conclusion about a putative link between parasite-induced olfactory modulation and fly behavioural changes that enhances parasite transmission. The paper should focus on the impact of the trypanosome infection in the mammalian host on tsetse fly attraction with the possible involvement of specific odour components modulated by the parasite infection in the host.

Reviewer #2

(Remarks to the Author)
Dear Dr. Johannes Stortz,

The authors carried out most of my suggested modifications, and the manuscript looks very comprehensive, with a broad variety of experiments that enlighten the filed of neurobiology of disease vectors.

I am still hesitant with the "Experiment with engorgement and parasite transmission" as the experimental design does not encompass any comparative condition, i.e. controls (gene knockouts or probing frequency btw infected and uninfected flies), that could be used to infer feeding differences.

Having said that, the figure does not depreciate the quality of the manuscript. So, I will let you make a decision whether or not the authors should remove the figure, as I am satisfied with the current manuscript.

Reviewer #3

(Remarks to the Author)
The paper investigated how Trypanosoma congolense infection changes the chemosensory system and host seeking behavior of Glossina pallidipes, member of the savannah (morsitans) group and primary vector for African trypanosomiasis (nagana). The authors report that the parasite can modify the vector's olfactory and behavioral preferences which modify the host attraction or feeding behavior to favor parasite spread.

Similar findings have been reported for other vector-pathogen systems e.g. malaria parasites in mosquitoes. Such manipulation in African trypanosomes is limited and offers potentially valuable contribution by linking changes in chemosensory system to ecological outcomes.

The authors addressed ALL the concerns raised with point numbers 4 and 8 reviewed as advised by other reviewers.

The study used an interdisciplinary approach integrating vector biology, parasitology and disease ecology, with the findings promising and having great potential. However, with additional experiments on functional validation to support the data, the study could make a solid contribution to control of Tsetse vectors.

Reviewer comment	Response
Reviewer 1: We thank you for the positive critical comments, suggestions that have significantly improved the clarity and connectivity of the story. We have addressed each point raised as detailed in the table below. Changes made are marked in red in the marked up manuscript.	
Line 120- 139. *Glossina pallidipes* interrupted feeding experiment: This experiment is highly unclear in its rationale, set-up and relevance in the context of this paper and how it links with the main hypothesis to be addressed.	Thanks. We have revised our text to clearly incorporate the rationale, experimental logic, and its connection to the main hypothesis. Once a tsetse fly becomes infected, i.e *T. congolense* colonize the mouthparts (proboscis), the fly remains infective for life (Van den Abbeele et al., 1999; Rotureau & Van den Abbeele, 2013; Matetovici et al., 2016.). This means that any behavioral change that increases host contact, such as interrupted feeding may have a cumulative and amplified effect on transmission. Interrupted feeding experiment directly supports our central hypothesis that trypanosome infection enhances transmission potential not only through physiological and sensory changes but also via altered feeding behavior, as previous research has shown, infection alters feeding behavior. By demonstrating that a single infected fly can infect multiple hosts under interrupted feeding conditions, the experiment provides mechanistic and epidemiological evidence that such behavioral changes can significantly increase transmission efficiency. Furthermore, the result from this experiment has practical implications for vector control, suggesting that targeting infected flies, through altered olfactory or behavioral traits-could reduce transmission**.**
Reference is made to Muita et al., 2025 but not clear why; this reference is not taken up in the reference list.	Muita et al., 2025 is now updated and has been included (Ref 37).
It is stated that the authors wanted to verify the maximum number of hosts that a single *G. pallidipes* fly can infect during an interrupted feeding cycle, mimicking host defense using a	Under field conditions, tsetse flies are frequently interrupted during blood feeding due to host defensive behaviors from tsetse bite, through tail flicking, body part movement, or grooming. As a result, the fly may move between hosts before fully

mouse model. What is meant here? (1) Is this to verify whether mechanical transmission occurs when the fly feeding on a trypanosome-infected host is interrupted and jumps from one host to another. Please clarify.	engorged. To mimic this natural scenario under controlled laboratory conditions, we designed an experiment in which a single fly was sequentially exposed to multiple mice for short, fixed feeding periods (1 minute per host).
A single fly was allowed to feed on a maximum of five mice, consisting of one *T. congolense*-infected and four healthy mice restrained individually. Each fly was offered one minute/mouse: did the authors check whether the fly was actually probing during this short time period on each individual mouse?	We agree that confirming active probing/feeding within the 1-minute exposure period is important. We confirmed if flies fed in that 1-minute time by dissecting the flies gut after the fly is fully fed on the last healthy mice. We also used microscopy to ascertain the infection status of mice fed on by each fly and we found 17 out of 20 had active parasites and were able to infect at least one mouse. Flies with active parasites, demonstrated that they fed on infected mice and managed to pick the trypanosome parasites in that 1-minute window time, we have added that information as supplementary file 3: Table S3. Furthermore, if flies could not pick parasite in that 1-minute feeding time, we would not find any infected mouse.
I guess a non-infected fly is used in this experiment. If not, and an infected fly is used, it is not clear from the Materials and methods how the authors selected for a metacyclic-infected G. pallidipes without dissecting it. This whole section needs to be rewritten to make the rationale and methodology more clear and how it fits within the paper.	We used non-infected flies. The flies were fed on infected mice, as we wanted to see how many hosts can potentially be infected by a single fly, so we used tsetse fly fed on infected mice and forcedly interrupted and allowed to feed on healthy mice, on each mouse 1 minute
Line 150-167: Headspace volatiles of *T. congolense* and uninfected mice. à to enable us to efficiently conduct laboratory infections and feeding experiments. Not clear what the authors mean here. How will the	The purpose of analyzing headspace volatiles was to confirm that *Trypanosoma congolense* infection modulates host odor profiles in our mouse model experiment**,** consistent with our previous findings in livestock (Getahun et al., 2022). This experiment was important to validate the mouse as an appropriate

headspace volatiles will help to efficiently conduct laboratory infections and feeding experiments? Please revise.	experimental model for subsequent olfactory and behavioral assays. The volatile analysis demonstrated trypanosome infection-induced odor changes, that impact tsetse-host interaction.
It is not clear whether the control mice are truly controls that can be compared with: uninfected mice of the same age/sex/ as the T. congolense-infected mice and that were maintained under similar conditions and where the volatile-capturing occurs at the same time/place as the infected mice	We confirm that the same mice were sampled before infection and after infection, thus using a paired experimental design**.** This controls for individual variability (e.g., age, sex, genetic background), as each animal serves as its own control. All collections were conducted under identical environmental conditions.
A single mouse per trial was placed inside a volatile collection glass jar during 12 hours to capture the volatiles. This seems to me very stressful for the mouse being isolated in a glass jar for 12 hours. Will this stressful situation not affect significantly the volatiles that are 'released' by the animals?	We acknowledge the concern regarding potential stress effects. To minimize stress, the glass collection chambers were designed to allow continuous airflow of charcoal-filtered air, preventing accumulation of CO_2 and maintaining adequate ventilation. Importantly, both infected and uninfected mice were subjected to identical experimental conditions, meaning that any potential stress-related effects on volatile emissions would be consistent across treatments and therefore unlikely to bias comparative results.
What is the observed variability in the volatile profiles within the uninfected and T. congolense-infected mice. Figure 1A en B only shows the profile of one individual each	Thanks for this useful question. The odor analysis was conducted across three mice biological replicates (each sampled before and after infection)**.** The observed changes in mice volatile profiles were consistent qualitatively across replicates, particularly for key compound (Dihydro beta ionone) associated with *T. congolense* infection status in cattle, but varies in relative abundance from 0.98, 4.8 to 11%, which may depend how the host reacts to the infection. We infected 4 mice 3 of them showed the target compound, but one did not.
Line 170-190: Laboratory behavioral assays. First assay: small cage (10 cm × 10 cm × 15 cm) with a both sides a restrained mouse (infected versus non-infected). It is not clear from the description and data whether	Thanks for this and we have now clarified it better. The assay we used is a free-flight cage bioassay**,** which differs from a Y-tube olfactometer in that flies are not constrained to a directional choice but can move freely

this experimental set-up was first validated for reproducibility and variability by using i) at both sides a non-infected mouse and ii) at both sides an infected mouse? In both case you would expect that there are no significant differences in the response index at both sides.	and respond naturally to host cues. This reduces the likelihood of positional bias. The positions of infected and uninfected mice were randomly assigned between trials. Twenty replicates were conducted to account for variability.
Related to this set-up, could an increased body temperature and/or CO2 production by the infected mouse (this animal has an increased respiration and body temperature due to severe parasite-induced sickness) result in more fly attraction compared to the uninfected mouse irrespective of other volatiles. I think the used experimental set-up cannot exclude for this possibility.	We agree with the reviewer, we cannot rule out this, and we acknowledged that in our manuscript.
It is not clear from the methodogical section how metacyclic-infected flies were selected prior to the tsetse feeding experiments (for this you need live flies and not dissected ones). As the authors correctly describe in the introduction, *G. pallidipes* is a highly refractory tsetse fly vector and only very few flies will develop a final metacyclic infection in the mouthparts after an infective bloodmeal. So, what is the achieved infection rate and how are metacyclic-infected flies selected without killing them. A T. congolense mouthpart infection does not automatically mean there are metacyclics present.	We did not select metacyclic flies to use in the feeding experiment but instead we used non-infected flies fed on infected mice. With our attempt to get metacyclic stage we only got 1 out of 200 *G. pallidipes* at 21 days, demonstrating the high refractory nature of *G. pallidipes*, thus we mimicked the process by allowing teneral *G. pallidpes* to feed on infected mice for 1 minute and test to transmit mechanically to multiple healthy mice
Line 191-222: Field trapping + dissection + tissue collection &	The main objective is to study how trypanosome infection and its parasitemia

storage in liquid nitrogen.? It is not clear from the text what the rationale /main objectives are for this section. Field flies are captured and dissected for scoring the trypanosome infection. Then, tissues for each different infection-status category were subjected to transcriptome analysis by RNA-seq.	level modulate sensory pathways of *G. pallidipes*. Field-collected *G. pallidipes* were used in this study for head-transcriptomic analysis. The main rationale of this section is to correctly identify the flies having mature and immature stages of trypanosomes in different tissues. This is only possible after conducting field-trapping, dissection and microscopic examinations. The total RNA was extracted from the collected samples in triplicates and processed for RNAseq.
Line 238-205: RNA extraction and sequencing. Total RNA was isolated from only one single female *G. pallidipes* head tissue. Are these females from the field-collected flies or are they experimentally infected flies? In case of the former, age is determined by the wing fray method so variability in fly age range from 32-50 days. And what about variability in nutritional status. Could these two variables have an impact on the transcriptome outcome which is not related to the trypanosome-infection status? It would be highly complementary to have well-controlled *G. pallidipes* flies in the lab conditions (with a different infection-status) to include in this RNA-seq approach. Here, potential variables such as age and nutritional status that could affect the transcriptome are under control.	We agree with the reviewer that controlled laboratory infections would have been better. However, in our experiment only ~1 out of 200 experimentally exposed flies progressed to the metacyclic (infective) stage, making it extremely difficult to obtain sufficient biological replicates for robust transcriptomic analysis. Given this challenge, we used field trapped flies, which naturally encompass successful parasite development under ecologically relevant conditions including those shaped by environmental, host-derived, and physiological factors. We got flies with various stages of parasite, parasitemia load we also used control from the same ecologies. These are field-collected females, age was determined using wing fray a method recommended by FAO, we acknowledge that field-collected flies may vary in age (approximately 32–50 days) and nutritional status due to feeding on diverse hosts, and these factors could potentially influence the transcriptome independently of trypanosome infection. However, both infected and un-infected (control) flies were collected from the same ecology and were processed under identical conditions. Therefore, environmental and physiological variability, including age and nutritional status, are expected to be randomly distributed across groups. In addition, downstream analyses were based on normalized expression values, which helps

	mitigate systematic variation between samples.
Line 336: Data analysis: Transcripts were considered differentially expressed between the comparison pairs if they had a log2 fold >1 and an adjusted p-value <0.05. In Fig. 3 (line 425) most of the differentially expressed genes have extremely high log2 Fold changes. Was there a threshold handled for minimum of reads for a specific gene? Extreme log2fold changes are often observed for genes that are not or hardly expressed in one or both conditions (e.g. in both conditions there is a low number of gene reads/million total reads for that gene) resulting in unsignificant but high fold-changes if you compare them. E.g. calculating the fold change of a gene with 1 read/million versus 10 reads/million results in a high fold change although the gene can be considered not significantly expressed in both conditions. Therefore, often a threshold of a gene read/million is handled of > 50 or even more (to be observed at least in one of the conditions that are compared) to calculate the log2fold changes of significantly expressed genes.	During the transcriptome analysis, counts were filtered to remove the genes with zero or N/A which introduces elevated noise signals. The extreme fold changes were observed due to high sample variability between and within samples (as shown in the PCA plot) and this as well might have been contributed to by the lower replicate number since we used only triplicates. Few samples had missing counts in at least one out of the three groups. All the obtained reads per sample were normalized according to their library sizes and the reads for each identified gene therefore effect reads of a gene. Re-analysis involved the use of stringent filtering but due to our low sample size of three per group resulting in only nine samples, we did not remove any outliers. Supplementary data containing the list of affected genes per group are attached as additional supplementary excel files 5 and 6. Prior to differential expression analysis, we applied a filtering threshold of 0.5 counts per million (cpm) per library, retaining only genes expressed above this level across samples. This step was implemented to remove very low expressed genes that could otherwise inflate fold-change estimates. In addition, the differential expression analysis was performed using the edgeR framework**,** which incorporates normalization and dispersion estimation to account for variability

Can the authors give more clarifications here for the observed very high log2 fold changes? These basic data are not shown. For the RNA-seq. data: please show (in supplementary data-table + excel file) the full basic RNA-seq data set and differential expression analysis of the different tissues and fly groups	in low-count data and reduces the likelihood of false positives arising from low read counts. Interpretation of high log2 fold changes**:** We acknowledge that some genes still display high log2 fold changes. These are primarily driven by large relative differences between conditions, and in some cases may still involve genes with relatively low absolute expression levels. We have now clarified this point in the manuscript and advise cautious interpretation of such genes.
Line 103-119: Not clear what the relevance of this information is for the paper. It mainly describes how mice are infected with a cryostabilate of *T. congolense* to make new stabilates.	We have improved the relevance of the information provided for clarity. It's now detailed on how the parasites were multiplied in mice model, cryopreserved and used for laboratory infection experiments.
Line 140-149: Laboratory fly infection and feeding experiment. This paragraph describes how the flies were given a T. congolense infected bloodmeal (on infected mice) and were dissected to determine the infection status. It is not clear i) what the feeding experiment is referring to and ii) what the tsetse infection data after dissection are used for. Please revise.	Section revised to increase clarity and confirm using field-collected flies. The feeding experiment we are referring to here is the procedure where the flies obtain a meal from an infected mice and checked for progression of parasites in its midgut to the mouthpart regions. Tsetse fly laboratory-infection data is only useful after dissection to guide in determining the trypanosome infection rates in the flies. We aimed to do the experiment under controlled experiment but *G. pallidipes* were highly refractory, only 1 out of 200 showed infective stage.
Line 309. On feeding days, each infected fly infects up to 3 cattle and increases control 21 days post-infection to represent elevated aggressiveness. Is this evidence-based – on which data is this based? Please give a reference.	In our interrupted feeding experiments, a single infected *Glossina pallidipes* was able to transmit trypanosomes to up to three hosts within one feeding cycle. We attempted to extend this by offering additional hosts; however, after feeding on three mice, flies were fully engorged and no longer attempted to feed. This physiological limitation constrained our experimentally observed upper bound, which may depend on the host used. Nevertheless, published studies for example, Thuita et al. 2008 reported a single fly infected nine vervet monkeys. Based on

	this broader evidence, we incorporated a conservative upper limit of five hosts per feeding cycle in the model at later stages of infection (≥21 days post-infection, at time trypanosomes colonize mouth part), reflecting increased probing persistence of infected tsetse, infection-induced behavioral changes host defensive behavior, while remaining well below the maximum reported in Thuita et al. 2008. This choice balances our experimental findings with published evidence, while avoiding overestimation of transmission potential. Supporting data are supplied as supplementary file 3: Table S3)
Fig.2 A. > variation in the GMF group? B. Please explain SRP_RNA in the legend	There was no clear variation between GMF and GOF clustering (infected groups) as shown PCA in figure 2 whereby these two trypanosome-infected groups clustered together whereas the control group of CFs grouped uniquely into one cluster. The non-uniform or observed wider variability in how the infected groups clustered can be explained by the degree of their infection status due to variations in vector's defense mechanisms or permissibility to trypanosome establishment. This is corrected and SRP_RNA in the legend stands for Small Recognition protein _RNA. The figure has been updated and section B removed from this section, but mapping statistics are incorporated in methods section.
410-412: More genes are expressed within the vector when the trypanosome breaks midgut barriers to colonize the midgut, compared to the expression during its progression to the mouthpart metacyclic stage? What do the authors mean with 'breaking' the midgut barriers; what are these midgut barriers that a trypanosome has to 'break?	Thanks for your question: Breaking the midgut barriers in this context mean overcoming the self defense mechanism put in place by the vector against parasite establishment in its gut. Understanding tsetse fly's immune barrier in the midgut forms an integral part of vector biology with the semi-permeable chitinous layer Peritrophic matrix (PM) and cardia. These structures form immune barrier, and the parasites must cross it to progress to mouthpart or salivary gland. We have clarified in the revised manuscript
421-423: Most genes that were initially upregulated to promote *T. congolense* midgut establishment became	Moved to discussion section as advised.

downregulated in the GMF vs GOF comparison, suggesting a shift toward resource conservation that enables a more balanced co-existence between the parasite and the vector? à shift to discussion; not clear on which basis this is stated?	
Reviewer 2: We thank reviewer for his/her positive critical comments, suggestions that have significantly improved the clarity and connectivity of the story. We have addressed each point raised as detailed in the table. Changes made are marked in red in the marked up manuscript	
Lines 388-393: Mapping statistics and Fig 2b: This has no biological meaning for the manuscript's hypothesis. It should be transferred to the methods or authors can write a specific results' subsection pertaining to the sequencing results (#reads, mapping, filtering).	Figure deleted and related data deleted.
Lines 416-418: Authors need to state (1) What is the information that supports this statement? (2) Which genes are those? (3) Why they facilitate parasite progression?	We have labelled the volcano plot by replacing the gene ids with the actual gene names for our genes of interest. The downregulated genes are labelled in blue and upregulated ones labelled in red. A combination of these genes might be associated with increased motility, and an example is downregulation of peptidoglycan recognition protein, a major component of tsetse fly innate immunity and has also been reported as trypanocidal and thus its downregulation is associated to enhanced trypanosome susceptibility. We have updated the result section to incorporate the said genes and attached a supplementary file with the selected and their functions (Supplementary file 6: Table S6).
Lines 419-424: Authors need to describe/mention in text (and/or figure 3) the putative functions of such genes. Access number only does not suffice. Tables comprehensively describing the expression levels and functions	Differential expression results with tables showing the level of expression are provided as supplementary file 5: Table S 5 with their respective gene ids, and the putative functions of the select genes of interest in this study supplied as supplementary file 6: Table S6.

of these genes need to be provided. Figures 2 and 3: Tables comprehensively describing the expression levels and functions of these genes need to be provided.	
Lines 458-463: Even though these genes were differentially regulated, this discussion about gene expression supporting parasite development is off-topic. Authors should focus on neuro-receptos/transmittors/modulators, as the hypothesis of the manuscript inquiries about such classes of genes.	We included the differentially expressed non-sensory genes because they provide important physiological context that may indirectly influence sensory function and host-seeking behavior. Trypanosome immune evasion strategy is vital in understanding tsetse behavior. We correlated based on behavioral experiments conducted that parasites shift vector's odor sensitivity and signal transduction affecting the expression levels of the olfactory receptors differently. This justifies our choice to describe the immune effectors described in this section. For example, parasite establishment and persistence require substantial host resource allocation, which may alter energy balance and neuromodulatory signaling, both of which are closely linked to feeding motivation and responsiveness to host cues. Such systemic effects can modulate the sensitivity or responsiveness of olfactory and other sensory systems, even if the genes themselves are not directly involved in sensory reception.
Lines 502-502: I don't believe the qRT-PCR validates the RNA-Seq experiments as these used different experimental designs. The experimental set up as well as the comparisons in figure 6 are not the same as in Figure 5. Therefore, this is not a fair validation. RNA-Seq addressed the effects of parasite development and localization in head gene expression. qRT-PCR addressed the effects of parasite load in head gene expression. These independent experiments are not overlapping, answer	Thanks we have corrected as the follow: The qRT-PCR data should be interpreted as complementary evidence, providing targeted, quantitative insight into the relationship between parasite burden and the expression of key odorant receptors identified in the RNA-Seq dataset. Importantly, the RT-qPCR results show consistent directional trends for selected genes, supporting the biological relevance of the RNA-Seq findings while extending them by linking gene expression to infection intensity. The RNAseq and qPCR results have been described independently.

different questions, and should be discussed as such independently.	
In that regard, Lines 529-531: This explanation has no scientific basis - low-transcript abundance and normalization effects does not affect gene expression output.	Information reframed to correctly capture the intended message These differences may be attributed to methodological differences in how the transcripts are quantified between RNA-seq and RT-qPCR. Given the high sequence similarity in the odorant receptor gene family, the presence of so many isoforms of the same gene in RNA-seq quantification might have inaccurately influenced the fold change direction, but RT-qPCR uses gene-specific primers and thus provides isoform-specific quantification.
Figure 6: Authors should revisit these results/raw data/analyses. It is unusual to see every single tested gene being up-regulated in high infection. The libraries showed a more variable pattern of genes being up or down-regulated. Authors should choose two unrelated genes (serina protease and aconitase, for instance) and run the qRT-PCRs as controls.	Both suggested serine protease and aconitase are not ideal for infection-related studies involving tsetse flies and trypanosomes. Both suggested genes are linked to immunity and metabolic regulations and will compromise normalization accuracy and in fact from our results serine protease was among the most affected genes and therefore are not stable enough for normalization process. In that regard, we ensured robust normalization, we evaluated multiple candidate reference genes and tested a group of 4 genes and chose GAPDH and EF1 following geNorm NormFinder, and BestKeeper algorithms. We are therefore more confident with the results we have compared to when using the housekeeping genes suggested.
Lines 537-576: These phylogenetic analyses do not fit in this manuscript pertained to the demonstration that parasite infection affects sensory system-specific gene expression and host-seeking behavior. Authors should use this information in another manuscript dealing with evolution of such genes.	Phylogenetic analysis served a functional annotation purpose, which was essential for interpreting our RNA-Seq data. Specifically, because the *Glossina pallidipes* genome is not fully annotated and need more improvements. This step was therefore critical for linking infection-induced transcriptional changes to biologically meaningful sensory pathways, rather than reporting uncharacterized transcripts in *G. pallidipes*. Importantly, the phylogenetic results enabled us to identify previously unannotated olfactory genes that are responsive to trypanosome infection,

	thereby filling gaps in the current annotation of *G. pallidipes* and strengthening the interpretation of infection-driven modulation of the olfactory system. Thanks,phylogenetics results are now well connected to the main hypothesis of the work in the main text with a proper introductory statement into this section.
In that regard: Statement in lines 549-553 - I don't see the point of building phylogenetic trees to explain gene expression results.	Please see our comment above
Lines 590-593: A heatmap figure (or make a figure) with these RH gene results should be included.	Figure 8 regarding RH genes was already included in the document. The differential expression of these RH genes was reported based on RT-qPCR results.
Lines 597-599: Again, qRT-PCR and libraries experiments were not designed in the same way. Therefore, they should not be comparable. Authors should describe them as independent results for the questions they address based on specific experimental designs.	The two experiments used separate designs and corrections have been made and described independently within the document, please see our previous comments for the two methods.
Lines 623-642: Experiment with engorgement and parasite transmission is off-topic in a manuscript designed to investigate vector neuromodulation by parasite. The experimental design does not encompass any comparative condition of differential feeding between infected and uninfected tsetse. This is another misplaced subsection that does not address the hypothesis of the manuscript. It should be removed.	The interrupted feeding and transmission experiment was included to provide a functional and epidemiological context for the observed neurosensory changes. Specifically, our RNA-seq and behavioral data suggest that trypanosome infection alters olfactory-driven host-seeking behavior. The interrupted feeding experiment was designed to test the downstream consequence of such behavioral changes, namely whether altered feeding dynamics could increase transmission efficiency. In natural settings, tsetse flies frequently experience interrupted feeding due to host defensive behaviors, which can lead to repeated host contacts. When combined with infection-induced behavioral changes (e.g., altered host attraction or feeding persistence), this may increase the number of hosts exposed during a single feeding cycle. Our results demonstrate that a single infected *Glossina pallidipes* can infect multiple hosts under such conditions, providing a mechanistic link

	between behavioral modulation and transmission amplification.
Lines 650-653: Are these differences (fig 9A) statistically significant? If so statistical test and p-values should be indicated for each comparison. If not significant, it should also be stated.	Yes, statistics have been added to the main text in results section.
Lines 662-663: Are these differences (fig 9B) statistically significant? If so statistical test and p-values should be indicated for each comparison. If not significant, it should also be stated.	Thanks, statistics have been added and raw data supplied as supplementary file 4: Table S4.
Lines 665-672: All these results (trap catches) do not address the main hypothesis of the manuscript and should be removed (publish elsewhere).	The results in these sections have been removed as advised and transferred to a different article.
Lines 768-772: I believe the authors implied that the tsetse HDAC can influence gene expression of Tryps. This is misleading and should be removed.	Thanks for pointing out this and has been deleted.
Lines 773-794: All this discussion has no place in the manuscript as it does not deal with neuromodulation of sensory systems by tryp infection. This should be removed, along with the correspondent result's subsection	Section and the subsequent results have been removed from the document.
Lines 377-378: Sentence “After filtering at 0.5 count per library, 377 retained data were normalized by EdgeR within the iDEP platform” should be transferred to Methods section.	Transferred to the methods section as advised.
Lines 404-405: Authors need to mention that the differentially expressed genes of tsetse’s heads are on display.	Stated as advised.

Lines 445-448: Reference #53 is related to midgut expressed genes. This is not a good comparison with head/brain gene expression. Author could compare results with other brain transcriptomes of vectors infected with pathogens, for instance.	The reference and statements relating to midgut effects have been corrected with literature and citation relating to the neuronal signal transduction.
Lines 451-452: Reference supporting statement needs to be included.	Supporting evidence added.
Reviewer 3: We thank reviewer for the insightful comments and suggestions that have significantly improved the clarity and connectivity of the story of this manuscript We have addressed each point raised as detailed in the table. Changes made are marked in red in the marked up manuscript.	
Format for reference citation in line 131 should be changed	Citation by Muita et al corrected.
4. The period (months and year) that the field trapping of tsetse flies was done in Kwale County should be specified	We captured the period details but following reviewer one's comment to delete this section, we removed this section on field data from this paper.
The sample size for the behavioral assays was limited as 20 replicated is too low and may be under powered for ecological inference and weaken confidence in conclusions	Our behavioral assays were conducted under highly controlled laboratory conditions, where environmental variability was minimized, age, sex controlled. Thus, respectfully disagree with the reviewer and 20 independent replicates is good enough for statistical inference and each replicate consisted of 20 lab-reared flies of same age. Importantly, the responses observed in our study were highly consistent across replicates, demonstrated by smaller standard error with clear directional trends in fly preference between infected and uninfected mice and urine. This consistency increases confidence in the robustness of the observed effect despite the moderate sample size.
There is no evidence of using synthetic compounds to validate behavioral assays. The authors to clarify why this wasn't done	Field behavioral assays conducted used synthetic compounds. All the tested phenolics, ionones and POCA under field conditions are synthetic compounds. Phenolics and ionones tested were significantly affected in

	trypanosome infected hosts and therefore of vital importance to this study.
The statistical results should state the False Discovery Rate (FDR) thresholds	Thank you for this important comment. All the enrichment tests were corrected for multiple testing using the Benjamini–Hochberg method threshold of < 0.05 and log2 fold change > 1. We had relatively low-abundance transcripts which would result in loss of biologically relevant signals in head transcriptomes if they were not retained due to masking effect by other highly expressed genes and opted to conduct exploratory analysis displaying significance based on p-values. Despite the consistent trends observed following RT-qPCR experiments, the reported RNAseq p-values should be interpreted with caution. Differential expression data supplied as supplementary file 5: Table S5.
The RNA-seq and RT-qPCR results are inconsistent as some OR genes show opposite regulation between methods	Despite the two methods complementing each other in most cases, some discrepancies are expected due to differences in their study designs, sensitivity, low transcript abundance and therefore the two methods have been described differently as advised by reviewer I. "Differences in regulation observed between RNA-seq and qRT-PCR for some OR genes likely reflect differences in experimental design, low transcript abundance, and biological variability; however, both datasets consistently support the conclusion that trypanosome infection modulates the olfactory system in *G. pallidipes*." Our RT-qPCR data is supportive but not strictly validating. We have included the description in the manuscript.

Dear Editor,

Thank you for the positive comments. We have addressed your comments and the comments from the three reviewers point by point as follows and indicate in red text in the marked-up document.

Reviewer comment	**Response**
Reviewer 1	
Although the authors made efforts to improve the manuscript based on the comments of the reviewers, there are still major flaws in the manuscript. Moreover, quite some relevant comments of the different reviewers are only somehow addressed in the rebuttal letter but did not result in improvement/substantial changes in the manuscript. This give me the impression that some highly relevant comments of reviewers are ignored for improving the manuscript. The whole experimental section on tsetse fly interrupted feeding and trypanosome transmission remains highly unclear in its rationale and set-up. The explanation that the authors give for linking with the main hypothesis of this paper (i.e. olfactory modulation by the parasite infection in the host) is not relevant/satisfactory and is missing a sound scientific logic. Moreover, the experimental set-up remains scientifically unsound. In my	We thank the reviewer for his/her positive feedback we have removed the section on fly infection and interrupted feeding from the entire document and related supplementary files as suggested.

view this whole section on fly infection and interrupted feeding should be removed as it makes no sense. It has already been demonstrated in the past that *T. congolense* infected flies have difficulties to feed because of a mechanical obstruction of the ingested blood flow by the attached parasite rosettes to the chitinous lining of the mouthparts. As tsetse flies are easily disturbed, the probability that a *T. congolense*-infected fly is interrupted and continue its feeding on another host is increased. This behavioural change by the parasite infection has nothing to do with olfactory modulation. So, the infected fly/interrupted feeding experiment included in this manuscript will not allow to make any interpretation/conclusion about a putative link between parasite-induced olfactory modulation and fly behavioural changes that enhances parasite transmission. The paper should focus on the impact of the trypanosome infection in the mammalian host on tsetse fly attraction with the possible involvement of specific odour components modulated by the parasite infection in the host.	

Reviewer 2	
The authors carried out most of my suggested modifications, and the manuscript looks very comprehensive, with a broad variety of experiments that enlighten the field of neurobiology of disease vectors. I am still hesitant with the "Experiment with engorgement and parasite transmission" as the experimental design does not encompass any comparative condition, i.e. controls (gene knockouts or probing frequency btw infected and uninfected flies), that could be used to infer feeding differences. Having said that, the figure does not depreciate the quality of the manuscript. So, I will let you make a decision whether or not the authors should remove the figure, as I am satisfied with the current manuscript.	Thanks to the reviewer for his/her positive feedback we have removed the engorgement and parasite transmission experiment in mice from the entire document as suggested.
Reviewer 3	
The study used an interdisciplinary approach integrating vector biology, parasitology and disease ecology, with the findings promising and having great potential. However, with additional experiments on functional validation to support the data, the study could make a solid contribution to control of Tsetse vectors.	We thank the reviewer for this insightful comment and we fully agree that functional validation of the identified/ their expression affected or candidate receptors would further strengthen the mechanistic interpretation of our findings. However, functional genetic manipulation and receptor validation in *Glossina pallidipes* remain technically challenging because this species is not yet a genetically tractable model insect, and robust tools such as stable transgenesis, CRISPR-based knockout systems, or heterologous receptor expression platforms are currently limited for tsetse flies. Although functional validation of candidate receptors was beyond the

	scope of the present study, we put the idea as the next step to explain the link between infection-receptor-behavior as following. We acknowledge the need for functional validation. Please, see line 1022-1023.
Editor	
We therefore invite you to revise and resubmit your manuscript, taking into account the points raised. In particular, please remove the feeding and transmission experiment (and the accompanying methods, discussion and conclusions) from your submission, as requested by the reviewers #1 and #2.	We have removed feeding and transmission experiment (and the accompanying methods, discussion and conclusions) from our submission as advised.
For all graphs depicting a single point value (e.g., mean) with error bars, you must add individual data points or convert the graph to a boxplot or dot-plot to show data distribution.	Thank you for that, we have already included individual data points graphs reporting mean values (Figure 1) with error bars and presented Figure 6 and Figure 8 using boxplot to improve transparency and visualization of data distribution. For the graph presenting percentage (Fig 9 A-B), inclusion of individual data points was not feasible because the values represent aggregated percentage outcomes rather than independent continuous replicate measurements. In this case, plotting individual points could be misleading and would not accurately reflect the structure of the underlying dataset. We therefore retained the percentage-based presentation while ensuring that the number of replicates(n=200) and statistical analyses are clearly indicated in the manuscript.

It's mandatory to provide access to the numerical source data for graphs and charts either through a repository or by providing the data in a Supplementary Data file (in excel format).	All data provides as supplementary data files and the raw transcriptome data supporting the findings of this study have been deposited in NCBI's SRA database with reference code PRJNA1348605 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1348605) stated in the data availability statement of the manuscript.