



kdr homozygous resistant *An. gambiae* displayed enhanced feeding success when exposed to permethrin Insect-Treated Nets

Adrian Diaz based on peer reviews by **Thomas Guillemaud** , **Etienne Bilgo**, **Niels Verhulst** and 1 anonymous reviewer

Malal Mamadou Diop, Fabrice Chandre, Marie Rossignol, Angelique Porciani, Mathieu Chateau, Nicolas Moiroux, Cedric Penner (2020) Sub-lethal insecticide exposure affects host biting efficiency of Kdr-resistant *Anopheles gambiae*. bioRxiv, ver. 4, peer-reviewed and recommended by Peer Community in Zoology. [10.1101/653980](https://doi.org/10.1101/653980)

Submitted: 29 May 2019, Recommended: 01 July 2020

Cite this recommendation as:

Diaz, A. (2020) kdr homozygous resistant *An. gambiae* displayed enhanced feeding success when exposed to permethrin Insect-Treated Nets. *Peer Community in Zoology*, 100003. [10.24072/pci.zool.100003](https://doi.org/10.24072/pci.zool.100003)

Published: 01 July 2020

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Malaria is a vector-borne parasitic disease found in 91 countries with an estimated of 228 million cases occurred worldwide during 2018. The 93% (213 million) of those cases were reported in the African Region (WHO 2019). Six species of *Plasmodium* parasites can produce the disease but only *P. falciparum* and *P. vivax* are the predominant species globally. More than 40 species of *Anopheles* mosquitoes are important malaria vectors (Asley *et al.* 2018). Intrinsic (genetic background, parasite susceptibility) and extrinsic (feeding host preference, host diversity and availability, mosquito abundance) factors affect the capacity of mosquitoes to vector the disease (Macdonald 1952). Malaria is prevented by chemoprophylaxis, vaccination, bite-avoidance and vector-control measures. The mainstays of vector control are long-lasting insecticide (pyrethroid) treated nets and indoor residual spraying with insecticides (Asley *et al.* 2018). The widespread use of pyrethroid insecticides forced the emergence of insecticide resistance in malaria vectors reducing the insecticidal effect. Mosquitoes can modify their behaviour avoiding insecticide contact and so potentially reducing vector control tools efficacy. In this sense, Diop *et al.* (2020) investigated whether pre-exposure to an Insecticide-Treated Net (ITN) modulates the mosquito ability to take a blood meal in *Anopheles gambiae*. By means of video recording experiments the authors analyzed how the feeding/biting behaviour was affected by kdr mutation genotypes (homozygous susceptible – SS-, heterozygotes -RS- and homozygous resistant -RR-) when exposed to two different insecticides (permethrin and deltamethrin). According to the results, the blood-feeding success

did not differ between the three genotypes in the absence of insecticide exposure. However, authors observed differences in the feeding duration and blood meal size. In example, RR mosquitoes spent less time taking their blood meal than RS and SS. On the other hand, RS mosquitoes took higher blood volumes than RR females. These differences can affect the mosquito fitness by decreasing/increasing the likelihood to be killed by the host defensive behavior or increase the oogenesis so enhancing fecundity. Regarding the effect of exposition to insecticides authors detected a strong relationship between *kdr* genotype and Knock Down (KD) phenotype when mosquitoes were exposed to Permethrin. Previously, the authors have evidenced that RR mosquitoes prefer a host protected by a permethrin-treated net rather than an untreated net and that heterozygotes RS mosquitoes have a remarkable ability to find a hole into a bet net (Diop et al. 2015, Porciani et al. 2017). With data here obtained, they demonstrated that *kdr* homozygous resistant *An. gambiae* displayed enhanced feeding success when exposed to permethrin ITN. The changes observed in the feeding/biting mosquito behaviour can affect their fitness shaping the evolution of the insecticide resistance in mosquitoes' natural populations. Moreover, this may also alter parasite transmission dynamics by modifying vector/host interactions and so vector capacity.

References:

World Health Organization (2019). World malaria report 2019. Geneva: World Health Organization; 2019. ISBN 978-92-4-156572-1

Ashley EA, Pyae Phyo A, Woodrow CJ (2018). Malaria. *Lancet*. 391(10130):1608-1621. doi: [10.1016/S0140-6736(18)30324-6]([https://dx.doi.org/10.1016/S0140-6736\(18\)30324-6](https://dx.doi.org/10.1016/S0140-6736(18)30324-6))

Macdonald G (1952). The analysis of equilibrium in malaria. *Trop Dis Bull* 49: 813-828.

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Diop MM, Moiroux N, Chandre F, Martin-Herrou H, Milesi P, Boussari O, et al. (2015) Behavioral cost and overdominance in *Anopheles gambiae*. *PLoS ONE*. 10(4):e0121755. doi: [10.1371/journal.pone.0121755](<https://dx.doi.org/10.1371/journal.pone.0121755>)

Porciani A, Diop M, Moiroux N, Kadoke-Lambi T, Cohuet A, Chandre F, et al. (2017) Influence of pyrethroid-treated bed net on host seeking behavior of *Anopheles gambiae* s.s. carrying the *kdr* allele. *PLOS ONE*. 12(7):e0164518. doi: [10.1371/journal.pone.0164518](<https://dx.doi.org/10.1371/journal.pone.0164518>)

Reviews

Evaluation round #3

Reviewed by anonymous reviewer 1, 03 June 2020

The authors fully took into account my last comments and I'm now fine with the final version of this MS.

I'm just wondering why the authors did not include the Table 1 (Summary Result of treatment comparisons for blood feeding success and biting behavior parameters) and Table 2 (Summary Result of genotype comparisons for blood feeding success and biting behavior parameters) in their MS (or at least as supplementary material) given in their answers to my comments. They did a great job in making these tables and these tables would probably be very useful for the readers. I would therefore encourage them to include these two tables (they will correspond to Tables 2 and 3) in the final version of their MS when they will format it before recommendation.

Evaluation round #2

DOI or URL of the preprint: [10.1101/653980](https://doi.org/10.1101/653980)

Version of the preprint: 2

Authors' reply, 10 May 2020

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Decision by [Adrian Diaz](#), posted 13 March 2020

Merits a revision

Dear Malal Mamadou Diop, Thanks so much for submitted the revised version of your manuscript "Sub-lethal insecticide exposure affects host biting efficiency of Kdr-resistant *Anopheles gambiae*" authored by Malal Mamadou Diop, Fabrice Chandre, Marie Rossignol, Angeliqne Porciani, Mathieu Chateau, Nicolas Moiroux, Cedric Pennetier. This version was reviewed by 3 reviewer and most of them agreed that you addressed most of the comments and suggestions made on the first round. However, one of the reviewer is not completely satisfied with one of your answers. I invite you to review this new comment and submit your preprint for a recommendation. Thanks so much for your support to PCI Entomology. Best

Adrian Diaz

Reviewed by [Thomas Guillemaud](#) , 13 March 2020

I'm satisfied with the authors' replies and the modifications of the MS.

Reviewed by anonymous reviewer 1, 27 February 2020

[Download the review](#)

Reviewed by [Niels Verhulst](#), 28 February 2020

I read through the manuscript and all my suggestions were either implemented in the manuscript or clarified in the rebuttal.

Evaluation round #1

DOI or URL of the preprint: <https://doi.org/10.1101/653980>

Version of the preprint: 1

Authors' reply, 05 February 2020

[Download author's reply](#)

Decision by [Adrian Diaz](#), posted 18 July 2019

Merits a revision

Dear Malal Mamadou Diop and authors.

Thanks so much for submitted your preprint to PCI Entomology.

Your preprint was reviewed by 4 specialist and all of them agree it is well written, contains important information that merits revision. However, a few major points were highlighted by reviewers and addressing them out will improve the quality of your work.

So I invite you to go through all the comments made by each of the reviewers and submit a revised version of your preprint.

Thanks so much for your support to PCI Entomology.

Adrian Diaz.

Additional requirements of the managing board:

As indicated in the 'How does it work?' section and in the code of conduct, please make sure that:

-Data are available to readers, either in the text or through an open data repository such as Zenodo (free), Dryad or some other institutional repository. Data must be reusable, thus metadata or accompanying text must carefully describe the data.

-Details on quantitative analyses (e.g., data treatment and statistical scripts in R, bioinformatic pipeline scripts, etc.) and details concerning simulations (scripts, codes) are available to readers in the text, as appendices, or through an open data repository, such as Zenodo, Dryad or some other institutional repository. The scripts or codes must be carefully described so that they can be reused.

-Details on experimental procedures are available to readers in the text or as appendices.

-Authors have no financial conflict of interest relating to the article. The article must contain a "Conflict of interest disclosure" paragraph before the reference section containing this sentence: "The authors of this preprint declare that they have no financial conflict of interest with the content of this article." If appropriate, this disclosure may be completed by a sentence indicating that some of the authors are PCI recommenders: "XXX is one of the PCI XXX recommenders."

Reviewed by Niels Verhulst, 14 June 2019

The effect of insecticide resistance on mosquito behavior is an interesting topic and the authors apply a very nice video assay to study these effects. It is interesting to see the opposite effects of the two insecticides and although the authors do hypothesise about the mechanisms, this remains to be investigated. I only have a few minor comments on this study as it is well designed, performed and written down.

Introduction/discussion: What is known about the average contact time with a bednet? Is there a difference between resistance and non-resistant mosquitoes and how would this affect the results obtained in this study?

Discussion: It would be good to discuss the recently published paper by Hause et al and compare to the results obtained here: Hauser, Gaël, Kevin Thiévent, and Jacob C. Koella. "The ability of *Anopheles gambiae* mosquitoes to bite through a permethrin-treated net and the consequences for their fitness." *Scientific reports* 9.1 (2019): 8141.

Line 98: How were the mosquitoes blood fed and on what source?

Line 323: Correct: suggestsThe

Figure 1: Some parts seem to be more sharp than others

Figure 2+3+4: Why were boxplots not used for the feeding success? And what are the confidence intervals based on? Batches of mosquitoes with a certain success? Although the number of mosquitoes can be found in table one, please, include number of replicates and mosquitoes in either the graph or legends of the graphs. Some y-axis include the 0, others not

Would it be possible to combine figure 3 and supp. figure 1 for a better overview?

Reviewed by Etienne Bilgo, 15 July 2019

Dear recommender,

The manuscript entitled:

"Sub-lethal insecticide exposure affects host biting efficiency of Kdr-resistant *Anopheles gambiae*" is well written and scientifically sound and the experiments appear to be thoughtfully executed and the analysis is suitable. Although it is acceptable in the present form and contents, I suggest the authors should clarify and implement some parts. I refer in particular to:

Abstract:

The authors should give a brief explanation of the consequences of the observed behavioral changes on malaria vector fitness and disease transmission that they have observed on behavioral study instead of saying that they are going to discuss that in the article. Among keywords, the authors should include Resistance/Susceptibility or even Kdr as keywords, too.

Methods:

Line 104: why did the authors use 7 to 9 days old mosquitoes for your bioassays, it is generally recommended to use 3-5 days old mosquitoes. Are there any particular reasons?

Line 120: Behavioral assay

Could the authors give the abiotic conditions of the Bioassay test? Is that during the night, Temperature, Relative humidity data?

I suggest associating the picture (the Photo) of the experimental design of figure 1 instead of having only the schematically representation of the experimental design.

Reviewed by **Thomas Guillemaud** , 15 July 2019

The paper by Diop et al concludes a series of papers that seek to understand the influence of the kdr resistance genotype and insecticide presence on the behaviour of *Anopheles Gambiae* mosquitoes related to parasite transmission by bite. This article focuses in particular on the effect of genotype and the presence of pyrethroids on nets on biting behaviour itself.

The article is very well written, extremely clear, and easy to follow. The introduction is complete and linear and logically leads to the presentation of the questions asked by the authors. The material and method is very comprehensive and the statistical analyses seem to be quite adequate. Data and analysis scripts under R are available under github. This allowed me to check some of the analyses and play with the data a little bit. The results are clear and complete and the discussion is simple and effective.

Overall I do not detect any significant problems with this article. We can always imagine some improvements, we can obviously regret some shortcomings, for example, the fact that the size of each individual has not been measured and then taken into account as a fixed effect in generalized models. It is unfortunate that the genotypes were not randomized over time (all RS mosquitoes are observed within the last 15 days), that the observations were clearly not made blind to treatments and genotypes, etc. (any explanation on these points is welcome in a response). But overall, this article is of good quality.

The only main issue I see is the lack of discussion on the KD results (the proportion of mosquitoes with knock-down status for the various kdr genotypes). These results look a bit strange as a high proportion of resistant mosquitoes (according to their kdr genotype) suffers from KD. Are these results expected? If not, a short discussion on this point could be written.

I list below a number of questions and comments that could improve the article:

Data in github:

-incorporate legend of data in your data file or in a separate file of metadata. As much metadata as possible is desirable.

-please provide the raw data, eg the blood volume and the mosquito weight

-github is an option, but it is a private company and it does not provide doi for data and scripts. A preferable alternative is for example Zenodo.org

-line 39: define endophagic

-line 41: 'such as xenobiotics', why xenobiotics? do you mean other insecticides?

- line 56: define exophily
- line 67: ref [25] is probably not appropriate for mosquitoes. It is general or beneficials oriented
- lines 80-82: is there any link to this committee or to its guidelines? If not, the reader can't do anything with this information
- line 87 onward. The strain *kdr* is called *kdr-kis* in previous papers. You may change this name to homogenize.
- line 89: what do you mean by *kdr-west*? Not referred before
- line 90: basic information on VKPer is missing: origin, date of isolation etc...
- line 90: how was made the selection of RR genotypes after each backcross? This is not described in ref [28]. Please provide details.
- line 93: Are references [30-31] correct for the 15 cM in *An. gambiae*?
- lines 98-99: mosquito rearing is made without blood meal? Please detail.
- line 104: the mated status of the female is not checked? In either case, indicate it
- line 104: 'a batch of 10 adults'. But some days you tested up to 16 adults. Please explain
- lines 116-117: '30 s' as median time of contact with insecticide-treated nets. I don't find this info in [24]. Fig F of supplementary information 2 of [24] suggests 500 seconds. Where am I wrong?
- line 132: is there randomization of genotypes through time. Apparently not. How was made this choice?
- line 148: Please indicate if KD mosquitoes are included in the feeding success measurement. success = Fed/tot or Fed/(tot-kd)? the script gives the answer but the reader may not read the script.
- line 165: replicates of what? Of the reading I suppose.
- line 168: It would have been better to measure the mosquito size and to use this measure among the explanatory variables. Do you have these data?
- lines 209-214: please provide the p-values (and not just <0.05)
- line 216: please provide the p-value (not just >0.05)
- line 222: 30.2% KD among RR. Isn't it too large for RR individuals?
- line 222: Statistical tests such as Fisher exact tests on the proportions could be done to know if KD is more frequent in SS than in RS and RR.
- line 312: correct diuresis/prediuresis
- line 323-324: correct 'This suggests the whole picture'
- Figures and supplementary figure: provide the p-values and not just ns, *, , *

Reviewed by anonymous reviewer 1, 12 July 2019

This preprint provides data that may be used to draw solid conclusions about the feeding behaviour of susceptible and *kdr*-resistant *Anopheles gambiae*. However, I found some major and minor flaws:

Main points

1- The authors did not test the relationship between the phenotype knockdown (KD) and the different parameters (feeding success, feeding duration, prediuresis duration and weight blood meal size). According to the data given in Table 1, there is a strong relationship between KD and the feeding success: KD females (overall genotypes) fed less successfully (13.6% (14/103)) than non-KD females (53.2% (217/408)). It would be interesting to see whether this relationship also holds between the KD phenotype and the other parameters studied (ie feeding duration, prediuresis duration and weight blood meal size).

Then, my question is: do the differences in feeding behavior better explained by the fact that females were KD before being tested or by their SS, RS or RR genotypes.

As expected and as recorded by the authors, SS females were more frequently KD than RS and RR females when pre-exposed to deltamethrin (this difference being more pronounced when they were pre-exposed to permethrin). The *Kdr* mutation (L1014F) might only change the probability of being KD when pre-exposed to these two insecticides. Once KD, SS, RS and RR females might encounter the same difference in feeding behavior. Or, do SS, RS and RR KD females still behave differently? If so, this would slightly change the

discussion and conclusion of their study.

2- In the discussion section, line 322, the authors indicate that "*Herein, we have completed the sequence by showing that permethrin exposure enhances the feeding success of RR mosquitoes*". As a consequence, they conclude, line 324 that "*The whole picture suggests that permethrin ITN may increase vectorial capacity of *An. gambiae* populations in areas where PYR resistance with *kdr* mutation is well established.*" This statement is not supported by their results. Indeed, according to Figure 3C and the corresponding analyses, exposure to permethrin did NOT significantly enhance the feeding success of RR mosquitoes. This should encourage them to revise their conclusions about the consequences they draw in terms of the evolution of resistance, the dynamics of malaria infection and the use of permethrin ITNs.

3- This assertion is repeated in the summary of the article. Line 23, the authors indicate that "*the permethrin ITN increased the blood success of RR mosquitoes*". Again, this is not in agreement with their results and must therefore be corrected.

4- Line 127. The same rabbit was used during all experiments. Using only one rabbit can actually have some effects on the successive tests notably if the tests were not performed randomly. Were the tests SS, RS and RR genotypes and using the different treatment (ie untreated, permethrin and deltamethrin) randomized to avoid biases related to the sequence of tests that were made during several days/weeks/months?

5- In Figure 3A and Figure S1B, the blood feeding rate of the SS genotypes pre-exposed to permethrin is ca 18%. However, according to the data given in Table 1, this blood feeding rate is actually 10% (10/50). This error might change the statistical analysis of the comparisons between SS pre-exposed to permethrin vs SS pre-exposed to deltamethrin and between between SS pre-exposed to permethrin and between RS pre-exposed to permethrin (the difference in blood feeding rates might in fact be significant). Please, modify Figure 3A and Figure S1B and, if appropriate, update the statistical analysis. BTW, I suggest the authors to double-check the other Figures and Tables to be sure that no other errors have been made.

6- In the introduction and in the discussion, the authors never talk about the cost of resistance: ie the fact that the *kdr* mutation is often associated with a reduction in fitness in the absence of insecticides in many different species - eg Foster et al. (1999) Bulletin of Entomological Research. doi: 10.1017/S0007485399000218 - including mosquitoes - eg Brito et al. (2013) PLoS one. doi: 10.1371/journal.pone.0060878 - and, within mosquitoes, in species belonging to the *Anopheles* genus - eg Platt et al. (2015) Heredity. doi: 10.1038/hdy.2015.33.

Actually, the authors perfectly know the cost of resistance. In their previous paper (Diop et al. (2015), PLoS One. doi: 10.1371/journal.pone.0121755), they reported that **Kdr* homozygous, PYR-resistant mosquitoes were the least efficient at penetrating an untreated damaged net, with about 51% success rate compared to 80% and 78% for homozygous susceptible and heterozygous respectively. *They likely attributed this reduced efficiency to *reduced host-seeking activity and considered it as a recessive behavioral cost of the mutation.*

In the present preprint, they cite Diop et al. (2015), but they only indicate - lines 321-322 - that RR mosquitoes have a remarkable ability to find a hole into a bed net, omitting the fact that this ability is lower than those of SS and RS mosquitoes.

In the end, I encourage the authors to revise their introduction, discussions, conclusions and abstract sections to better take into account both (i) the above comments (i.e. the fact that RR females do not have a significantly better feeding success when pre-exposed to permethrin than when exposed to untreated nets) and (ii) the differences in selective advantages and disadvantages conferred by the *kdr* mutation in the presence vs. absence of insecticides.

By the way, following the recent paper by Lenormand T, Harmand N, Gallet R (2018) Rethinking Ecology. doi: 10.3897/rethinkingecology.3.31992, I suggest them to not talk about the cost of resistance, but rather about differences in fitness in treated vs untreated environments.

Minor points

1- Line 90: can you provide some details about the VKPer strain (origin, lab maintenance...).

2- Line 104: did females were virgins or mated? (I guess this can make a difference in term of feeding behavior).

3- Line 118: after exposure the authors observed a 1-min latency period before releasing the insect in the

behavioural assay setup. Please, explain why and why 1-min?

4- Lines 257-258: is this conclusion valid for all 3 genotypes? 5- Figure 4: use the same scales for the y-axis - this would facilitate the comparisons between SS, RS and RR genotypes.

Typos

Line 95: change "crossing once" by "crossing".

Line 98: change "(light : dark)" by "(light:dark)".

Line 127: change "all the exepriments.The experimental" by "all the experiments. The experimental".

Line 131 "CO" is not defined in Figure 1.

Line 134: change "observational" tunnel by "OT".

Line 140: change "odours .The" by "odours. The".

Line 144: change "if is was feed " by "if it was fed".

Line 148: change "KD if they were" by "knockdown (KD) when".

Could be nice for readers to use homogeneously "insecticide exposure" or "insecticide pre-exposure" throughout the text. Idem for pre-exposed or exposed (choose one or the other and use it consistently throughout the text).

In the same way it would be simpler for the reader if the authors homogenize the way they refer to untreated nets and those treated with deltamethrin or permethrin.

Line 217: replace "knockdown" by "KD".

Line 219: replace "knock-down" by "KD".

Line 219: remove "(14/23)" (or give this ratio for all genotypes and treatments throughout the MS.

Line 220: remove "(14/23)" (or give this ratio for all genotypes and treatments throughout the MS.

Line 222: remove "(16/50)" (or give this ratio for all genotypes and treatments throughout the MS.

Line 234: change "was not different than" by "although lower, was not significantly different than".

Line 248: change "Figure 4B)" by "Figure 4B). For this latter parameter, the non significance was probably due to a lack of power".

Line 307: change "real need to decipher more deeply with consequences" by "the need to further investigate the consequences".

Lines 311:-312: change "pre-diuresisprediuresis" by "pre-diuresis".

Line 314: change "This results suggest" by "This result suggests".

Line 323: delete "This suggests".

Line 549: TP does not appear in the Figure 1.

Line 559: change "panel B and C" by "panels B and C".

Line 564: change "panel A, B and C" by "panels A, B and C".

Line 573: change "panels A,B and C" by "panels A, B and C".

Figure 1: indicate in the legend of the Figure what GF means.

References: please, correct the typos, homogenize the style and provide the doi for all references.