

Dear Recommender,

We are pleased to submit a revised version of the manuscript entitled 'Sub-lethal insecticide exposure affects host biting efficiency of Kdr-resistant *Anopheles gambiae* ' authored by Malal M. Diop *et al.* to be considered for recommendation in *PCI Entomology*.

We are very grateful to the referees for their meticulous and relevant comments on the manuscript. You will find below our response to each point raised by the referees.

Look forward to hearing from you at your earliest convenience,

Best regards,

Nicolas Moiroux on behalf of the authors

Round #1

Author's Reply:

Decision

by Adrian Diaz, 2019-07-18 17:44

Manuscript: <https://doi.org/10.1101/653980>

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.

Data and code are available on Zenodo: <https://zenodo.org/record/3629451#.XjBD6DmH670>

-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."

A conflict of interest disclosure has been added to the manuscript.

Reviewer 1

Reviewed by Niels Verhulst, 2019-06-14 12:34

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 hypothesis 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.

1/ 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?

In this study, we choose to expose the mosquitoes during 30s allowing a sufficiently high number of SS mosquitoes to survive the contact (or sufficiently delaying death) (Diop et al. 2015) and then, to observe the feeding behavioral sequence. Using a constant time of contact allowed us to clearly show the effect of both the LLINs and genotypes. However, it seems from what we can find in the literature that the contact time in 'natural' condition might depend on the resistance phenotype and/or genotype for the *kdr* mutation and the type of LLIN/insecticide:

In an irritancy bioassay in WHO cones, Chandre et al. (2000) measure the time between first landing and following take-off on papers impregnated with 1% permethrin. The median time for first take-off was 3.7s and 27.3s for the susceptible Kisumu strain and the resistant Kou strain, respectively. Parker et al. (2015) video-tracked multiple free-flying *Anopheles gambiae* (Kisumu strain) responding to human-occupied deltamethrin ITN in a large-scale system. During a period of 60 min, they estimated that the mean duration of each contact with the ITN ranged from 17.5s to 95.6s. In one of our previous studies team [Diop et al. 2015], we investigated the effect of the *Kdr* mutation on the ability of female *An. gambiae* to locate and penetrate a 1cm-diameter hole in a piece of netting. We estimated from the data that the median duration of cumulated contact with the treated net before passing through the hole vary according to the insecticide on the net and the *Kdr* genotype. Please refer to the following table:

treatment	genotype	N	time spent on the net before success (s)
Untreated	SS	50	161.5
	RS	31	13.9
	RR	31	337.08
Permethrin	SS	44	33.66
	RS	37	239.36
	RR	45	150.2
Deltamethrin	SS	53	132.3
	RS	33	25.92
	RR	49	182

It is therefore highly probable that in natural condition, mosquitoes experience different contact times with the LLIN depending on its genotype for the *Kdr* mutation and the type of LLIN. In order to discuss this point, we added the following sentences in the last paragraph of the *Discussion* section:

“However here, we exposed all our mosquitoes for a constant duration to the treated nets which does not reflect the variability that happens in natural conditions. Indeed, we can expect from the literature [Chandre 2000, Parker 2015, Diop 2015] that both the genotype for the kdr mutation and the type of pyrethroids on LLINs may affect the contact duration. It would be of great interest to decipher with the relationship between the time of contact with the insecticide and the feeding sequence.”

The following references have consequently been added in the reference section:

Chandre F, Dabire RK, Hougard JM, Djogbenou LS, Irish SR, Rowland M, et al. Field efficacy of pyrethroid treated plastic sheeting (durable lining) in combination with long lasting insecticidal nets against malaria vectors. *Parasit Vectors*. 2010;3(1):65.

Parker JEA, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall PJ. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Sci Rep [Internet]*. 2015 Sep 1;5.
Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4642575/>

Diop MM, Moiroux N, Chandre F, Martin-Herrou H, Milesi P, Boussari O, et al. Behavioral cost & overdominance in *Anopheles gambiae*. *PLoS ONE*. 2015;10(4):e0121755.

2/ Discussion: It would be good to discuss the recently published paper by Hauser 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.

In an experimental setup where insecticide exposure of Kisumu SS anopheles occurred during blood feeding (bite through the net on a human arm), Hauser *et al.* found that mosquitoes exposed to permethrin+PBO had lower feeding successes, shorter feeding durations and lower blood meal sizes compared to those exposed to untreated nets. This results are in accordance with what we found here with the same strain of insecticide-susceptible mosquitoes. We discuss our results in regards of those of Hauser *et al.* by modifying a paragraph of the *Discussion* section as follows:

"[...] They both induced a decrease of feeding and prediuresis durations. This is in agreement with the results of Hauser et al. [ref] showing that mosquitoes biting through permethrin+PBO nets (Olyset Plus) had less feeding successes, shorter feeding duration and lower blood meal sizes compared to those biting through untreated nets. As discussed above, short feeding durations and small blood-meal lead us to expect that [...]"

Moreover, reference to Hauser et al. was added line 277 of the initial submission to support the statement that *"[large blood meal] could [...] increase their fecundity compared to RR."*

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

Mosquito colonies (Kisumu and KdrKis strains) were fed on rabbit ears. We modified line 98 as follow:

"Mosquitoes were reared at $27 \pm 1^\circ\text{C}$, 70-80% relative humidity under a 12h:12h (light : dark) photoperiod in the insectary and fed on rabbits."

4/ Line 323: Correct: suggests The

Done

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

A revised version of Figure 1 is now provided that is of better quality.

6/ 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?

Feeding success of each individual mosquito was recorded as a binomial variable (0/1) and individual data cannot be represented with boxplots. We therefore plot the proportion of fed mosquitoes (feeding success rate) with bar plots and the 95% confidence interval of this proportion (as indicated in the Figure legend).

We calculated the binomial confidence interval of feeding rates and knock-down rates with the Wilson's score method using the 'binconf' command from the 'Hmisc' package as indicated in the method section (lines 188-189 of the initial submission).

7/ 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.

Number of mosquitoes are now added on the figures. Axes were automatically produced by the software. We modified them in order to have equal axis for each parameter on Figure 4 (that allow easier comparison between genotypes) and to include zeros in both Figure 2 and 4. R codes have been updated. Number of mosquitoes are now indicated in the legends.

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

Supplementary Figure-1 was provided to facilitate comparisons between genotypes in each treatment. However, data shown in Supplementary Figure 1 are the same that those shown in Figure 3 (and for a part, in Figure 2) but in a different order. We therefore truly believe that Supplementary Figure 1 and Figure 3 should not be combined in one figure.

Reviewer 2

Reviewed by Etienne Bilgo, 2019-07-15 00:48

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:

1/ 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.

The last sentence of the abstract was modified as follow:

"Our study demonstrates a complex interaction between insecticide exposure and the kdr mutation on the biting behavior of mosquitoes, which may impact substantially malaria vector fitness and disease transmission."

2/ Among keywords, the authors should include Resistance/Susceptibility or even *Kdr* as keywords, too.

"Resistance" and "kdr" have been added as keywords.

Methods:

3/ 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?

*By using 7-9 days old *An. gambiae*, we expected to enhance the probability that these females were physiologically active for host-seeking. Indeed, it has been shown that 7, 8 and 9 years old females display higher rate of host-seeking activity than 4-5 years old individuals (Jones and Gubbins, 1978).*

We add the following sentence in the same paragraph (see also response to comment 17 of reviewer 3):

*"By using 7-9 days old *An. gambiae*, we expected to enhance the probability that these females were inseminated (32) and physiologically active for host-seeking (33)."*

Ref:

*Jones MDR, Gubbins SJ. Changes in the circadian flight activity of the mosquito *Anopheles gambiae* in relation to insemination, feeding and oviposition. *Physiological Entomology*. 1978 Sep 1;3(3):213–20.*

4/ Line 120: Behavioral assay. Could the authors give the abiotic conditions of the Bioassay test? Is that during the night, Temperature, Relative humidity data?!

The feeding experiments were done under insectary condition (temperature at $27 \pm 1^\circ\text{C}$, and relative humidity 70-80%). The experiments were carried out in a dark room during the night (according to the Light:Dark photoperiod of the insectary) following the same feeding rhythm we are using at the insectary to get success on rearing the mosquito strains.

The following sentence was added at the end of the *Behavioral Assay* paragraph:

“The feeding experiments were done under insectary condition (temperature at $27 \pm 1^\circ\text{C}$, and relative humidity 70-80%), in a dark room during the night (according to the Light:Dark photoperiod of the insectary).”

5/ 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.

Unfortunately, we have disassembled the behavioral setup and yet we can't find a photo of the experimental setup (the experiments were carried out in 2014). In case we find a picture of the experimental set-up, we will make it available to the readers.

Reviewer 3

Reviewed by Thomas Guillemaud, 2019-07-15 08:46

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.

1/ 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.

We agree with the limitations pointed by Dr. Guillemaud. We answer this comment in 3 points:

1- It would have been better to obtain individual measure of the size of each individual mosquito (e.g. by measuring wing size). Unfortunately, we chose to use an easier/faster method to get a proxy of mosquito size. We randomly selected 5 anopheles females from each rearing cages used during the experiment and weighed them together. The weight was then divided by 5 to obtain the average weight that was used as a proxy for the size of the mosquitoes coming from each cage. Using a linear model of the average weight of mosquito per cage fitted with the genotype, the treatment and interaction as fixed effects, we found that the variability of mean body weight among cages was low and stable regardless the genotype (as shown in the following Table 1). Moreover, there was no difference in mean weight between genotypes in all treatments indicating that mosquito body weight was relatively constant (Table2) over the study.

treatment	genotype	emmean	lower.CL	upper.CL
Control	RR	0.913	0.859	0.966
	RS	0.957	0.891	1.022
	SS	0.95	0.908	0.991
Olyset	RR	0.95	0.901	0.999
	RS	0.957	0.881	1.032
	SS	0.967	0.923	1.011
permanet	RR	0.944	0.891	0.998
	RS	0.949	0.884	1.014
	SS	1.001	0.952	1.051

Table 1: Estimated marginal means (emmean) and 95% confidence interval obtained from a linear model of the average weight of mosquito per cage.

treatment	contrast	estimate	SE	df	t.ratio	p.value
Control	RR - RS	-0.04383	0.042	47	-1.044	0.5535
	RR - SS	-0.03683	0.0336	47	-1.096	0.5212
	RS - SS	0.007	0.0385	47	0.182	0.9819
Olyset	RR - RS	-0.00667	0.0449	47	-0.148	0.9879
	RR - SS	-0.017	0.0328	47	-0.518	0.8628
	RS - SS	-0.01033	0.0434	47	-0.238	0.9692
permanet	RR - RS	-0.00467	0.042	47	-0.111	0.9932
	RR - SS	-0.05695	0.0362	47	-1.573	0.2672
	RS - SS	-0.05229	0.0408	47	-1.282	0.4123

Table 2: Estimated difference of average weight between genotype according to a linear model.

2- It is true that RS mosquitoes were tested only at the end of the experiment, with the risk to induce a bias. Indeed we felt to synchronize the rearing of both Kis, KdrKis and the production of their F1 progeny. Both rearing of the KdrKis (RR) strain and crossing with the Kisumu (SS) strain are difficult tasks that need to be successful simultaneously. In order not to add another bias relative to the treatments randomization, we therefore choose to produce all RS mosquitoes in the same time and in sufficient number to randomize over the treatments. However at least, we randomized treatments and genotypes over the week as far as possible within the limit of insectaries constraints.

We had the following paragraph in the Discussion section to discuss these limitations (points 1 and 2):

“This work has some limitations. First, we were not able to randomize genotypes over time because of rearing constraints. Consequently all RS mosquitoes were tested during the last month of experiments. This did not induce any effect when analyzing the treatment effect relative to the genotype but might have introduced a bias while comparing RS with RR and/or SS mosquitoes (as experimental period is possibly a confounding factor for RS genotype). The second limitation relies on body size measurement. Indeed, we chose to use an easy method to get a proxy of mosquito size. We randomly selected 5 anopheles females from each rearing cages used during the experiment, weighed them together and used the mean weight to adjust blood-meal size. An individual wing length measurement would allow to avoid any bias in developmental variability within each rearing cage.”

3- Observations were not made blind to treatments and genotypes. However, the observations of feeding behavior were made by video-tracking with therefore a very poor risk of human bias.

2/ 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.

Indeed, we observed moderate KD rates (16/50) in RR mosquitoes exposed to deltamethrin treated nets whereas we did not observe any KD among RR mosquitoes exposed to a permethrin-treated net. This is actually not surprising, as deltamethrin is expected to induce higher knock-down rates than permethrin against resistant populations of *Anopheles gambiae* (Hougard 2003). This observation is also true when looking at mortality in experimental hut trial in area with high frequencies of the *kdr* mutation in the vector population (N’Guessan 2010, Toe 2014, Toe 2018). This difference between permethrin and deltamethrin effect may be linked to the different chemical properties of permethrin

and deltamethrin that induce two types of bursting activity of sodium channels (type I and Type II pyrethroids). Consequently, to our knowledge, our study is the first one deciphering with the relationship between the *kdr* genotype and KD rates after a forced contact with both permethrin and deltamethrin treated net.

As recommended, we analyzed the relationship between KD rates and *kdr* genotype. For this task, we fitted a binomial model of the KD with the genotype and the treatment (Olyset or Permanet) and interaction as explanatory variables. The results of this model show that the relationship between KD and genotypes displayed different trends relative to the insecticide. When exposed to permethrin, KD rates significantly varied among the genotypes whereas when exposed to deltamethrin, KD rate did not varied significantly among the three genotypes.

The following § was added in the *Method* section (*Statistical analysis*):

*“KD (coded as 1 for KD mosquitoes and 0 for others) was analysed using a binomial model with the *kdr* genotypes (SS, RS or RR), type of pre-exposure (Olyset or PermaNet) and their interactions as fixed terms. Because the dataset showed data separation, we fitted the model using the bias-reduction method developed by Firth (41). We used the ‘brglmFit’ function of the ‘brglm2’ package (42,43) for this task.”*

The following sentence was added in the *Results* section (*Impact of insecticide exposure on knockdown rates*):

“Kdr genotype was highly correlated with KD rates for mosquitoes exposed to permethrin ($OR_{RR-RS} = 0.019 [6.1 \times 10^{-4}, 0.597]$, $OR_{RR-SS} = 0.003 [9.7 \times 10^{-5}, 0.095]$, $OR_{RS-SS} = 0.160 [5.41 \times 10^{-2}, 0.471]$) but not for mosquitoes exposed to deltamethrin ($OR_{RR-RS} = 0.80 [0.29, 2.22]$, $OR_{RR-SS} = 0.67 [0.25, 1.83]$, $OR_{RS-SS} = 0.85 [0.31, 2.34]$)”

The following sentence was added in the *Discussion* section:

*“As expected, we found a strong relationship between *kdr* genotype and KD phenotype when mosquitoes were exposed to Permethrin. However with deltamethrin, we were not able to find such a relationship and mosquitoes carrying the *kdr* mutation experienced moderate levels of KD. Indeed, deltamethrin is expected to induce higher knock-down rates than permethrin against resistant populations of *Anopheles gambiae* (Hougard 2003). This observation is also true when looking at mortality in experimental hut trial in areas with high frequencies of the *kdr* mutation in the vector population (N’Guessan 2010, Toe 2014, Toe 2018). This difference between permethrin and deltamethrin effect may be linked to the different chemical properties of permethrin and deltamethrin (type I and Type II pyrethroids) as describe above.”*

Moreover, as recommended by reviewer 4 (comment 1), we analyzed and discuss KD in relation to feeding success and behavior in the revised manuscript.

References cited in the answer:

Hougard J-M, Duchon S, Darriet F, Zaim M, Rogier C, Guillet P. Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. Bull World Health Organ. 2003;81(5):324–33.

N'Guessan R, Asidi A, Boko P, Odjo A, Akogbeto M, Pigeon O, et al. An experimental hut evaluation of PermaNet(®) 3.0, a deltamethrin-piperonyl butoxide combination net, against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in southern Benin. *Trans R Soc Trop Med Hyg.* 2010 Dec;104(12):758–65.

Toe KH, Müller P, Badolo A, Traore A, Sagnon N, Dabiré RK, et al. Do bednets including piperonyl butoxide offer additional protection against populations of *Anopheles gambiae* s.l. that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Faso. *Medical and Veterinary Entomology.* 2018;32(4):407–16.

Toé KH, Jones CM, N'Fale S, Ismail HM, Dabiré RK, Ranson H. Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed Net Effectiveness, Burkina Faso. *Emerg Infect Dis.* 2014 Oct;20(10):1691–6.

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

3/ 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.

Metadata for the dataset have been added in the code.

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

Done

5/ 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

Done, data and code are now available on Zenodo:
<https://zenodo.org/record/3629451#.XjBD6DmH670>

6/ line 39: define endophagic

Done

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

We mean “pollutants in mosquito environment”. We added the following reference to support the statement:

Poupardin R, Riaz MA, Jones CM, Chandor-Proust A, Reynaud S, David J-P. Do pollutants affect insecticide-driven gene selection in mosquitoes? Experimental evidence from transcriptomics. *Aquatic Toxicology.* 2012 Jun 15;114–115:49–57.

8/ line 56: define exophily

We defined the induced exophily in the revised manuscript as “*i.e. the proportion of mosquitoes that exit early and are found in exit traps relative to the untreated hut*”.

9/ line 67: ref [25] is probably not appropriate for mosquitoes. It is general or beneficials oriented

Indeed, we removed it as the next one is sufficient to support both sentences.

10/ 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

It is a legal requirement as far as we worked with alive mammals (i.e. rabbit). The following web page of the French ministry of scientific research gives the links to (in French) (i) the national charter on ethic for animal experimentation, (ii) the legal acts about animal experimentation and (iii) the list of the ethic committees empowered to deliver ethic agreements.

L'utilisation des animaux à des fins scientifiques [Internet]. Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation. [cited 2019 Oct 11]. Available from: [//www.enseignementsup-recherche.gouv.fr/cid70597/l-utilisation-des-animaux-a-des-fins-scientifiques.html](http://www.enseignementsup-recherche.gouv.fr/cid70597/l-utilisation-des-animaux-a-des-fins-scientifiques.html)

11/ line 87 onward. The strain kdrkis is called kdr-kis in previous papers. You may change this name to homogenize.

Done.

12/ line 89: what do you mean by kdr-west? Not referred before

We replaced "Kdr-west allele (L1014F)" by "Kdr allele (L1014F)" as it is useless and confusing to describe the second mutation at the same locus (L1014S), i.e. Kdr originated from East-Africa in this paper.

13/ line 90: basic information on VKPer is missing: origin, date of isolation etc...

We rephrased this sentence as follows: *"The VKPer strain, originated from a rice growing area named Vallée du Kou, less than 40 Km north of Bobo-Dioulasso (Burkina Faso) was used to obtain Kdr-kis. VKPer displayed the same expression level of metabolic resistance enzyme as Kisumu. Both Kisumu and Kdr-kis strains are maintained at the insectary of IRD (Institut de Recherche pour le Développement) – WHO collaborating center FRA-72 in Montpellier, France."*

14/ line 90: how was made the selection of RR genotypes after each backcross? This is not described in ref [28]. Please provide details.

Through larval selection with permethrin. The information can be find in the following sentence from the *Mosquito Strains* § of the *Methods* section in Alout *et al.* (2013):

"Resistant strains were obtained through at least 15 successive backcrosses with the Kisumu strain and selection with propoxur insecticide for the Acerkis strain and permethrin insecticide for Kdrkis, so that the three strains share common genetic background at the exception of the locus carrying the insecticide resistance genes"

15/ line 93: Are references [30-31] correct for the 15 cM in *An. gambiae*?

According to Berticat et al. (ref [30] of the initial submission), "For a given allele of an introgressed strain, we can define the probability P that, at the end of i backcrosses, this allele is still associated with the selected resistance allele, i.e. no recombination event has occurred between the two genes. If r is the recombination rate between both genes, then $P = (1-r)^i$. This allows the computation of the genetic distance around the selected gene which has not been replaced by the [kisumu] genome, e.g. around $1 - (e^{(\ln(\alpha)/i)})$, α being the risk level. This leads to 19 and 18 cM for 14 and 15 backcrossing generations, respectively, at the 0.05 risk level."

Ref. [30] is therefore correct. We agree that ref [31] should be removed as it only cites [30].

16/ lines 98-99: mosquito rearing is made without blood meal? Please detail.

Please see response to comment 3 of reviewer 1.

17/ -line 104: the mated status of the female is not checked? In either case, indicate it

The mated status was not checked. However, using 7-9 days old females, we expect to reach high levels of insemination. In insectary from Burkina Faso, the inseminated rate of 5 days old females *An. coluzzii* reached 80% (Sawadogo et al. 2013). The following sentence was added to the paragraph (see also response to comment X of reviewer 2):

*"By using 7-9 days old *An. gambiae*, we expected to enhance the probability that these females were inseminated (32) and physiologically active for host-seeking (33). However, mated status was not checked."*

Ref:

Sawadogo SP, Diabaté A, Toé HK, Sanon A, Lefevre T, Baldet T, et al. Effects of age and size on *Anopheles gambiae* s.s. male mosquito mating success. *J Med Entomol.* 2013 Mar;50(2):285–93.

18/ -line 104: 'a batch of 10 adults'. But some days you tested up to 16 adults. Please explain

When having designed the study, we planned to use batch of ten mosquitoes. However we had no choice to adapt the protocol according to the insectary production. This led to test 8 mosquitoes per days in average (2 to 16 per day). The method section was written based on the initial protocol leading to this error in the current manuscript. The sentence was modified as follow:

"On each experimental day, an average of 8 (2 to 16) female mosquitoes (7 to 9 days old) never fed with blood were randomly collected..."

19/ -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?

Supplementary figure 2 of [24] gives behavioral parameters for mosquitoes exposed to untreated nets, not the permethrin treated net. Median time of contact with treated nets was not shown in this latter article. However, we calculated from this dataset that the median time of contact with the permethrin net, before passing through the net hole was 33.6s as shown in the response to comment 1 of reviewer 1.

20/ -line 132: is there randomization of genotypes through time. Apparently not. How was made this choice?

We answered in response to comment 1.

22/ -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.

The sentence was modified as follow:

*"A mosquito (**whatever its KD status**) was scored as successful if it was fed at the end of the 10-min trial (whatever the amount of blood it took) and unsuccessful if it did not."*

23/ -line 165: replicates of what? Of the reading I suppose.

True, we update the sentence accordingly.

24/ -line 168: It would have been better to measure the mosquito size and to use use this measure among the explanatory variables. Do you have these data?

Please see answer to Comment 1.

25/ -lines 209-214: please provide the p-values (and not just <0.05) -line 216: please provide the p-value (not just >0.05)

Instead of the p-values, we now indicate supplementary Tables 1, 2, 4 and 5 that give the detailed results.

26/ -line 222: 30.2% KD among RR. Isn't it too large for RR individuals?

Please see response to comment 2.

27/ -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.

Please see response to comment 2.

28/ -line 312: correct diuresisprediuresis

Done

29/ -line 323-324: correct 'This suggestsThe whole picture'

Done

30/ -Figures and supplementary figure: provide the p-values and not just ns, *, *

Done

Reviewer 4

Reviewed by anonymous reviewer, 2019-07-12 17:12

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.

As pointed by the reviewer, it seems to be a strong relationship between KD phenotypes and feeding success. This was an expected results and we didn't test it. We therefore fitted a new model. We added KD phenotype as an explanatory variable (and interactions) in the binomial model of feeding success fitted only on mosquitoes exposed to the insecticides treatments (Permethrin and Deltamethrin). This allow us to compare feeding success between KD and non-KD mosquitoes of each genotype and for each treatment. We found that SS mosquitoes exposed to both treated nets and RS mosquitoes exposed to Deltamethrin experienced lower feeding success when KD (compared to non-KD). We were not able to find a significant difference for other combinations (for RR mosquitoes exposed to permethrin, the comparison was not possible since no mosquitoes were recorded as KD). See Supplementary Table 3.

Although our analysis of feeding success (fitted on all mosquitoes whatever their KD phenotype) is of great interest because overall feeding success is an important epidemiological parameter, it does not allow us to differentiate between the effect of KD and other pleiotropic effect of *kdr* on feeding.

Therefore, in order to preclude any effect of the KD phenotype on feeding success, we run a supplementary analysis with only non-KD mosquitoes. The results of this new analysis show the same trends that original analysis and therefore confirm our conclusions (Supplementary Figure 1 & 3).

Regarding now other behavioral parameters, as stated in the method section, we recorded behavioral parameters (feeding duration, number of probing events, probing duration, prediuresis duration and weight blood meal size) only in fed mosquitoes (those that complete the behavioral sequence of feeding). Among mosquitoes that fed successfully (N=231) only 14 were subsequently recorded as KD. This number of KD mosquitoes (belonging to 3 genotypes and 2 treatments) was unfortunately too small, not allowing to compare between KD and non-KD mosquitoes as we did for feeding success.

Regarding our conclusions relative to these behavioral parameters, they are less likely to be biased by the phenotype as KD mosquitoes represented only 6% (14/231) of the sample. To make sure, we re-run the analyses of feeding duration, number of probing events, probing duration, pre-diuresis duration and blood meal size on the dataset minus these 14 KD individuals. Changes are minor compared to analyses including KD mosquitoes and do not affect our discussion and conclusion (Supplementary Tables 8 to 14).

We added the following § in the method section (§ Statistical analysis):

*“In order to discriminate between the effect of KD phenotype and other pleiotropic effects of the *kdr* mutation on feeding success and behavioural parameters, we fitted all previously described models on the dataset but for KD mosquitoes. To complement the later analysis, we tested the effect of the KD phenotype on feeding success. For this task, we added KD phenotype and its interactions as explanatory variables in the binomial model of feeding success fitted only on mosquitoes exposed to the insecticide treatments (Permethrin and Deltamethrin). This allowed us to compare feeding success between KD and non-KD mosquitoes of each genotype and treatment.”*

The following was added in the Results section (§ Impact of insecticide exposure on feeding success):

At the end of the 1st §:

“When excluding KD mosquitoes from this analysis, all trends were kept (supplementary Figure 1) except that feeding success of SS mosquitoes was no longer reduced by permethrin exposure when compared to UTN ($OR_{perm-UTN}=0.78$ [0.18, 3.40]; supplementary Figure 1A). Indeed among SS mosquitoes, non-KD had higher feeding success than those recorded as KD ($OR_{nonKD-KD}=4.71$ [1.06, 20.9]; supplementary Table 1). The same was true for SS mosquitoes exposed to deltamethrin ($OR_{nonKD-KD}=22.5$ [2.59, 195]; supplementary Table 3).”

At the end of the 2nd §:

“We observed the same trends when excluding KD mosquitoes from the analysis (supplementary Figure 3).”

The following was added in the Results section (§ Impact of insecticide exposure on biting behaviour):

“Excluding 14 KD mosquitoes from the analyses of the biting behaviour parameters do not significantly change the results (supplementary Tables 8 to 14).”

The following was added in the Discussion section:

*“We found that KD reduced the feeding success of mosquitoes exposed to PYR insecticides, particularly in SS mosquitoes. However when analyzing feeding success and behavior of non-KD mosquitoes carrying the *kdr* mutation, we observed the same trends than we get when including KD mosquitoes. This indicates that observed differences in feeding success and behavior are therefore directly linked to the presence of the mutation and not only a consequence of the KD phenotype.”*

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.

We agree with the reviewer that the sentence is not fully supported by our results. Indeed, according (strictly) to our results, it is not permethrin ITN that enhances the feeding success of *An. gambiae* in the presence of resistance (because there is no significant difference in blood feeding success of RR mosquitoes exposed to untreated or permethrin treated net). The valid statement is that resistance with *kdr* mutation enhances the feeding success of *An. gambiae* exposed to permethrin ITN (as when exposed to permethrin ITN, RR mosquitoes had a higher feeding success than SS). We therefore change the sentences accordingly:

"Herein, we have completed the sequence by showing that PYR resistance with kdr mutation enhances the feeding success of An. gambiae exposed to permethrin ITN. The whole picture suggests that kdr mutation may increase vectorial capacity of An. gambiae populations in areas where permethrin ITNs are implemented."

Nevertheless, this modification doesn't affect our conclusions.

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.

We also change the sentence in the abstract accordingly:

"Exposure to deltamethrin ITN decreased the blood feeding success rate of RR and RS mosquitoes, whereas in presence of permethrin ITN, the kdr mutation increased the blood-feeding success of mosquitoes."

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?

We randomized treatments and genotypes over the week when possible giving the constraints imposed by the insectary and the breeding farm. An exception was made for the RS mosquitoes that were tested at the end of the experiment but with caution to randomize the treatments over the weeks (see answer to comment 1 of reviewer 3). In order to deal with possible unexplained variability, we used mixed-effect model with the date of the experiment as a random intercept. Date of the experiment are available in the joined dataset.

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 1S1B 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.

We thanks the reviewer to invite us to check every percentages. We did so and confirmed the 10/50 equals 20%, as shown in the figure.

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.

We thanks the reviewer for this comment. We disagree with the fact that we would not have discussed the differences in selective advantages or disadvantages conferred by the *kdr* mutation in the presence vs. absence of insecticides. Indeed, we believe that our discussion clearly go onto this subject. Particularly, the 2nd paragraph discussed how the genotype for the *kdr* mutation may affect the fitness in an untreated environment.

Regarding our statement that *“that RR mosquitoes [...] have a remarkable ability to find a hole into a bed net”*, it is exaggerated and do not correspond to the main results of the cited study that is that Heterozygotes RS mosquitoes have a higher ability to find a hole in an ITN than homozygotes genotypes. We therefore modify the sentence as follow:

“we evidenced that RR mosquitoes prefer a host protected by a permethrin-treated net rather than an untreated net [31] and that heterozygotes RS mosquitoes have a remarkable ability to find a hole into a bet net [24].”

Minor points

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

Please see response to comment 13 of reviewer 3.

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

Please see response to comment 17 of reviewer 3.

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?

It was one of operational aspect of the experiment: it's the time elapsed between exposure room to move to the experimental room to release the mosquito inside the behavioral assay.

4- Lines 257-258: is this conclusion valid for all 3 genotypes?

Yes. We added "*whatever the genotype*" at the end of the sentence

5- Figure 4: use the same scales for the y-axis - this would facilitate the comparisons between SS, RS and RR genotypes.

Figure 4 has been modified accordingly.

Typos

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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.

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

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

Done

Line 323: delete "This suggests".

Done

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

Corrected

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

Done

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

Done

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.

GF was replaced by TP that is defined in the legend.

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

Done