Round #1

Author's Reply:

by Stephanie Bedhomme, 2020-08-17 14:30
Manuscript: https://biorxiv.org/cgi/content/short/2020.06.08.139741v1

major revisions needed

Dear authors,

Three reviewers have now read your manuscript entitled "A novel assay for tracking carboxylesterase gene amplifications conferring organophosphate resistance in the mosquito Aedes aegypti: from experimental evolution to field application". The three of them acknowledged the quality and interest of your work but the three of them also pointed some problems, related both to the content and to the clarity of the manuscript.

I agree with the vast majority of the points they raised and would like to add that the manuscript lacks a bit of focus: the title announces a methodological paper ("A novel assay…") but the paper is actually combining field sampling and population analysis, artificial selection, gene expression and genomics and finally the development of a novel assay.

> → *We agree that the initial title was not well reflecting the content of the manuscript. We therefore modified the title as follows "A genomic amplification affecting a carboxylesterase gene cluster confers organophosphate resistance in the mosquito Aedes aegypti: from genomic characterization to high-throughput field detection". We believe that this new title better reflects the content of the manuscript while still remaining concise.*

It is not always easy to follow the logic of the choice of the populations for each analysis (e.g. why not using the "experimentally evolved population" for the whole genome sequencing?) and it gives the impression that a lot of data have been acquired but not exploited.

> → *We agree that the reasons for which the whole genome sequencing was performed on a different line as the one used for our laboratory selection experiment was not well justified in the previous version of the manuscript. In fact, we choose to perform the whole genome sequencing on the field resistant Thai population Nakh-R for several reasons:*
>
> *i) Previous studies conducted by our team detected the over-expression of this CCE gene cluster and the potential presence of a genomic amplification at this locus in the Nakh-R population (see Faucon et al 2015 and 2017).*
>
> *ii) As pointed out by reviewer 3, laboratory selection can lead to false positives because of drift effects. As the Thai resistant population Nakh-R was collected from the field, this allowed us to confirm that the results obtained from laboratory selection were not the consequence of genetic drift. Indeed, as genetic drift is a random effect, it would have been very unlikely that the same genomic amplification appeared randomly in our selected Laos composite population and also in a Thai field population less subjected to drift effects and showing a different genetic background.*

iii) At the time of the study, we did not have the financial resources to sequence the whole genome of the laboratory-selected, the control lines and a fully susceptible line (~3000 euros each) while the whole genome of the Nakh-R population and the full susceptible strain Bora-Bora were already generated by our team. However, the Nakh-R population was likely resistant to multiple insecticides (see Faucon et al 2017) suggesting that limiting the design as this pairwise comparison was not relevant. In addition, the Nakh-R population was sampled from a unique location meaning that this population was less representative of the diversity of duplication haplotypes that can be found in the SEA region. As a consequence, we decided to create the Laos composite population (made from populations sampled from various locations) and use it for our experimental selection experiment in order to clearly confirm the association of this CCE genomic amplification with organophosphate resistance.

In conclusion, although the logic of choice of a different strain for whole genome sequencing was not easy to follow in the previous version of the manuscript, we believe that such choice strengthens our conclusions, especially regarding the potential effect of genetic drift in our selection experiment (see our answer to reviewer 3). A sentence was added to the manuscript to better justifies the use of the field Nakh-R population for whole genome sequencing ("Whole genome sequencing" section).

For example, (1) the whole genome sequencing is only used for the coverage data and not for the sequences themselves when the sequences would probably give access to a more complete picture of the resistance mechanisms at work (SNPs for example),

→ *As described in the manuscript, previous studies on organophosphate resistance in this mosquito species pointed out the absence of the key target-site mutation G119S affecting the acetylcholinesterase (the known biochemical target of organophosphates) in this mosquito species because of strong genetic constraints (Weill et al 2004). This fact together with previous studies combining biochemical and molecular data clearly supported the potential key role of esterase-mediated detoxification in resistance of Ae. aegypti to organophosphate insecticides. Then, our RNA-seq data pointed out the over-transcription of multiple CCE genes in the malathion-selected line while our DNA-seq and qPCR data confirmed that this over-transcription was the consequence of a genomic duplication spanning this CCE gene cluster. Although we cannot exclude that other resistance mechanisms can contribute to the overall resistance phenotype observed (this is now clearly mentioned in the discussion section), our data strongly support the central role of this CCE gene duplication in organophosphate resistance. As this genomic duplication was not characterized and no diagnostic assays was developed to track it in natural populations, we made the choice to focus the manuscript on this specific genomic amplification.*

In addition, we totally agree that the analysis of polymorphism data such as SNPs can allow identifying resistance mechanisms and loci under selection. However, using such approach would necessitate the whole genome sequencing of multiple resistant and susceptible lines in order to reach enough power to differentiate between loci affected by drift, differential SNPs associated with hitchhiking effects and those functionally linked to the resistance phenotype. Furthermore, the pool-seq dataset that we generated did not provide access to individual genotypes which limits the methodological approaches that can be used for identifying regions under selection. Finally, as opposed to long read sequencing, short read sequencing was not the approach of choice for characterizing polymorphisms variations in duplicated regions as all reads obtained from duplicated copies map to the same locus in the reference genome. It is therefore difficult to exploit polymorphism data in duplicated

regions and to decipher if all copies are identical or not, or if a significant selection signature can be observed at this locus. Although of interest, answering these questions clearly necessitate additional sequencing work.

For all these reasons, we made the choice to not use polymorphism data generated by whole genome sequencing in the present study and limit the use of these data to coverage analysis.

(2) among the genes that present a differential expression, a lot are left aside and the study then focuses on three of them that had previously already been identified as involved in insecticide resistance. There is thus a high risk to ignore other genes involved in resistance.

→ *Although we cannot exclude that other resistance mechanisms can contribute to the resistance phenotype (e.g. cuticle modification), organophosphate resistance in Aedes mosquitoes has been mainly associated with increase expression of detoxification enzymes as the target-site mutation classically associated with organophosphate resistance is absent in this mosquito species (see above). In addition, previous molecular and biochemical studies showed that organophosphate resistance through increased detoxification or sequestration by carboxylesterases is frequent in mosquitoes.*

As pointed out by reviewers, among all genes found differentially transcribed in the resistant line as compared to both the non-selected line and the fully susceptible line, it is likely that some are false positives as a consequence of genetic drift, making the interpretation of their over-transcription difficult in absence of additional data suggesting their association with insecticide resistance in this mosquito species. Among the 24 over-transcribed genes that were associated with known physiological resistance mechanisms, only 7 genes (1 GST, 1 P450 and the 5 contiguous CCEs) were associated with detoxification. As opposed to CCE genes, the GST and P450 genes were also found over-transcribed in two other lines selected with insecticide from different families suggesting their non-specific association with the resistance phenotype observed. In support of this, the P450 and the GST genes were not previously found over transcribed in the field Nakh-R population resistant to organophosphates (Faucon et al. 2017).

As a consequence, instead of over-interpreting gene expression data (likely leading to a lack of focus of the manuscript), we choose to adopt a more conservative approach and focus the study on this CCE genomic amplification as multiple findings supported its key role in the resistance phenotype. Nevertheless, the discussion section has been modified in order to better acknowledge the potential contribution of other mechanisms in the resistant phenotype observed.

All these points have to be addressed before I can decide whether this manuscript can be recommended in PCI Evolutionary Biology.

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.

→ *Supplemental data are available in the "data/code" section in the BioRxiv version in this google drive link:*

https://drive.google.com/drive/folders/1hfgTbDI_KvlCUxuldKGScq7Psx7hKUbE?usp=sharing

-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 scripts used in this study have been deposited to Zenodo. The link to have an access is now specifying in the manuscript section "Data availability statement".*

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

→ *Details on all experimental procedures used in this study are already available to readers in the manuscript.*

-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" paragraph has been added to the manuscript*

Reviews

Reviewed by anonymous reviewer, 2020-07-29 06:10

I enjoyed reading this interesting study that identifies and characterizes an evolutionary route to insecticide resistance in the mosquito *Ae. Aegypti*. I have some suggestions, both general and specific that I hope will help improve the manuscript.

First, I found it a little difficult to keep track of all the populations/ individuals, both environmental and evolved, and what analysis was performed on which. I'd suggest going through the manuscript, and making sure that this is very clear. In some cases, appears that multiple naming conventions are used. For example, which population is NakR (see Fig 3)? Is this the Thai resistant population? Also, is the "Laos resistant population" the same are the experimentally evolved population G5-Mala?

→ *Yes, the Thai resistant population is Nakh-R population. This information was clarified in the caption of the Fig 3. The Laos resistant population is the experimentally evolved population. We clarified this point all along the manuscript, in particular in the method section.*

It's also not clear to me why some of the analyses appear to be done on G5 evolved populations and other analyses are done on G6 populations. This isn't outlined in the methods – can you clarify what was done here?

→ *Because multiple analyses were performed on the selected line (RNA-seq, multiple bioassays and CNV analysis) it was not possible to get enough biological material to perform all these analyses from a single generation. Therefore, we decided to performed bioassays on unexposed G5 individuals in order to asses constitutive inherited resistance phenotype after 4 generations of selection while CNV analysis by qPCR was performed on*

G5 survivors in order to better support their association with the resistance phenotype observed. RNA-seq was performed on unexposed G6 individuals in order to avoid gene induction/repression effects following insecticide exposure and only capture constitutive inherited differential expression. These differences in the nature of individuals used for CNV and RNA-seq might partially explain the lower transcription levels observed for CCE genes from RNA-seq (up to 10-fold) as compared to CNV data obtained from pools of individuals (up to 32-fold). One should note that this higher CNV ratio observed in G5 survivors also supports the association between these CCE genes and insecticide survival (see reviewer 3' comment about confirming the association of this CCE gene amplification with the resistance phenotype). The different generations used for each experiment are now clearly stated in the method section and a justification sentence was added in the RNA-seq section.

Second general suggestion/ question is regarding how comprehensive the TaqMan multiplex assay that the authors have developed is at detecting resistance in the field. The authors provide evidence that the assay does a great job of detecting individuals with CNV in three CCE genes identified as being important. However, are there other undetected evolutionary routes to resistance occurring these environmental populations that will go undetected? The authors have sampled a larger number of populations in SEA and assayed for CNV in CCE genes, but how many of those mosquitoes that don't have CNV in CCE genes are also resistant?

→ *As we cannot exclude that other resistance mechanisms can contribute to the resistance phenotype (see answer to editor above), the present study was restricted to characterizing the role of this CCE genomic amplification in organophosphate resistance. Therefore, the high-throughput diagnostic assay developed was not presented as a molecular assay allowing to track organophosphate resistance in this mosquito species but rather as a new tool to efficiently track this specific resistant mechanism. In addition, no bioassay data and dead/survivor samples were available for most field populations provided by our partners restricting their use to investigate the presence of other potential mechanism playing a significant role in the resistance phenotype.*

<u>Some more specific comments</u>:

Line 164: What does "calibrated" mean in this context?

→ *This means that individuals were reared in the same insectary conditions (including larval density and feeding regime) leading to individuals of the same size for bioassays and molecular analyses. This has been clarified in the manuscript.*

Figure 1: Did the G5-NS increase significantly compared to G1? Was there a statistical test performed here?

→ *The slight increase from G0 to G5-NS was not significant (P>0.05). A sentence was added in the manuscript to clarify this point.*

Paragraph starting at line 336. The authors focus on over-transcribed genes that are potentially associated with known resistance mechanisms. Is there any possibility that under-transcribed genes could have an effect on resistance?

→ *In line to our answer to the editor above, although we cannot exclude that a decrease expression of particular genes might also contribute to the resistance phenotype, to our knowledge such mechanism has never been pointed out in mosquitoes. In addition, as stated in the introduction section, Ach target-site G119S mutation is not present in this mosquito species due to genetic constraints supporting the overexpression of detoxification enzymes as a key resistance mechanism to organophosphates in this mosquito species. Finally, as pointed out by another reviewer, we cannot exclude that some differentially transcribed gene identified are not the consequence of genetic drift during the selection process. As a consequence, instead of over-interpreting gene expression data (likely leading to a lack of focus of the manuscript), we choose to adopt a more conservative approach and focus the study on this CCE genomic amplification as multiple findings supported its key role in the resistance phenotype. Nevertheless, the discussion section has been modified in order to better acknowledge the potential contribution of other mechanisms in the resistant phenotype observed.*

Also, were there any genes out of the candidate ones identified here that also had mutations that could be important? (Later, on line 670, the authors suggests that SNPs have been shown to be important for resistance in other studies – is this a possible additional mechanism in this study as well?)

→ *Non-synonymous SNP in insecticide target genes (known as target-site mutations) are frequently involved in resistance but such mutation does not occur in this mosquito species because of genetic constraint (see above and in the manuscript). SNP variations affecting transcriptional regulation or enhancing the affinity of an enzyme for its substrate may be also involved in resistance to insecticide. However as explained above, we believe that the genomic data generated for this study were not powerful enough to investigate SNP data in link with the resistance phenotype. Indeed, identifying SNP variations conferring resistance is not trivial as this requires distinguishing between differential SNPs resulting from genetic drift or genetic hitchhiking and those functionally associated with resistance through an adequate experimental design (i.e. including multiple resistant and susceptible lines or using functional validation tools) which was out of the scope of the present study. Finally, it cannot be excluded that non-synonymous mutations affecting these CCE genes may also contribute to resistance as multiple non-synonymous mutations associated with insecticide resistance were previously identified in these genes (see Cattel et al 2019, Evolutionary Applications) but we believe that investigating this in the present study was out of reach given the nature of NGS data and the experimental design.*

Figure 4B: We would expect to see both haplotypes in G1, correct? Type B must just be at very low frequency and so just wasn't detected in the sample. Or was haplotype B the result of a de novo mutation?

→ *Yes, as our experimental design rather selects for existing haplotypes than de novo mutations, we would expect to see both haplotypes in G1. Therefore, the non-detection of haplotype B in G1 was likely the result of sampling effect because individual carrying this duplication haplotype were present at a very low frequency (only 2 individuals out of 28 being positive for haplotype A).*

Lines 480-482: “No significant differences were observed between field populations and the different lines (G1, G5-NS, G5-Mala), suggesting that insecticide selection rather select for positive individuals than for individuals carrying a higher number of copies.” Maybe copy number does matter and the data reflect balancing selection for an intermediate copy number, not an absence of selection on copy number.

→ *We agree that an intermediate copy number may be favored through balanced selection but deciphering this would require additional experiment which could include the monitoring of copy number polymorphisms in regards of different selection pressures and/or the phenotypic comparison of different fixed lines each carrying different CCE copy number.*

Line 543: “integrated strategies aiming at preventing” should be changed to “integrated strategies aimed at preventing”

→ *We believe that this sentence was correct.*

Line 545: “threaten their efficacy.” Should be changed to “threatens their efficacy.”

→ *Done.*

Line 595: “including AAEL00113” Shouldn’t this be “including AAEL005113”?

→ *Yes, this is correct. This has been corrected in the manuscript*

Line 631-634: “… our experimental evolution approach demonstrated that the frequency of these resistance alleles increases rapidly in populations submitted to insecticide selection pressure while a susceptible allele is favored in absence of selection.” I don’t think the data actually demonstrate this. As far as I can tell there is no significant difference between the control and the G5-NS.

→ *Yes, this is correct. This sentence has been removed from the manuscript.*

Lines 672-673: “Further work is required to precise the interplay…” change to something like “Further work is required to precisely understand the interplay…”

→ *Done.*

Line 680: “In term of…” -> “In terms of…”

→ *Done.*

Reviewed by Diego Ayala, 2020-08-05 16:15

The manuscript by Cattel et al., has investigated the gene amplification profiles of carboxylesterase genes related to insecticide resistance in Aedes aegypti, major vector of arboviruses. This is an original and comprehensive study about the evolution of the metabolic resistance of organophosphates, worldwide used for vector control. The authors carried out a compelling work combining fieldwork, laboratory experiments and molecular works. Finally, the authors provide a new molecular tool to monitor insecticide resistance associated to copy number variation of a carboxylesterase gene (AAEL023844).

Overall, the study is well written and the main ideas are clearly structured and addressed. I have no found major issues and I enjoyed reading the manuscript. I really appreciated the discussion section, which examines different evolutionary aspects of the CCE, such as the duplication origin or the evolutionary dynamics of the amplifications. I mention that, because reading the title, you can expect "only a novel assay". Moreover, I do not think that the term "experimental evolution" can be applied to this work. To my knowledge, the authors applied the standard method to select insecticide resistance in mosquitoes, and they do not follow up CNV across generations with different malathion doses, or carry out fitness experiments.

→ *Although artificial selection can be considered as a form of "experimentally-controlled evolution", we agree that it does not fully complies with the term "experimental evolution" as classically used in other studies (mainly focusing on de novo mutations using pure lines). This term has thus been removed from the title of the manuscript. The title has also been modified to better reflect the whole content of the manuscript (see answer to editor above).*

Here, I list a number of minor points, which would help to improve the clarity of the manuscript. I refer to the version from bioRxiv prepint doi.org/10.1101/2020.06.08.139741:

.- line 100 : CCEae3A ◊ CCEAE3A

→ *Done.*

.- line 131 : Ae ◊ Aedes

→ *Done.*

.- lines 131- 132. How many breeding sites per site? Could a low number of breeding sites limit the genetic diversity of each population due to the presence of siblings in the same breeding site? Could the authors include this information in Table S1.

→ *11 different populations originating from different locations of Laos were used. Each population was composed of individuals sampled from 2 to more than 10 breeding sites within a 100 m distance. We believe that this standard sampling strategy was adequate for a relatively good sampling of the diversity occurring in Laos. This information has been added in Table S1.*

.- line 139. How were the adult females collected? larvae? adults?

→ *This information has been added to the manuscript.*

.- line 145: Could you define 'population'? it means you already pooled the larvae of the different breeding sites ? for how many generations ?

→ *Yes, the term population refers to individuals from multiple breeding sites located within a 100 m distance. F0 adults from all populations were pooled in equal proportion in order to generate the Laos composite population..*

.- line 150: 'thirty-three-days' , should it be 'three days' ?

→ *Done.*

.- line 154-155 : if the females freely mated before the insecticide selection assays, could it affect the dynamics of malathion resistance ? Why do you not expose the males? Less resistant?

→ *This is right, performing insecticide selection separately on virgin males and females and allowing survivors of both sexes to mate would likely faster the selection of resistance alleles. However, this procedure is significantly heavier as it requires separating males from females before they can mate. This would also requires using different insecticide doses for males and females (males are less tolerant) which is probably not representative of the insecticide selection pressure occurring in the field. In addition, using such procedure would likely increase drift effects which may lead to more false positives. In our case, mass selection was performed on ~1000 adults of mixed sex with a single insecticide dose leading to 90% female mortality in G1. A few surviving males were also observed (this point is now stated in the manuscript). It is true that some surviving females may have mated with susceptible males leading to the transmission of susceptible alleles in the offspring, but our data show that such procedure was still efficient at selecting resistance alleles initially present at low frequency in only four generations.*

.- line 305: Why the malathion results for G5-Mala are not equal between Fig 1 and Fig 2

→ *These bioassays have been performed on the same generation but with different batches of individuals. In figure 1 (monitoring through the selection process) the mean mortality rate observed is 52% and while the mean mortality shown in figure 2 (cross-resistance comparative bioassays) is 47%. This relatively small difference (5%) likely reflects the genetic variability still occurring in the G5 resistant population.*

.- line 401: Could you provide the % of each haplotype ? In Fig 4, it seems 100% Haplotype A

→ *This information has been added to the manuscript. Yes, all G1 positive individuals were carrying haplotype A. However, haplotype B was likely present in G1 but at a frequency being too low for its detection across 28 individuals.*

.- line 401-403: Could you provide the % of the two haplotypes for G5-NS ?

→ *Done.*

.- line 452: "Thai population resistant", missing reference

→ *A reference was added to the manuscript.*

.- line480: I guess that it is with the Sybergreen method ?

→ *Yes, this has been clarified in the manuscript.*

.- line 539: Why different numbers between TaqMan and Sybergreen ?

→ *The higher CNV obtained with the Sybrgreen qPCR assay as compared to the Taqman assay may be the consequence of a higher specificity of the Taqman assay as slightly different amplification primers were used to amplify the gene AAEL023844 in addition to TaqMan hybridization probes during the amplification process. Although this difference does not affect our findings, this suggests that the Taqman assay should be preferred for tracking this CCE genomic amplification in the field.*

.- Discussion:
- Do the authors consider that all the duplications are closely located such as in An. gambiae (Assogba et al., 2015)? If it is not the case, could it affect their results?

→ *Although our genomic data and the current Ae. aegypti genome assembly do not allow confirming the precise location of duplicated copies, a tandem duplication is probable. Anyway, because the high-throughput diagnostic tool developed to track this gene amplification is based on copies quantification rather than on the genomic structure of this amplification (e.g. amplification of duplication boundaries), this should not affect its efficiency to track this resistance marker in the field. Further studies using long read sequencing and/or genome mapping techniques will allow clarifying the genomic structure of this genomic amplification.*

- As the organophosphates are less and less used for vector control and replaced by the pyrenothroids, how do the authors explain the presence across the SEA sampled pops (lines 635-636) ? are they involved in other detoxification mechanism that could explain their persistence ?

→ *Organophosphates insecticides have been used worldwide since the 60' for controlling mosquitoes, especially in South-East Asia where they were still massively used until recently for larviciding (temephos) and spatial spraying against adults (malathion) such as in Laos. In addition, although these insecticides are now officially banned in many countries, they are probably still unofficially used locally. Finally, organophosphates are still authorized and heavily used in agriculture against pest insects which can still represent a significant selection pressure for mosquitoes in particular areas. All these factors may explain why organophosphate resistance are still circulating in mosquito populations from SEA (and probably in other regions of the world). Although it cannot be excluded that this CCE gene amplification is not involved in resistance to other insecticides, our data suggest that it does no confer cross-resistance to pyrethroids (at least to deltamethrin). Following the dynamics of this CCE gene amplification in regards of the implementation of alternative vector control strategies will be of interest to further assess its role in resistance to other insecticides and its associated genetic costs.*

- The authors say that the "carrying high gene copy number are not preferentially selected" (line 653-654), but looking at the Figure 6, the copy number are higher in natural populations than in G5-Mala.

→ *Although we agree that some field-collected individuals show higher copy number than in the G5-mala resistant line, our statistical analysis did not support a significant*

difference of copy number (for any CCE gene) between field populations and the G5-mala selected line. This was probably the consequence of the high copy number polymorphism observed within both laboratory and field populations.

Tables and Figures
.- Figure 6: If it is possible, change the colors since they are the same than for Haplotypes in the other Figures.

→ *Done.*

.- Table S1: Include geographical coordinates of each village

→ *Done.*

Reviewed by anonymous reviewer, 2020-07-23 16:53

This study investigates the importance of amplifications in CCE genes for resistance against organophosphate insecticides. The authors first find that selection with malathion in a line of Aedes aegypti mosquitoes leads to an increase in resistance over 5 generations. Using RNAseq, they then identify a set of seven detoxification genes over-expressed in the selected line and focus the study on a cluster of upregulated CCE genes, finding that these genes are the subject of amplifications in wild populations from South East Asia and in their selection lines, with the frequency of the amplified haplotypes increasing after selection. As part of this work, the authors have developed a Taqman assay to detect the presence of the CNV.

There are some nice results in this paper, but I find that throughout the manuscript the results are interpreted with too much conviction. I think the CNVs which the authors have found probably are as important as they claim, but this is mainly based on accumulated evidence from previous research. One of the main claims of the paper is that it "confirms" the importance of the CCE genes and their associated CNVs in insecticide resistance. This conclusion is too strong given the results. While I do believe that the CNVs are indeed associated with resistance, the results of a selection experiment with just one replicate for each line does not constitute proof. The possibility that the CNVs increased in frequency due to drift (given the strong bottleneck between generations) cannot be excluded. A better test would be to genotype the mosquitoes used in the bioassays to see whether the CNVs are at a higher frequency among the survivors. This would provide much more convincing evidence that the CNVs are indeed associated with resistance. This could even be done for the other organophosphates (assuming the sample size is large enough), which would then provide evidence that the CNVs are at least partly responsible for the cross-resistance observed in the study. Since this would provide clearer evidence of an association with resistance, it is not clear to me why this was not done.

→ *We agree that despite the large number of mosquitoes used for selection, our experimental selection design using a single replicate does not allow to fully control for genetic drift effects. However, the fact that this same CCE gene amplification was previously associated with organophosphate resistance in natural Ae. aegypti populations (see Faucon et al 2015, 2017, Poupardin et al 2014, Gouindin et al 2017, Marcombe et al 2009, …) but also in Aedes albopictus (Grigoraki et al 2016 and 2017) clearly refutes the role of genetic drift in its increase frequency in our selected line. We agree that it would have been interesting to*

compare the frequency of this marker in dead and survivors of our laboratory lines following insecticide exposure but this would still not have constituted a proof of its role in resistance as a marker could have no functional role in resistance but still be segregated between dead and survivors as a consequence of being genetically linked with another resistance locus. A better way to perform such genotype-phenotype association study would be to use individuals or lines obtained from controlled crosses with a susceptible strain (at least F2) in order to break (by recombination) genetic links between markers and segregate individuals with different insecticide doses before investigating their CCE amplification genotype. While we such experiment will likely confirm the association of this marker with resistance, we thought that performing such heavy experiment was not necessary given the multiple evidences (including functional validation) supporting its role in organophosphate resistance. In conclusion, while we agree that our study does not constitute an irrefutable proof of the role of this CCE gene duplication in resistance we believe that the data we generated together with the published literature on this subject constitute enough evidence to support the role of this marker in organophosphate resistance in this mosquito species.

Other points:

line 52: I don't understand the second half of this sentence. What does "their" refer to? Duplications? Insecticides? Why are either useful for monitoring resistance alleles? One is a resistant allele itself, and the other exerts the selective pressure to maintain the allele in the population.

→ *Yes, "their usefulness" in the second half of the sentence refers to duplications. For now, duplications are not classically used as resistance monitoring tools in the field in conjunction with bioassays (as opposed to target-site resistance mutations). Therefore, the confirmation of the role of gene duplications in insecticide resistance provides new opportunities to better track resistance in the field. This is especially true for organophosphate resistance in Ae. aegypti for which the selection of ace1 target-site mutation is submitted to a strong genetic constraint (Weill et al 2004) and no high-throughput DNA diagnostic tool exists to monitor metabolic resistance alleles.*

line 53: Replace "aimed at characterising" with either "aims to characterise" or "aimed to characterise".

→ *Done.*

line 56: I would replace "confirmed" with "was consistent with". Given that there was only one replicate of each of the selected and unselected lines, it is very difficult to prove that the increase in frequency of the CNV was not the result of random drift.

→ *Please refer to our previous answer about the unlikely role of genetic drift in the increased frequency of this CCE genomic amplification during selection and its interpretation given the multiple evidences existing in the literature.*

line 59: What does "large" mean in this case? Are you referring to the size of the genome covered by the amplification? Or to the number of extra copies? Is a CNV covering three genes really that large?

→ *According to our data, the size of this amplification is ~100 Kb. This sentence has been modified for better clarity.*

line 60: This sentence uses the word "amplification" in one place and "duplication" in another. Is the distinction important to the meaning of the sentence? If so, please clarify this distinction.

→ *As stated in the introduction section, the general term 'genomic amplification' was used when referring to a duplication event for which more than one duplicated copy are present. The term 'duplication haplotypes' was used when referring to structural variants (different boundaries and/or duplicated copy arrangements).*

lines 61-62: What do you mean by "high copy number variations"?

→ *This term was replaced by high copy number polymorphism for better clarity.*

line 65: change "shall" to "will"

→ *Done.*

lines 80-90: Can also add the neofunctionalisation of detox genes (Zimmer et al 2018)

→ *This has been added to the introduction section with reference Zimmer et al 2018.*

line 87: change "mosquitoes genera" to "mosquito genera"

→ *Done.*

lines 100 and 103: Is there a reason why CCEae3A is capitalised differently in these two sentences. It seems to me that in both cases, the names refer to genes (not proteins) and so should be written Cceae3a. However, I don't think it really matters as long as it is consistent (or there is an important distinction underlying the different capitalisation). On the other hand, on lin 106, I think this is referring to the protein, and so it is fine to leave it fully capitalised.

→ *In agreement with existing conventions, CCEAE3A has been written in capitalized italic when referring to the gene and not in italic when referring to the protein. This has been corrected through the whole manuscript.*

line 108: "this CCE amplification" is confusing here because it was last mentioned at the start of the paragraph, between which there are two more sentences referring to another species.

→ *This has been clarified in the manuscript.*

line 117: Again, this wording is too strong. Firstly, as before, with just a single replicate of each line, it cannot be excluded that the increase in frequency of these genes was a result of drift.

→ *Please refer to our previous answer about the unlikely role of genetic drift in the increased frequency of this CCE genomic amplification during selection and its interpretation given the multiple evidences existing in the literature.*

Second, even if that is not the case and these CNVs are associated with malathion resistance, it does not mean that they are the cause of the cross-resistance. Other resistance alleles could have also been selected, which could have caused the cross-resistance.

→ *This sentence has been toned down to better reflect this point.*

line 156: Here and elsewhere: correct spelling is "Three-day-old" when used as an adjective.

→ *This has been corrected throughout the whole manuscript.*

line 156: also "non-blood-fed"

→ *This has been corrected throughout the whole manuscript.*

line 168: change "other insecticide" to "other insecticides".

→ *Done.*

line 175: what are "calibrated" third instar larvae?

→ *This means that individuals were reared in the same insectary conditions (including larval density and feeding regime) leading to individuals of the same size for bioassays. This has been clarified in the manuscript.*

line 215: Please clarify that these 100 individuals were pooled into a single library for sequencing.

→ *Done.*

line 225: remove "were considered".

→ *Done.*

line 235: change "prior amplification" to "prior to amplification"

→ *Done.*

lines 236-237: why were no technical replicates run for individual samples?

→ *Preliminary qPCR experiments revealed that technical replicates were highly repeatable with very low CT variations. Therefore, we preferred maximizing the number of biological samples analyzed instead of performing multiple technical replicates per sample. In addition, the CNV threshold of 2.5-fold adopted for scoring an individual as positive for CCE duplication by qPCR was adopted in order to limit the number of false positives associated with qPCR technical variation.*

lines 237-238: commas used for decimal points here, but points used elsewhere.

→ *This has been corrected.*

line 247: 2.5 is an oddly specific threshold. On what grounds was it chosen?

→ *As mentioned above, the CNV threshold of 2.5-fold adopted for scoring an individual as positive for CCE duplication by qPCR was adopted in order to limit the number of false positives associated with qPCR technical variation (estimated < 2-fold from replicated negative control samples). Although this relatively high threshold may lead to a slight underestimation of CNV frequency, we though that adopting a more conservative approach would better support the interpretation of results. This is now better explained in the manuscript.*

line 247: It's not clear to me how the two different structural variations were distinguished? Is it that if all three genes were amplified, then haplotype A was assigned, but if only the first two genes were amplified, then haplotype B was assigned? If so, what would happen if, say, only one gene was found to be amplified, but not the other two?

→ *Yes, if all three genes were amplified, then haplotype A was assigned, but if only the first two genes were amplified, then haplotype B was assigned. If only one gene was found to be amplified, then another haplotype would have been assigned but this never occurred in our samples. This has been clarified in the method section.*

line 340: What are the "candidate genes"?

→ *Candidate genes were defined as any gene encoding a protein potentially associated with known insecticide resistance mechanisms (target-site resistance, cuticle alteration, detoxification, sequestration or altered transport). This has been clarified in the manuscript.*

line 351: "Invalidating" is a strong word here. I suggest "and may thus not be specifically associated with malathion resistance".

→ *This has been corrected in the manuscript.*

Figure 3B: Using raw coverage is not ideal here since the library size (total number of reads obtained from sequencing) may have been different for the two lines. Coverage should be normalised to the genome-wide average to properly compare these two plots.

→ *Although figure 2 does not intend to precisely quantify the mean CNV obtained between the two populations but rather to compare their coverage profiles at this locus (constant for Bora-Bora and showing a dramatic increased for Nakh-R). This figure has been modified in order to show normalized read coverage.*

line 382: change CEE to CCE

→ *Done.*

line 387: remove comma.

→ *Done.*

line 390: What is the evidence for this "slight elevation"? Was it significant?

→ *This slight elevation was not significant. This information has been added to the manuscript.*

lines 392-393: But this was not significant.

→ *Yes, this has been clarified in the manuscript.*

line 397: But the CNV is present, so how can we explain the lack of over-expression?

→ *This is an interesting point. As opposed to CNV obtained by qPCR, RNA-seq data indicate that CCEAE1A is not significantly over-transcribed in the G5-Mala population as compared to both G6-NS and the susceptible strain Bora-Bora. This difference may be due to sampling effects as CNV quantification was performed on G5-survivors while RNA-seq was performed on unexposed G6 individuals in order to avoid gene induction/repression effects caused by insecticide exposure. Therefore, as supported by the high CNV variation observed in figure 4, it is likely that a few G5 individuals surviving malathion were carrying this duplicated gene (haplotype A) while these individuals were barely absent in unexposed G6 individuals used for RNA-seq. Although we did not test this, it is possible that this gene is down-regulated in the Mala line leading to a reduction of costs associated with CCEAE1A over-expression. Though answering these questions would be interesting, we believe that this is not of key importance for the study as this gene appears to have a minor role in the resistance phenotype and is not targeted by our Taqman high-throughput diagnostic assay.*

line 399: I would say "at least two"

→ *This has been corrected in the manuscript.*

line 403: How did you distinguish the haplotypes? What were the criteria for calling haplotype A and haplotype B? How are the frequencies expressed? Are they the allele frequencies? If so, then the sum of the two haplotypes (33 + 66) should equal the cumulated frequency (84). Or are they the proportion of individuals that carry at least one copy of the haplotype, in which case this should be specified.

→ *See our previous answer above for the calling of different haplotypes. Following this calling procedure, among the 25 G5-Mala individuals tested we found 14 individuals with the haplotype B, 7 individuals with the haplotype A and 4 individuals not carrying CCE no amplification. This means we found 84% (21/25) individuals positives individuals for amplification (both haplotypes confounded). Among these, positives we found 67% (14/21) with the haplotype B and 33% (7/21) with the haplotype A. More details have been added to the manuscript to clarify this point.*

If the presence of haplotype B is determined based on the absence of a CNV in Cceae1a, is it possible to identify A/B heterozygotes? Or will the presence of A necessarily mask the presence of B? If it is not possible to identify both haplotypes in the same individual, then I again don't understand how the cumulated frequency can be smaller than the sum of the two haplotypes.

→ *See our previous answers about the calling of each haplotype and the estimation their population frequencies. In regards of differentiating between heterozygotes and homozygotes, it is true that our qPCR assay does not allow to do this. As a consequence, the data are presented as "population haplotype frequencies" rather than "population genotype frequencies".*

As pointed out in the manuscript, this CCE genomic amplification shows a high complexity both in terms of structural variations and copy number polymorphism. To our knowledge, developing a high-throughput diagnostic assay able to distinguish between homozygotes and heterozygotes for such complex genomic amplification is not trivial as even a multiplex PCR assay based on the amplification of duplicated copy boundaries would even not be able to do this correctly (e.g. same signal obtained for a heterozygote carrying one allele with 1 copy and another allele with 3 copies as for a homozygote carrying 2 alleles of 2 copies each). Though the full resolution of the genomic structure of this amplification might allow developing a better diagnostic assay, this will necessitate a significant research effort that was out of the scope of the present study. In addition, we believe that developing such assay would probably not provide a major improvement for resistance tracking in the field.

line 476: I can't find Figure S1 in the supplementary materials. Maybe I'm looking in the wrong place, in which case I apologise.

→ *Fig S1 has now been added to other supplementary materials in the google drive and can be accessed using the weblink provided in the manuscript: https://drive.google.com/drive/folders/1hfgTbDI_KvlCUxuldKGScq7Psx7hKUbE*

lines 480-482: I don't understand this sentence. Which comparison revealed no significant difference (also, please show the results of the statistical test, at least the p-value)?

→ *Please see our previous answer to reviewer 2 regarding statistical tests performed on individual CNV. P values of statistical tests have been added to the manuscript.*

The different lines were very different to each other, as were the populations, so how is it possible that NONE of the possible comparisons between field populations and the lines were significant?

→ *Please see our previous answer to reviewer 2 regarding statistical tests performed on individual CNV. The absence of significant effect between populations can be explained by the high copy number polymorphism observed within populations.*

Also, if selection is not selecting for higher numbers of copies, it seems very surprising that copy number could ever reach 80.

→ *Only two individuals were found with such high copy number in the field while the majority of positive individuals carried less copies. In addition, our selection experiment (which can only select for copy number alleles existing in G1) did not show a significant*

difference in copy number for any gene between the G5-Mala line and the G1 or G5-NS lines. P values have been added to the manuscript.

line 515: "no false negative was observed" is redundant given the first part of this sentence.

Also, could you comment on whether any false positives were observed?

→ *As stated in the manuscript, although the sybrgreen qPCR assay slightly over-estimated copy numbers, all individuals identified as positive using this assay were also found positive using the TaqMan assay (no false positives). Reciprocally, all individuals identified as negative using the sybrgreen qPCR assay were also negative using the TaqMan assay (no false negatives).*

line 554: As above, "confirmed" is too strong a word here.

→ *See our previous answers. We believe that "confirmed" is not too strong here.*

lines 567-568: This does not follow from the previous sentences, where CCEs were not mentioned.

→ *As this sentence is related to phenotypic data (bioassays), we believe that the term 'resistance alleles' as in the first sentence of the discussion is more appropriate here.*

lines 570-573: How can the level of resistance to temephos be compared to the other insecticides? Since the assays used are necessarily different (larval vs adult) with different concentrations of different insecticides, it is not clear whether a lower mortality against one insecticide can be equated with "higher" resistance. This is particularly the case since the baseline mortality to temephos was already much lower than it was to the other inseciticides. Thus, what would a similar level of resistance look like in terms of a change in mortality?

→ *You are right. Though mortality differences between two lines are usually maximized when using a dose close to the LD50 (as for temephos), comparing dose-response curves for each insecticide and calculating LD50 and RR50 would have probably been more informative. In addition, it is right that the comparison between adult and larval bioassays should be interpreted with caution. Considering this, the discussion sentence related to the different resistance levels of the selected line to temephos and other orgnaophosphates has been removed from the manuscript.*

line 578: It could still have a key role in resistance while offering cross-resistance to other insecticides.

→ *This is right, this sentence has been modified as "Though their role in insecticide resistance cannot be excluded, the over-transcription of this P450 and this GST in two other sister lines selected with insecticides from different families and showing no increased resistance to malathion does not support their key role in resistance to this insecticide"*

line 605: Why is "loci" italicised?

→ *This was corrected throughout the whole manuscript.*

line 630: change "is" to "might be".

→ *The end of this sentence has been deleted according to a comment from another reviewer.*

lines 632-633: I would again say that this is too strongly worded. Only one replicate line has shown that the frequency of the CCEs increased. Certainly the second claim of the sentence, stating that the wild-type allele is selected in the absence of insecticidal selection, cannot be made, since the difference between the unselected line and the parental generation was not significant.

→ *Please refer to our previous answer about the unlikely role of genetic drift in the increased frequency of this CCE genomic amplification during selection and its interpretation given the multiple evidences existing in the literature. The first part of the sentence has been tone down while the second part of the sentence about the wild type allele has been deleted from the manuscript.*

line 635: As above, the conclusion of "significant fitness costs" is not supported. At best, it can be written as a speculative suggestion.

→ *This has been modified.*

line 654: I don't understand the logic. If there is a balance between the fitness cost of high copy number and the benefit of increased resistance, you would still expect to see lower copy number in unselected samples, since the cost of higher copies would be higher and there would be no benefit.

→ *This sentence has been modified for better clarity.*

line 681: change "term" to "terms"

→ *Done.*

line 682: change "such CNV marker" to "such a CNV marker" or "a CNV marker such as this"

→ *Done.*