nature portfolio

Peer Review File

Varroa mites escape the evolutionary trap of haplodiploidy

Corresponding Author: Dr Nurit Eliash

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors investigate inheritance in Varroa mites using a 3-generation pedigree and whole-genome resequencing. They find that males are diploid clones of their mothers and that they transmit either maternal allele to their daughters randomly.

This is an impressive study involving a painstaking rearing protocol, a lot of genome sequencing, and discovery of segregating sites in a genetically depauperate inbreeding lineage. The results are remarkable. The authors have filled in a new square on the bingo card of imaginable genetic systems. I congratulate them on their achievement.

The manuscript is in excellent shape in almost every respect. I have just one area of concern. It seems to me that the authors have misidentified the short-term advantage of diploid arrhenotoky. The short-term advantage they cite is that “production of clonal males increases the females’ fitness by assuring transmission of both her genetic variants.” This does not seem plausible to me. Why should the female care whether the meiotic lottery happens in her own germline or in that of her clonal sons? Either way, each allele winds up in about half of her F2 descendents through sons. In any event, even if I’m missing something there, it seems to me that there are two larger short-term advantages:
1) She insulates her sons against the phenotypic effects of deleterious recessive alleles, including de novo recessive lethal mutations.
2) She hedges her bet on her son’s (and thus her daughters’ mate’s) genetic quality. A haplodiploid female producing a sib-mating brood including a single son will have hemiclonal F2 daughters: they will all have her son’s haploid genome, including whatever de novo deleterious mutations it may contain. She can hedge this bet by having two sons, but that comes at the expense of her fecundity — she gets more F2 descendents if she only has one son and produces an extra daughter instead. Producing a diploid son yields all the genetic benefits of a second son with none of the demographic cost.

Reviewer #2

(Remarks to the Author)
In the paper “Varroa mites escape the evolutionary trap of haplodiploidy”, Eliash et al., elegantly use a pedigree-based sequencing approach to show that the Varroa mite, an important honey bee parasite, is not haplodiploid but diplodploid. The current hypothesis, based on phylogenetic evidence, has been that Varroa mites are haplodiploid, in which males are haploid, and arise from unfertilized eggs, and females are diploid, arising from fertilized eggs. This genetic system has important implications for the level of genetic variation, with haplodiploids having smaller effective population sizes.

By tracking allele-inheritence through three generations of pedigrees, Eliash et al. show Varroa males are diploid, and produced by diploid arrhentoky, in which both maternal alleles are inherited and expressed. Furthermore, these maternal alleles are transmitted at random and with equal probability. Finally, using an analytical modelling approach, they show that Varroa mites lose less heterozygosity when inbreeding, resulting in higher effective population sizes.

This paper changes the current view of Varroa biology, and can help to explain its success as an invasive pest of honey bees. Furthermore, given the importance of genetic diversity in pest management systems, for instance in the evolution of pesticide resistance, or the development of genetic biocontrol, the observation of diploid arrhenotoky and the resulting higher level of genetic diversity in Varroa is an important finding. It also shows how careful observation can extend our knowledge on fundamental biological processes, such as reproduction.

The study is carefully designed and executed, and the results are sound. The results support the claims and conclusions

made by the authors, and I did not see any major flaws in the analysis, interpretation or conclusions. The methodology is sound and has been carefully described, and allows for replication of the results.

However, there are a few minor inconsistencies that need to be resolved, before this paper can be published:
• Figure 1 and the legend are confusing. While they are correct, the description of panel A and B refer to the F1, while I was drawn to the mite drawings for F0, which seem to indicate the opposite pattern. This can be prevented by indicating the genotype below the chromosome cartoon, and I would find it helpful to have the sexes indicated in each generation (or draw a box around all the females and one around the males for instance).The color coding gives this information, but the difference between F0 and F1 is confusing, especially as the main conclusions are drawn from the F1, it is better to give F1 more prominence in the figure.
• The legend of Figure 2 (l.623-624) is not correct. It does not describe the boxplot that is given in the figure.
• The y-axis of Figure 2 is labeled: “proportion of heterozygous sites”, but the scale gives percentages, not proportions.
• Figure 4: add male and female sign to the mites in the top row. And remove the box surrounding the blue chromosome in the male and in the right most fertilized egg.
• In Suppementary Table S2, the header is missing.
• In l. 126 (amongst others, this occurs throughout the manuscript), the authors speak of “Both alleles”, which to me implies a single locus, with two alleles. In reality, they investigated many SNPs for the presence of alleles from both parents. Similarly, in line 132, “males not only inherited two alleles, but randomly transmitted each allele to their daughters..”. In reality, they randomly transmitted both alleles across all investigated sites. Carefully look at the formulation, here and throughout the paper.
• In the methods (l. 268-269), it is not clear how the mites were maked on their dorsal idiosoma. What is PC-1 MR POSCA? Did the authors use a marker, or a pen? There seems to be some information missing.
• Similarly, it is not clear how the mites were marked onan acetate sheet (l.280).
• Table 1 only shows the VAF threshold, and not the QAF. These happen to be of similar values as the VAF, but they are different units, so add a column indicating the QAF.

Bart Pannebakker

Reviewer #3

(Remarks to the Author)
Review of “Varroa mites escape the evolutionary trap of haplodiploidy”
Summary
This is a study of the genetics of Varroa destructor, showing through a combination of controlled pedigree breeding and transcriptomics, that this species does not conform to either of the two suggested previous genetic systems of these species, haploid arrhenotoky or paternal genome elimination, but instead a novel genetic system, diploid arrhenotoky. In addition, they perform simulations to show that this genetic system loses genetic diversity more slowly than standard haploid arrhenotoky.
This is an important study that will be of importance to a broad range of researchers. As Varroa is a global threat to apiculture properly understanding its genetics is of central importance, both for modelling the efficacy of possible control schemes and making inferences about population structure. In addition, these findings will also be of great interest to those working on the origins and consequences of different genetic and sex determination systems, particularly in mites. It is rare that new genetic systems are discovered, especially in better studied species.

The core experiments that the authors perform are straightforward, and the results are clear and well presented. The inferences that the authors make from their experiments appear sound, although those working more directly with genetic data might be better placed than I to evaluate subtle flaws in their methodology. The authors also perform numerical simulations to show that diploid arrhenotoky loses genetic variation at a slower rate than under haploid arrhenotoky. Whilst I do not doubt their qualitative results, I think both the results in the main text, as well as their derivations in the Supplementary Material, could be made much clearer to support these points.

General points
1. Previous evidence. (L82) The authors seemingly dismiss previous karyotyping studies stating that: “In Varroa, haplodiploidy was inferred from two karyotyping studies31,32. Yet, as those studies examined Varroa embryos only, sex could not be directly determined.” I agree that karyotyping studies could be wrong, but I have two queries. (1) When these previous authors are finding haploid embryos, what is happening? Even if sex could not be determined, one would not expect to find haploid embryos based on the results presented here. (2) Varroa is highly stereotypical in the order in which sons and daughters are laid (the son first and then the daughters). Therefore, even if sex could not be directly assayed in these previous studies, I think most researchers would agree that comparing first laid vs second or third laid embryos, is a very good proxy for sex (e.g. Rehm & Ritter, 1989). Once again, I don’t think this takes away from the novelty here, but it is important to piece together the correct story. Perhaps Varroa destructor shows a distinct inheritance system to its sister species Varroa jacobsoni and, as these two were only distinguished properly in the 2000’s (Anderson & Trueman, 2000), these previous studies might have been working on a separate species. Alternatively, perhaps Varroa destructor has geographic variation in the form of arrhenotoky it exhibits? I think some comments trying to make sense of this previous data would be valuable.

2. Invasion success. (L107) “making its success in spite of genetic handicaps somewhat of a mystery”. Throughout, the authors imply that Varroa’s success adapting to different environments is a surprise given their genetics and sib-mating, but I

can't help feel somewhat conflicted. Is it obvious that this population structure and genetics reduces the rate of adaptation substantially? We know of lots of species – particularly pest and parasites – that exhibit highly inbred arrhenotoky and nonetheless are very successful at adapting to new environments (Wrensch et al., 1994 give the example of quill mites).

3. Benefits to diploid arrhenotoky. (L158): "In the short-term, the production of clonal males increases the females' fitness by assuring transmission of both of her genetic variants". I do not think this is correct. This implies that a female is more related to her sons under diploid than haploid arrhenotoky, which is not true. Secondly, whilst males are diploid in this system, due to the asymmetric transmission genetics, per individual they have the same evolutionary worth but distributed over two gene copies, rather than one.

4. Terminal fusion. (L193) The authors mention that central fusion may be a mechanism of producing heterozygous diploid males in mites. Whilst this is plausible, I think it is worth mentioning other alternatives, as central fusion seems somewhat rare. An alternative is terminal fusion with an inverted meiosis. This seems to be the mechanism in some of the thelytokous mites and, given many mites have holokinetic chromosomes, inverted meiosis is not so implausible (Wrensch et al., 1994). Is there any such cytogenetic evidence for either an inverted meiosis or holokinetic chromosomes in Varroa?

5. Diploid arrhenotoky. At no point in the article do the authors refer to other systems of diploid arrhenotoky that have been described, yet there is at least one known in the scale insects (Nur, 1972; Ross et al., 2010). Importantly, these diploid males appear to be formed from gamete duplication, and thus the males are entirely homozygous. There may be other further systems that I do not know of. Whilst I do not think that in anyway takes away from the novelty of what is discovered here, I think this is worth mentioning briefly. Perhaps could be introduced when some of the potential mechanisms of diploid asexual male production are discussed and the hypothesis of central fusion is described.

6. Sex determination. (L195) The authors briefly mention some of the challenges of sex determination, but I think they could add a bit more detail, without necessarily adding more words. Notably, the challenge with some known mechanisms of haplodiploid sex determination (e.g. complementary sex determination) is that they struggle with inbreeding. As the asymmetry between males and females is generated by heterozygosity at particular locus, then inbreeding and the subsequent homozygosity generates diploid males. Here we would have the potential opposite problem, diploid, heterozygous males would be expected to be female if this were the sex determination system. However, we know that Varroa is highly inbred and therefore would not likely have this system regardless. Other such systems as seen in parasitoid wasps use asymmetry in paternal-origin vs maternal-origin genomes, this could also work as a mechanism of sex determination in diploid arrhenotoky (e.g. Zou et al., 2020).

7. Figure 3 & Ne. It is currently very unclear what quantity is being plotted in Figure 3. The figure is described as showing the effective population size, yet no explicit expression is provided – either in the figure legend or in the Supplementary Material –linking the quantities calculated (e.g. identity probabilities, correlations, or path coefficients) to a formal definition of effective population size. In addition, the Methods state that the figure illustrates how effective population size changes with the degree of inbreeding, whereas the x-axis shows generations, creating a mismatch between the description and the presentation.
Because multiple definitions of effective population size exist (e.g. inbreeding vs. variance effective size; Caballero 1994), the authors should explicitly state: (1) which definition of effective population size they are using, and (2) how the quantities tracked in the Supplementary Material map onto that definition.
Alternatively, it may be clearer to instead present the decline in heterozygosity over generations, which is a directly interpretable population-genetic quantity. Presenting results in terms of heterozygosity decline would also make the predictions more obvious testable, for example by sequencing females from experimentally infected colonies over time and comparing observed heterozygosity trajectories to model expectations.

9. Comparison with diploidy. It would strengthen the paper to include a comparison with the equivalent diploid scenario, both to aid an understanding of the derivation, and to give the reader a feel for the relative importance of the effect of diploid arrhenotoky vs complete diploidy.

10. Notation and accessibility. The Supplementary Material uses Wright's path analysis framework to derive the decline in heterozygosity, but the presentation is very difficult to follow. The main issue is the heavy use of notation without an initial roadmap. It would greatly improve readability if the authors included, at the start of the Supplementary Material: (1) a table or boxed summary of symbols, (2) a clear statement of which biological quantities are being tracked, and (3) a short paragraph explaining the overall logic of the derivation before introducing the formalism. This would make the derivations far more accessible to readers who are not already deeply familiar with path analysis.

11. Demographic structure. The demographic setup underlying the model is difficult to interpret biologically. For example, early the authors state: "Each female has P probability of laying eggs in an empty blood cell and produces n daughters and 1 son. Assuming females laying eggs randomly in K blood cells…". It is unclear how to interpret this biologically. Does each female distribute her n offspring amongst these K cells? Does she lay K broods? If so, it seems like the intrinsic growth rate should be higher? Additionally, it sounds like multiple females can infect the same cell? But is there a limit upon the number of females that can infect a cell? Is there a distribution of brood numbers per cell? Because these assumptions directly determine the genetic structure of the population, the authors should provide a clearer biological description of the reproductive process and how it maps onto the mathematical model.
12. Grounding in simpler, known cases. Given the complexity of the derivations, it would help the reader if the authors showed how their framework connects to well-established results in simpler scenarios. For example, the first generation following infestation, when a simple family structure exists, should correspond to classical results for inbreeding and

heterozygosity decline in small family groups. Demonstrating that the model reproduces such known special cases would: validate the approach, and help readers understand how the current results extend previous theory. Explicitly identifying these limiting or special cases would make the work much easier to evaluate.

Minor points
Citation 2 – I think could easily cite a major popgen textbook, e.g. Hartl & Clark.
L31: “a major parasite” or “the major parasite”
L114: sp. “transmited”
Figure 1: Minor thing, but perhaps little arrow could be placed to emphasise that daughters are a mix of their parental genes, but sons are exclusively from mothers.
SML34: “number of blood cells where females can lay eggs” – Is this meant to be “brood cells” or am I missing something? Same with L36 & L37?

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I have reviewed the revision made by the authors following my comments on their original version. I am glad to see that all my comments have been addressed, and I have just one remark left:

The legend to the boxplot (Fig 2) is still not describing the boxplot properly. Please indicate what the box, whiskers and lines represent.

Other than that, I have no additional remarks to this version.

Reviewer #3

(Remarks to the Author)
This paper examines the genetic system of Varroa destructor. The authors show that, contrary to the assumed haploid arrhenotoky, this species displays a diploid form of arrhenotoky. Moreover, unlike a previously described form of diploid arrhenotoky, males retain the heterozygosity of their mothers. The authors show that both maternal gene copies appear to be expressed and transmitted evenly. They also present a theoretical analysis suggesting that this inheritance system increases effective population size relative to standard arrhenotoky.

I have no doubt that this paper will be important, both because of its practical relevance and because it describes a remarkable inheritance system. It is likely to stimulate further work on Varroa, and more broadly, to encourage closer study of the genetics of other species currently assumed to have conventional haplodiploidy.

I previously reviewed this manuscript. The authors have addressed my earlier comments on the main text well. In particular, they have added a useful discussion of previous cytogenetic results, possible mechanisms of clonal reproduction, and implications for sex determination. These points are now presented clearly. The new figures describing the system are also very helpful.
However, I still have some concerns about the theoretical analysis. The authors have made the analysis clearer, and I have no doubt about the qualitative conclusion that diploid arrhenotoky should lead to a higher effective population size than haploid arrhenotoky. As I mentioned previously, I think this point could have been made with a much simpler model, more directly comparable to classical results. Nevertheless, I understand the motivation for modelling a scenario closer to the biology of Varroa.

My main concern is with the comparison to diploidy in the Supplementary Figure. The authors state that the diploid values are substantially higher than those for both forms of arrhenotoky. This seems surprising, especially because the differences appear to be extremely large. It is not obvious to me why an otherwise comparable diploid life cycle should generate effective population sizes that are 100- or 1000-fold higher than the arrhenotokous cases. Moreover, whereas the arrhenotokous values appear to plateau with increasing census size, the diploid value continues to increase. This suggests either that there may be a computational issue, or that the diploid case is not being modelled with the same mating ecology as the arrhenotokous cases. I cannot identify the precise source of the discrepancy without reconstructing the full analysis, but I think this result requires careful checking.

If the authors can readily identify and correct this issue, or justify the diploid result with reference to comparable results from previous work, then that would address my concern. However, I would not want this point to delay publication of an otherwise excellent paper. If the issue is not straightforward to resolve, I suggest that the authors either remove the effective population size analysis from the current manuscript, or replace it with a simpler model or simulation that can be more easily checked. This would not substantially weaken the paper, because the main biological claims do not depend on the theoretical analysis. The verbal argument is already clear: males carry two maternal gene copies and transmit them evenly, which should help retain heterozygosity, especially when each mother mates with only one male. A more detailed and fully validated treatment of effective population size in this system could then form the basis of a valuable future theoretical paper.

RESPONSE TO REVIEWERS' COMMENTS

We are submitting a significantly revised manuscript that includes edited modelling results, which we hope will address concerns previously raised by the reviewers. You can see the full list of changes, and point-by-point responses in the responses to the reviewers below (in blue).

Reviewer #1 (Remarks to the Author):

The authors investigate inheritance in Varroa mites using a 3-generation pedigree and whole-genome resequencing. They find that males are diploid clones of their mothers and that they transmit either maternal allele to their daughters randomly.

This is an impressive study involving a painstaking rearing protocol, a lot of genome sequencing, and discovery of segregating sites in a genetically depauperate inbreeding lineage. The results are remarkable. The authors have filled in a new square on the bingo card of imaginable genetic systems. I congratulate them on their achievement.

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1) She insulates her sons against the phenotypic effects of deleterious recessive alleles, including de novo recessive lethal mutations.
2) She hedges her bet on her son's (and thus her daughters' mate's) genetic quality. A haplodiploid female producing a sib-mating brood including a single son will have hemiclonal F2 daughters: they will all have her son's haploid genome, including whatever de novo deleterious mutations it may contain. She can hedge this bet by having two sons, but that comes at the expense of her fecundity — she gets more F2 descendents if she only has one son and produces an extra daughter instead. Producing a diploid son yields all the genetic benefits of a second son with none of the demographic cost.

We entirely agree with this assessment and thank the Reviewer for helping us clarify this mechanism. Our original phrasing, "assuring transmission," was imprecise regarding the expected mean of allele transmission, but it stemmed from our calculations regarding genetic drift and variance during the F1 bottleneck.

Under standard haplodiploidy, if a foundress lays one son and 4-5 daughters, which is typical in *Varroa*, there is a high variance in allele transmission; if the single haploid son fails to inherit a

specific maternal allele by chance, there is a distinct probability that the allele is permanently lost from the sib-mating lineage. Under diploid arrhenotoky, because the son is a clone, he perfectly preserves both maternal alleles through the F1 bottleneck (dropping the probability of allele loss in his sperm pool to zero). We recognize now that this reduction in variance, namely protecting alleles through the bottleneck without changing the expected mean transmission, is exactly the demographic "bet-hedging" the Reviewer elegantly described.

We have revised the Discussion to incorporate both of the Reviewer's excellent suggestions. We now explicitly state that diploid arrhenotoky provides short-term advantages by (1) shielding sons from the phenotypic effects of deleterious recessive alleles, and (2) acting as a bet-hedge against genetic drift during the F1 bottleneck, allowing the female to minimize variance in her F2 genetic representation without the demographic cost of producing a second son.

Reviewer #2 (Remarks to the Author):

In the paper "Varroa mites escape the evolutionary trap of haplodiploidy", Eliash et al., elegantly use a pedigree-based sequencing approach to show that the Varroa mite, an important honey bee parasite, is not haplodiploid but diplodploid. The current hypothesis, based on phylogenetic evidence, has been that Varroa mites are haplodiploid, in which males are haploid, and arise from unfertilized eggs, and females are diploid, arising from fertilized eggs. This genetic system has important implications for the level of genetic variation, with haplodiploids having smaller effective population sizes.

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This paper changes the current view of Varroa biology, and can help to explain its success as an invasive pest of honey bees. Furthermore, given the importance of genetic diversity in pest management systems, for instance in the evolution of pesticide resistance, or the development of genetic biocontrol, the observation of diploid arrhenotoky and the resulting higher level of genetic diversity in Varroa is an important finding. It also shows how careful observation can extend our knowledge on fundamental biological processes, such as reproduction.

The study is carefully designed and executed, and the results are sound. The results support the claims and conclusions made by the authors, and I did not see any major flaws in the analysis, interpretation or conclusions. The methodology is sound and has been carefully described, and allows for replication of the results.

However, there are a few minor inconsistencies that need to be resolved, before this paper can be published:

• Figure 1 and the legend are confusing. While they are correct, the description of panel A and B refer to the F1, while I was drawn to the mite drawings for F0, which seem to indicate the opposite pattern. This can be prevented by indicating the genotype below the chromosome cartoon, and I would find it helpful to have the sexes indicated in each generation (or draw a box around all the females and one around the males for instance).The color coding gives this information, but the difference between F0 and F1 is confusing, especially as the main conclusions are drawn from the F1, it is better to give F1 more prominence in the figure.

Figure 1.
We have revised the figure and legend to improve clarity and to align the panel order with the Results text (a-d). In the updated figure, the two cross types are shown as separate columns, with panels “a” and “c” representing the representative family on one chromosome and panels “b” and “d” showing the pooled genotype proportions across all families and all seven chromosomes. We added a title above each cross, indicating the referred F1 crosses: cross 1 (AB × AA; panel B) and cross 2 (AA × AB; panel D). We also clarified in the legend that sites were first filtered for informative genotypes in F0 and F1 and that the genotypes of those same sites were then examined in F2 females and males. We added sex symbols again at the bottom panels, to help orientation. Finally, we updated the notation for F0 males: because they were not collected, their genotypes are now shown in gray rather than as inferred allele combinations. We hope these changes make the logic of the analysis and the figure organization clearer to the reader.

• The legend of Figure 2 (l.623-624) is not correct. It does not describe the boxplot that is given in the figure. We have corrected the figure’s legend

• The y-axis of Figure 2 is labeled: “proportion of heterozygous sites”, but the scale gives percentages, not proportions. We have changed the y-axis to *proportions*, instead of *percentages*

• Figure 4: add male and female sign to the mites in the top row. And remove the box surrounding the blue chromosome in the male and in the right most fertilized egg. We have corrected the figure as suggested

• In Suppementary Table S2, the header is missing. We confirmed the presence of a header in table S2.

• In l. 126 (amongst others, this occurs throughout the manuscript), the authors speak of “Both alleles”, which to me implies a single locus, with two alleles. In reality, they investigated many SNPs for the presence of alleles from both parents. Similarly, in line 132, “males not only inherited two alleles, but randomly transmitted each allele to their daughters..”. In reality, they randomly transmitted both alleles across all investigated sites. Carefully look at the formulation, here and throughout the paper.
We agree that the phrasing "both alleles" or "two alleles" implies a single locus with two variants, whereas our data track allelic states across thousands of SNP sites genome-wide.

We have revised the relevant sentences throughout the manuscript to reflect this distinction. We replaced *"...both of their mothers alleles" with " …both copies of their mother's genome"* throughout the text.

• In the methods (l. 268-269), it is not clear how the mites were maked on their dorsal idiosoma. What is PC-1 MR POSCA? Did the authors use a marker, or a pen? There seems to be some information missing. We have added clarifying wording in the Methods: *"the mites were marked on their dorsal idiosoma using a water-based paint marker pen (PC-1MR POSCA, Japan)"*

• Similarly, it is not clear how the mites were marked onan acetate sheet (l.280).
We have added clarifying wording in the Methods: "*In detail, six hours before infestation, the location of cells containing a 5th instar larva (about to be sealed) was marked. This was done by overlaying and pinning an acetate sheet on the wooden frame of a low-mite hive. Each target cell was located visually, and its position was traced on the acetate sheet by drawing a small circle at the corresponding cell location with a permanent marker."*

• Table 1 only shows the VAF threshold, and not the QAF. These happen to be of similar values as the VAF, but they are different units, so add a column indicating the QAF. We apologize for the lack of clarity. Although VAF and QAF are computed from different quantities (read counts vs. summed base-quality scores), both are expressed as frequencies on a 0–1 scale (see Methods, lines 349–355). Therefore, the same numerical thresholds apply to both measures. To clarify this, we have updated the column title in Table 1 to "VAF *and QAF* thresholds" and added a note to the caption explaining that these thresholds are used identically for VAF and QAF.

Reviewer #3 (Remarks to the Author):

Review of "Varroa mites escape the evolutionary trap of haplodiploidy"
Summary
This is a study of the genetics of Varroa destructor, showing through a combination of controlled pedigree breeding and transcriptomics, that this species does not conform to either of the two suggested previous genetic systems of these species, haploid arrhenotoky or paternal genome elimination, but instead a novel genetic system, diploid arrhenotoky. In addition, they perform simulations to show that this genetic system loses genetic diversity more slowly than standard haploid arrhenotoky.
This is an important study that will be of importance to a broad range of researchers. As Varroa is a global threat to apiculture properly understanding its genetics is of central importance, both for modelling the efficacy of possible control schemes and making inferences about population structure. In addition, these findings will also be of great interest to those working on the origins and consequences of different genetic and sex determination systems, particularly in mites. It is rare that new genetic systems are discovered, especially in better studied species.

The core experiments that the authors perform are straightforward, and the results are clear and well presented. The inferences that the authors make from their experiments appear sound,

although those working more directly with genetic data might be better placed than I to evaluate subtle flaws in their methodology. The authors also perform numerical simulations to show that diploid arrhenotoky loses genetic variation at a slower rate than under haploid arrhenotoky. Whilst I do not doubt their qualitative results, I think both the results in the main text, as well as their derivations in the Supplementary Material, could be made much clearer to support these points.

General points

1. Previous evidence. (L82) The authors seemingly dismiss previous karyotyping studies stating that: “In Varroa, haplodiploidy was inferred from two karyotyping studies31,32. Yet, as those studies examined Varroa embryos only, sex could not be directly determined.” I agree that karyotyping studies could be wrong, but I have two queries. (1) When these previous authors are finding haploid embryos, what is happening? Even if sex could not be determined, one would not expect to find haploid embryos based on the results presented here. (2) Varroa is highly stereotypical in the order in which sons and daughters are laid (the son first and then the daughters). Therefore, even if sex could not be directly assayed in these previous studies, I think most researchers would agree that comparing first laid vs second or third laid embryos, is a very good proxy for sex (e.g. Rehm & Ritter, 1989). Once again, I don’t think this takes away from the novelty here, but it is important to piece together the correct story. Perhaps Varroa destructor shows a distinct inheritance system to its sister species Varroa jacobsoni and, as these two were only distinguished properly in the 2000’s (Anderson & Trueman, 2000), these previous studies might have been working on a separate species. Alternatively, perhaps Varroa destructor has geographic variation in the form of arrhenotoky it exhibits? I think some comments trying to make sense of this previous data would be valuable.

We thank the reviewer for this important observation. We address both points below.

(1) What is happening when previous authors find haploid embryos?

We take these cytological observations seriously and agree they require explanation rather than dismissal. We have therefore added a dedicated paragraph to the Discussion that directly confronts this tension:

“Our finding that adult Varroa males are functionally diploid creates an apparent tension with the karyotyping studies of Steiner et al. (1982) and De Ruijter & Pappas (1983), which identified haploid (n = 7) embryos at developmental stages corresponding to the timing of male offspring. Notably, both studies are independently corroborated: they were conducted on different continents with different staining methods, and the association with male egg-laying order provides a meaningful indirect link to sex. It is therefore possible that a somatic ploidy reduction occurs at an early point during male development. This would have a partial precedent in phytoseiid mites, where paternal genome elimination (PGE) produces functionally haploid male somas from diploid embryos[1]. However, the Varroa case would be mechanistically distinct from PGE: our pedigree data show that males transmit both alleles in a true Mendelian manner, with no evidence of parent-of-origin-specific allele loss. Whether the embryonic n = 7 reflects a genuine ploidy transition during male development, through genome reduction, diploidization, or

differential endoreduplication[2,3], or a cytological artifact of the preparation method, cannot be resolved from the available data and warrants dedicated investigation."

(2) Egg-laying order as a proxy for sex

We fully agree with the reviewer that egg-laying order constitutes a meaningful, if indirect, proxy for sex in Varroa, and we apologise that our original phrasing appeared to dismiss this. We have revised the Introduction to acknowledge this explicitly:

"In Varroa, haplodiploidy was inferred from two cytogenetic studies that identified haploid (n = 7) and diploid (2n = 14) embryos in reproducing adult female mites[4,5]. De Ruijter & Pappas (1983) further observed that early-laid eggs, corresponding to the male, which is mostly the first offspring produced, yielded haploid embryos, while later eggs were diploid. Together with reports that unmated females can produce only males[6], these findings provided reasonable, if indirect, evidence for arrhenotokous haplodiploidy rather than paternal genome elimination (PGE). "

Regarding the possibility that the studies examined different species

We consider it unlikely that the earlier cytogenetic studies and our pedigrees were conducted on different species. Although *V. destructor* and *V. jacobsoni* were only formally distinguished in 2000, taxonomic and review papers agree that pre-2000 reports of "*V. jacobsoni*" on *Apis mellifera* in fact correspond to *V. destructor* [7–9]. The karyotyping studies we cite were carried out on mites collected from *A. mellifera* colonies in South America and Greece, regions where only *V. destructor* has been reported. Thus, the most parsimonious interpretation is that all studies concern the same species. To make this explicit for readers, we added a brief clarifying sentence to the Introduction:

"Although these studies referred to their material as V. jacobsoni, subsequent taxonomic work has shown that pre-2000 reports of "V. jacobsoni" on Apis mellifera actually correspond to V. destructor [7–9], so they are directly comparable to our data. "

Regarding the reviewer's suggestion of geographic variation in the form of arrhenotoky within *V. destructor:* while sex determination systems can vary between closely related species, intraspecific variation in such a fundamental reproductive mechanism (particularly a switch between haplodiploidy and functional diploidy) would be extraordinary and is without precedent in the literature, in both vertebrates and invertebrates [10–12]. We therefore consider this an unlikely explanation. We note that the reviewer's suggestion is nonetheless an interesting hypothesis, but we have no data to address it at this stage and it is beyond the scope of the current manuscript.

2. Invasion success. (L107) "making its success in spite of genetic handicaps somewhat of a mystery". Throughout, the authors imply that Varroa's success adapting to different environments is a surprise given their genetics and sib-mating, but I can't help feel somewhat conflicted. Is it obvious that this population structure and genetics reduces the rate of adaptation

substantially? We know of lots of species – particularly pest and parasites – that exhibit highly inbred arrhenotoky and nonetheless are very successful at adapting to new environments (Wrensch et al., 1994 give the example of quill mites).

We note that the Introduction already acknowledges that haplodiploidy is not straightforwardly disadvantageous, stating:
"*Haplodiploid species have a smaller effective population size relative to diplodiploid systems, though haplodiploidy may confer long-term advantages in inbreeding systems such as more effective purging of deleterious alleles"* (L~90–93).

The reviewer is correct, however, that the phrasing at L107: *"making its success in spite of genetic handicaps somewhat of a mystery",* overstates the case and implies haplodiploidy is incompatible with success, which is not our intention. We have revised the sentence in place to remove this framing:
"Yet, despite strong circumstantial evidence for arrhenotokous parthenogenesis in Varroa, its actual mode of genetic inheritance has never been directly tested, limiting our understanding of how this parasite maintains evolutionary potential under repeated bottlenecks."

3. Benefits to diploid arrhenotoky. (L158): “In the short-term, the production of clonal males increases the females’ fitness by assuring transmission of both of her genetic variants”. I do not think this is correct. This implies that a female is more related to her sons under diploid than haploid arrhenotoky, which is not true. Secondly, whilst males are diploid in this system, due to the asymmetric transmission genetics, per individual they have the same evolutionary worth but distributed over two gene copies, rather than one.

The reviewer is entirely right on both counts. Under diploid arrhenotoky, a female's relatedness to her sons is 1.0 (identical to haploid arrhenotoky) so the original phrasing incorrectly implied a transmission advantage at the individual level that does not exist. Furthermore, although males carry two alleles, each is transmitted with probability 0.5 to daughters, so the per-allele evolutionary worth is unchanged relative to haplodiploidy. The advantage of diploid arrhenotoky operates at the population level, not the individual level, and is already correctly captured in our modeling (Fig. 3): diploid males slow the loss of heterozygosity under sib-mating by reducing the correlation between uniting gametes compared to haploid males. We have revised the relevant sentences in the Discussion accordingly:

"In the short-term, the production of diploid clonal males slows the loss of heterozygosity under sib-mating relative to ancestral haplodiploidy, by introducing Mendelian segregation through the male lineage and thereby reducing the correlation between uniting gametes (Fig. 3). This strategy can overcome the 'paradox of sex' by blending the benefits of sexual and asexual reproduction. Namely, females produce diploid sons that inherit both copies of their genome, while daughters are produced sexually via meiosis."

4. Terminal fusion. (L193) The authors mention that central fusion may be a mechanism of producing heterozygous diploid males in mites. Whilst this is plausible, I think it is worth

mentioning other alternatives, as central fusion seems somewhat rare. An alternative is terminal fusion with an inverted meiosis. This seems to be the mechanism in some of the thelytokous mites and, given many mites have holokinetic chromosomes, inverted meiosis is not so implausible (Wrensch et al., 1994). Is there any such cytogenetic evidence for either an inverted meiosis or holokinetic chromosomes in Varroa?

We agree that central fusion is not the only plausible automictic mechanism. Terminal fusion combined with inverted meiosis has been proposed for, and supported in, some thelytokous mites and other lineages with holokinetic chromosomes, where it can restore diploidy in unfertilized eggs while retaining substantial heterozygosity [2,13]. We have therefore broadened the relevant part of the Discussion to state that both central fusion and terminal fusion with inverted meiosis are possible mechanisms by which unfertilized eggs could give rise to diploid males in Varroa, without favouring one over the other in the absence of direct cytogenetic evidence for this species. We have broadened this paragraph substantially, also in response to comments 5 and 6, and refer the reviewer to the revised fourth paragraph in the Discussion for the full text.

With respect to the specific question about Varroa, there is currently no direct cytogenetic evidence for holokinetic chromosomes or inverted meiosis in this species. The only detailed karyotype available [4] describes chromosomes with well-defined centromeres and monokinetic behaviour, rather than holokinetic chromosomes. At present we therefore treat central fusion and terminal fusion with inverted meiosis as alternative, mechanistically plausible scenarios suggested by work in other taxa, and we refrain from favouring one over the other in Varroa until dedicated cytological data become available.

5. Diploid arrhenotoky. At no point in the article do the authors refer to other systems of diploid arrhenotoky that have been described, yet there is at least one known in the scale insects (Nur, 1972; Ross et al., 2010). Importantly, these diploid males appear to be formed from gamete duplication, and thus the males are entirely homozygous. There may be other further systems that I do not know of. Whilst I do not think that in anyway takes away from the novelty of what is discovered here, I think this is worth mentioning briefly. Perhaps could be introduced when some of the potential mechanisms of diploid asexual male production are discussed and the hypothesis of central fusion is described.

We thank the reviewer for pointing out these important examples from scale insects. We now explicitly acknowledge them and contrast them with the Varroa system. Nur (1972) and later syntheses by Ross et al. (2010) describe diploid arrhenotoky in scale insects, in which males develop from unfertilized eggs but become diploid via fusion of haploid cleavage nuclei (“gamete duplication”), producing homozygous or functionally haploid males[14,15]. In those systems, males do not transmit two distinct alleles in a Mendelian fashion. This contrast with scale insects is already addressed in our response to comment 5 above, and can be found in the revised fourth paragraph in the Discussion.

6. Sex determination. (L195) The authors briefly mention some of the challenges of sex determination, but I think they could add a bit more detail, without necessarily adding more words. Notably, the challenge with some known mechanisms of haplodiploid sex determination (e.g. complementary sex determination) is that they struggle with inbreeding. As the asymmetry between males and females is generated by heterozygosity at particular locus, then inbreeding and the subsequent homozygosity generates diploid males. Here we would have the potential opposite problem, diploid, heterozygous males would be expected to be female if this were the sex determination system. However, we know that Varroa is highly inbred and therefore would not likely have this system regardless. Other such systems as seen in parasitoid wasps use asymmetry in paternal-origin vs maternal-origin genomes, this could also work as a mechanism of sex determination in diploid arrhenotoky (e.g. Zou et al., 2020).

We agree that some common haplodiploid sex-determination systems are unlikely to apply to Varroa. In particular, complementary sex determination (CSD) requires diploid heterozygotes at a sex locus to develop as females, with haploids and diploid homozygotes developing as males. Under inbreeding this leads to many diploid males, which are usually inviable or sterile and increase extinction risk [16,17]. In Varroa, populations are highly inbred, males are fully viable, and our data show that adult males are often heterozygous at many loci while still developing as males, so a CSD-type mechanism is very unlikely. We have added a clarification to the Discussion stating explicitly that classical complementary sex determination does not fit the biology of Varroa:

"*Our results are not compatible with classical complementary sex determination: Varroa highly inbred populations would generate many diploid males, and our diploid heterozygous males would be predicted to develop as females under that system."*

We also agree that mechanisms based on parent-of-origin asymmetry between maternal and paternal genomes, as seen in parasitoid wasps, are a more plausible analogy. For example, in *Nasonia vitripennis* a paternally expressed factor instructs female development [18]. We now mention such parent-of-origin–based systems, alongside epigenetic modification, genomic imprinting, and endosymbionts, as possible models for sex determination in Varroa:

"*A maternal factor is therefore more likely responsible, for example epigenetic modification, genomic imprinting, interactions with endosymbionts, or female control over fertilization and diploidy restoration."*

We have also briefly noted, for completeness, that diploid arrhenotoky in scale insects differs mechanistically from Varroa, as it produces homozygous or functionally haploid males via gamete duplication rather than the heterozygous, Mendelian-transmitting males we describe here.

7. Figure 3 & Ne. It is currently very unclear what quantity is being plotted in Figure 3. The figure is described as showing the effective population size, yet no explicit expression is provided –

either in the figure legend or in the Supplementary Material –linking the quantities calculated (e.g. identity probabilities, correlations, or path coefficients) to a formal definition of effective population size. In addition, the Methods state that the figure illustrates how effective population size changes with the degree of inbreeding, whereas the x-axis shows generations, creating a mismatch between the description and the presentation.

We have now updated the figure to focus on effective population size rather than reduction in heterozygosity, and revised the methods to reflect that. However, the effective population size is directly linked to heterozygosity using this relationship:

$$N_e = -\frac{1}{2(\frac{p_t}{p_{t-1}} - 1)}$$

where p_t is heterozygosity at generation t (see Mathematical Modelling in Methods), so the results equivalent whether expressed as Ne or as heterozygosity decline. The figure now appears as Supplementary figure 1.

Because multiple definitions of effective population size exist (e.g. inbreeding vs. variance effective size; Caballero 1994), the authors should explicitly state: (1) which definition of effective population size they are using, and (2) how the quantities tracked in the Supplementary Material map onto that definition.
Alternatively, it may be clearer to instead present the decline in heterozygosity over generations, which is a directly interpretable population-genetic quantity. Presenting results in terms of heterozygosity decline would also make the predictions more obvious testable, for example by sequencing females from experimentally infected colonies over time and comparing observed heterozygosity trajectories to model expectations.

We used Wright's classic approach which produced the famous equations for diploid and haploid effective population sizes found in every population genetics textbook:

- Diploid Species: Ne = (4 * Nm * Nf) / (Nm + Nf)
- Haplodiploid Species: Ne = (9 * Nm * Nf) / (4 * Nm + 2 * Nf)

This has now been clarified in the SM "model overview" section, namely that effective population size is calculated by comparing relative loss rate in heterozygosity over a generation to the rate in random mating diploid population which is -1/2/N. So -1/loss rate gives the effective population size which is the size as if the population was random mating. Specifically, we use the inbreeding effective population size (Caballero 1994) [19], defined as the size of a randomly mating diploid population that would lose heterozygosity at the same per-generation rate

9. Comparison with diploidy. It would strengthen the paper to include a comparison with the equivalent diploid scenario, both to aid an understanding of the derivation, and to give the reader a feel for the relative importance of the effect of diploid arrhenotoky vs complete diploidy.

We now included the complete diploidy scenario – it indeed has much higher effective population size than either haplodiploidy or the Varroa mechanism, as first discovered by Wright.

10. Notation and accessibility. The Supplementary Material uses Wright's path analysis framework to derive the decline in heterozygosity, but the presentation is very difficult to follow. The main issue is the heavy use of notation without an initial roadmap. It would greatly improve readability if the authors included, at the start of the Supplementary Material: (1) a table or boxed summary of symbols, (2) a clear statement of which biological quantities are being tracked, and (3) a short paragraph explaining the overall logic of the derivation before introducing the formalism. This would make the derivations far more accessible to readers who are not already deeply familiar with path analysis.

We have now added Box S1 to summarise notation, an introduction paragraph to explain the overall logic of the derivation, and a detailed demonstration of the analysis using haplodiploidy scenario (the simplest scenario) as example.

11. Demographic structure. The demographic setup underlying the model is difficult to interpret biologically. For example, early the authors state: "Each female has P probability of laying eggs in an empty blood cell and produces n daughters and 1 son. Assuming females laying eggs randomly in K blood cells…". It is unclear how to interpret this biologically. Does each female distribute her n offspring amongst these K cells? Does she lay K broods? If so, it seems like the intrinsic growth rate should be higher? Additionally, it sounds like multiple females can infect the same cell? But is there a limit upon the number of females that can infect a cell? Is there a distribution of brood numbers per cell? Because these assumptions directly determine the genetic structure of the population, the authors should provide a clearer biological description of the reproductive process and how it maps onto the mathematical model.

We have now set up the demographic setup more carefully.
This includes a clear explanation that

1. Each female randomly picks a brood cell and lay all her offspring in the brood cell.
2. Multiple females can infect the same cell. In the previous derivation, we ignored cases with more than two females infesting the same brood cell, because the probability of these cases is relatively small. In the revision, we improved the derivation by considering all possible cases.
3. In the previous analysis, the number of cells is determined by carrying capacity K. In the revision, we now separately model the number of brood cells (K) and the carrying capacity (C). We set C=2K in the analysis, to reflect the observation that on average about 2 females co-infest a brood cell.
4. We did not model the brood numbers. Instead, we assume all the brood cells are identical.

To help readers understand how the mathematical model is linked to the demographic setup, we added two paragraphs to show in details how to calculate the probability of each case for the correlation between uniting gametes f and for the correlation in gametes between two females f_f.

12. Grounding in simpler, known cases. Given the complexity of the derivations, it would help the reader if the authors showed how their framework connects to well-established results in simpler scenarios. For example, the first generation following infestation, when a simple family structure exists, should correspond to classical results for inbreeding and heterozygosity decline in small family groups. Demonstrating that the model reproduces such known special cases would: validate the approach, and help readers understand how the current results extend previous theory. Explicitly identifying these limiting or special cases would make the work much easier to evaluate.

We now added a section to show the special cases in the SM.

Minor points
L31: “a major parasite” or “the major parasite”.
Because our study specifically addresses how invasive species maintain adaptability, we consider it important to emphasize the global invasive spread of Varroa. We have therefore revised the sentence to read: “...Varroa mites (*Varroa destructor*), the globally invasive honey bee parasite.”

L114: sp. “Transmited”.
We corrected to “transmitted”

Figure 1: Minor thing, but perhaps little arrow could be placed to emphasise that daughters are a mix of their parental genes, but sons are exclusively from mothers.
We have greatly edited Fig 1, with subsections and more visualizing helpers, which help in following the different sections.

SML34: “number of blood cells where females can lay eggs” – Is this meant to be “brood cells” or am I missing something? Same with L36 & L37?
We thank the reviewer for spotting this mistake, and we corrected “blood” to “brood”.

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RESPONSE TO REVIEWERS' COMMENTS

We are submitting a further revised version that addresses the remaining concerns raised in the last round of review. See our responses to the reviewers below (in blue).

We hope that these changes address the remaining concerns raised in the second round of review and that the revised manuscript is now suitable for publication.

Reviewer #2 (Remarks to the Author)

I have reviewed the revision made by the authors following my comments on their original version. I am glad to see that all my comments have been addressed, and I have just one remark left:

The legend to the boxplot (Fig 2) is still not describing the boxplot properly. Please indicate what the box, whiskers and lines represent.

Other than that, I have no additional remarks to this version.

We have updated the legend of Figure 2. It now indicates what the box, whiskers, lines and dots represent.

*"**Figure 2. RNA-seq analysis of heterozygous site proportion in male and female Varroa mites.** The two sexes show similar heterozygosity proportions in their RNA across five families (mean difference = 0.022 ± 0.10, paired t-test: t = 0.46, df = 4, P-value = 0.672). The y-axis shows the proportion of heterozygous sites of total sites analyzed. In each boxplot, the horizontal line indicates the median, the box boundaries represent the interquartile range (25th–75th percentiles), and the whiskers extend to the most extreme values within 1.5 × the interquartile range; individual points show the heterozygosity values for each mite. "*

Reviewer #3 (Remarks to the Author)

This paper examines the genetic system of Varroa destructor. The authors show that, contrary to the assumed haploid arrhenotoky, this species displays a diploid form of arrhenotoky. Moreover, unlike a previously described form of diploid arrhenotoky, males retain the heterozygosity of their mothers. The authors show that both maternal gene copies appear to be expressed and transmitted evenly. They also present a theoretical analysis suggesting that this inheritance system increases effective population size relative to standard arrhenotoky.

I have no doubt that this paper will be important, both because of its practical relevance and because it describes a remarkable inheritance system. It is likely to stimulate further work on Varroa, and more broadly, to encourage closer study of the genetics of other species currently assumed to have conventional haplodiploidy.

I previously reviewed this manuscript. The authors have addressed my earlier comments on the main text well. In particular, they have added a useful discussion of previous cytogenetic results, possible mechanisms of clonal reproduction, and implications for sex determination. These points are now presented clearly. The new figures describing the system are also very helpful. However, I still have some concerns about the theoretical analysis. The authors have made the analysis clearer, and I have no doubt about the qualitative conclusion that diploid arrhenotoky should lead to a higher effective population size than haploid arrhenotoky. As I mentioned previously, I think this point could have been made with a much simpler model, more directly comparable to classical results. Nevertheless, I understand the motivation for modelling a scenario closer to the biology of Varroa.

My main concern is with the comparison to diploidy in the Supplementary Figure. The authors state that the diploid values are substantially higher than those for both forms of arrhenotoky. This seems surprising, especially because the differences appear to be extremely large. It is not obvious to me why an otherwise comparable diploid life cycle should generate effective population sizes that are 100- or 1000-fold higher than the arrhenotokous cases. Moreover, whereas the arrhenotokous values appear to plateau with increasing census size, the diploid value continues to increase. This suggests either that there may be a computational issue, or that the diploid case is not being modelled with the same mating ecology as the arrhenotokous cases. I cannot identify the precise source of the discrepancy without reconstructing the full analysis, but I think this result requires careful checking.

If the authors can readily identify and correct this issue, or justify the diploid result with reference to comparable results from previous work, then that would address my concern. However, I would not want this point to delay publication of an otherwise excellent paper. If the issue is not straightforward to resolve, I suggest that the authors either remove the effective population size analysis from the current manuscript, or replace it with a simpler model or simulation that can be more easily checked. This would not substantially weaken the paper, because the main biological claims do not depend on the theoretical analysis. The verbal argument is already clear: males carry two maternal gene copies and transmit them evenly, which should help retain heterozygosity, especially when each mother mates with only one male. A more detailed and fully validated treatment of effective population size in this system could then form the basis of a valuable future theoretical paper.

We thank the reviewer for noticing this mistake. We mistakenly assumed that the correlation between gametes from different females (f_f) equals that between uniting gametes (f), which is only true for complete diploidy under a random mating population. We have now corrected this by separately model f_f and f. The effective population size under complete diploidy is roughly twice those under haplo-diploidy and diploid arrhenotoky, which is more biologically realistic than before. Our principal finding did not qualitatively change.

In accordance with this correction, we have changed Supplementary Figure 1. To also visually include the diploid scenario. And updated the text in the Mathematical modeling paragraph of the Method section:

“Supplementary Figure 1 plots Ne over generations for haplodiploidy, diploid arrhenotoky and complete diploidy under two demographic scenarios that differ in absolute colony size while keeping the ratio of carrying capacity (C) to brood cells (K) constant (C/K = 2). New values for all three systems at carrying capacity and at equilibrium are reported in the legend. Mathematical details are provided in Supplementary Information 1.”