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**The Recommender**  
*Dr. Caroline Nieberding*  
PCI Evolutionary Biology

Lyon, France, December 2017

Dear Dr. Caroline Nieberding,

Thank you very much for giving us the possibility to submit a revised version of our manuscript entitled “Insect and incest: field evidence for dangerous liaisons in a parasitoid wasp” by M. Collet, I. Amat, S. Sauzet, A. Auguste, X. Fauvergue, L. Mouton & E. Desouhant (<https://doi.org/10.1101/169268>) to *PCI Evolutionary Biology*. We have considered all referees’ comments and details of our responses are listed below. Please also note that we have slightly changed the title of the manuscript following a suggestion of the second reviewer (see below point 1 referee 2).

On behalf of all the authors,

Marie Collet

## Author responses to the comments given on manuscript

### Insects and incest: field evidence for dangerous liaisons in a parasitoid wasp

<https://doi.org/10.1101/169268>

**New submission:** <https://doi.org/10.1101/169268> (revision on BioRxiv with the same doi)

We are most grateful to the reviewers for their thoughtful comments. The revised manuscript has been edited to account for the referees' suggestions and comments. We also provide further clarifications to their queries below. The reviewers' comments are reproduced in blue and italics, followed by our responses in normal black font. We have also submitted our revised version in BioRxiv (<https://www.biorxiv.org/content/early/2018/01/17/169268>). We have numbered the reviewers' comments and the manuscript lines to ease the editor's work.

#### Reviewer 1 - Reviewed by anonymous reviewer, 2017-08-31 10:11

*Review of ms by Collet et al, about inbreeding avoidance in *Venturia canescens*; review for the PCI Evol Biol, August 2017.*

##### *General comments*

*This manuscript intends to assess whether matings of one wasp species (the model *Venturia canescens*) in the wild avoid the risk of inbreeding, and to examine several mechanisms that may have led to the evolution of inbreeding avoidance such as kin recognition and dispersal. Overall this manuscript appears to me as very original and carefully thought and designed. The main strengths include: 1) that this work combines careful field work -from which a robust sample size of 467 individuals (caught either in the wild or their direct F1 descendants) were sequenced for a total of 18 microsatellites for inferring inbreeding level, in two populations- , simulations run in R to assess the expected level of relatedness and other factors from these datasets, as well as lab-designed experiments in which some mechanisms leading to inbreeding avoidance are tested (the role of density and of relatedness in mate selection).*

*However, I have the problem that I have a hard time understanding: 1) how exactly the experiments were conducted, and this holds true mostly for the simulation work on the expected coefficient of relatedness and on the statistical treatment of the mate choice experiment (I detailed my concerns for the mate choice experiment below), and 2) the conceptual reasoning that put all experiments together.*

- 1. Regarding the first point, the simulation work could most likely be super straightforward to understand if there was simply more space dedicated to transmitting the procedure in the methods section (in the SOM or in the main text as authors prefer). This should be ok to implement as there is at this stage no length limitation (on the PCI website) for manuscripts.*

2. *Regarding the latter concern, I think that this could be solved by reorganizing the ideas such that what pops up to the mind of the reader in the first place is not brought in the manuscript in the last paragraph of the discussion (for example). I propose below a new “structure” to the presentation of the ideas in the introduction that would help me, and hopefully others, to better understand everything at once.*
  
3. *My second main concern is the novelty of this work: The study seems very novel in that the authors claim that the study of inbreeding avoidance in the wild has been so far rarely conducted except in large vertebrate taxa, but this would be much more convincing if it was stated as such more strongly and with a more detailed review of existing studies. If done, I believe that this study has the material for publication in a first rate quality journal. This is also why I wrote below a lengthy review trying to help revising the ms.*

We thank the referee for her/his complete review. We are answering to these three general comments via the specific comments below (remarks done in points 1, 2 and 3 will be commented in points 9, 4 and 7 respectively).

*Specific comments:*

*Here I list some of the places in the manuscript that justify the general comments written above. It is not exhaustive but it aims to help the authors understand where I had trouble and how, perhaps, this could be solved.*

***Abstract and introduction***

4. *\*The authors write: “Our results suggest that *V. canescens* tolerate sib mating in the field despite kin discrimination, and therefore call into question the common beliefs on inbreeding depression in species with single-locus complementary sex determination. This inbreeding tolerance also opens up the question of the maintenance of the kin discrimination in this species” Comment: The fact is when reading this section of the abstract and the introduction, the reader will immediately think that : **1)** the biology of the species may very well explain this apparent discrepancy (see specific questions below) but the lack of information on these aspects of the life history of *V. canescens* gives the feeling that one does not have all information we would need to be able to judge;*

We have taken this point into account, and more information on *Venturia canescens* life history and reproductive strategy is provided in the revised version (see also point 5 referee 1 for details; see L 150-166).

- 2) there are other options that are not discussed until very late in the manuscript (end of the discussion): the existence of kin recognition despite low risk in the wild for kin matings, may be explained also as **1)** a ghost from past adaptation (when effective sizes were smaller?) for which it would be important to have evidence of past demography for the species (using coalescent methods - the genome of the species has been sequenced)? **2)** and/or kin recognition is a mechanisms under selection for another reason that avoidance*

*of sibmating; for example, it may be advantageous to localize in space related individuals as they may bear similar genetic adaptations to similar environments (same host species, same food source, ...) such that kin recognition may be actually used for other reasons than the one mechanism under testing here, inbreeding avoidance.*

In the previous version, we already discussed these two points to explain the discrepancy between our field results (sib tolerance) and previous lab results that showed sib avoidance. However, we have now reorganized the discussion in order to emphasize alternative explanation about ghost of past adaptation (see L 429). Moreover, the genome sequencing is recent and is not yet annotated fully and therefore we don't have information on past demography in this species (Pichon et al, Science Adv, 2015). Nonetheless, we have kept in the last paragraph (L 490) the discussion on kin recognition as a mechanism under selection for reasons other than avoidance of sib-mating as a way to raise a new perspective at the end of manuscript.

*Third, there may also be no avoidance of kin mating because dispersal has already ensured that the average relatedness coefficient in the wild is below any sufficient inbreeding cost: both the dispersal and the cost of inbreeding for this species has been tested in the manuscript with simulations / analyses using the field data (very smartly)- but it does not come as an obvious alternative in the right place in the introduction; the cost of inbreeding (number of diploid males is provided at the end of the discussion rather than in the result section) and the dispersal effect to inbreeding avoidance is not brought upfront of the start of the introduction as it should be. Hence, all major concepts and mechanisms related to inbreeding avoidance and the evolution of kin recognition as one of the potential mechanisms explaining inbreeding avoidance (but also other patterns) are in the manuscript, but one needs some reorganization to show how smart this study was.*

Sex-biased dispersal and its potential effect on inbreeding avoidance is now discussed earlier in the discussion L432-437.

*I propose that instead of bringing all the ideas and results one by one, the introduction does a better job by showing that the authors have thought of all alternatives, and will test quite a few of them in the results. I hereunder propose such a skeleton for the intro if this may be useful: 1) Inbreeding avoidance has evolved in many organisms because it has costs (list them), yet it also entails benefits so its evolution is the results of the balance between costs and benefits. 2) Environmental factors affects the relative costs and benefits of inbreeding avoidance (list which ones: density, population structure, social structure of the species,...), hence a) we need field data to quantify better the relative cost to benefit of inbreeding avoidance, b) what we see now is the product of past selection and the original factors may well be no longer effective (such as recent increase in demographic size would make inbreeding avoidance mechanisms unnecessary). 3) Kin recognition (by means of chemical communication in many insects) is one major mechanism used for inbreeding avoidance, yet it is not the only one and kin recognition may also have been selected for other reasons (which ones). 4) Altogether, this is one of the very first study bringing these questions into the wild with robust field data and combined molecular and lab experimental work.*

We agree with this comment and we have reorganized the introduction as suggested.

5. *The justification of Venturia as a model species could be further improved by providing in the intro more information on the biology of the species that justify the relevance of*

*testing all the above points on inbreeding avoidance, cost, benefits and knowledge on kin recognition. The questions that need to be answered about the biology of the species (in the introduction of the MM): What are the chances that related individuals encounter each other for mating : does developmental time differ between sexes? Do mothers lay a clutch of eggs in the same place (group of host) or not? How long do mothers lay eggs ? Do they lay first male next female eggs? How many times mothers do mate (this info is provided later in the introduction)? All these pieces of information could allow predict the likelihood that in nature mating with kin is more or less likely, explaining that the effective rate of sib mating is obtained by random mating, or not, and that specific mechanisms should have evolved, or not.*

As requested, we have added more information on the biology of *V. canescens*, especially on reproductive behaviours (see Materials and Methods section, L150-166). For some requests, there is no published evidence due to a limited knowledge of this species in the field. Nevertheless, we have provided a general overview of the probability of encounters between partners (sibs or non-sibs). This species is monoandrous (L 150) and females can lay eggs during all their lifetime (around 3 weeks) leading to overlapping generations. We have also indicated that i) adults are good dispersers, ii) only one adult parasitoid can emerge from one parasitized host (*i. e.* *V. canescens* is a solitary parasitoid), iii) sex-ratio is at fisherian equilibrium, iv) females are not able to discriminate a patch with 4 hosts from a patch with more than 4 hosts (females probably do not encounter large host patches in the field; L153-156). There is no publication indicating whether a sequence of egg laying occurs in this species, but we generally observe the emergence of males only the first day of emergence, and males and females on following days. We cannot give any information about the oviposition strategy in the wild: we do not know what is a patch in the field: a fruit, a group of fruits, a tree...?

6. *We are waiting too long to know (when reading) What is the evidence for inbreeding depression in Venturia? The authors write : “Inbreeding depression arises for diploids that are homozygous at the sex locus: instead of developing normally into females, they turn into abnormal diploid males that are generally unviable or sterile “, and also “the low fitness of diploid males”...But the fitness of such males is 0 right- They can’t fertilize any eggs?*

The introduction has been reorganized as suggested. The readers should now find the information concerning inbreeding depression in *Venturia* in the right place (see L 123-126). We have corrected the “low fitness of diploid males” by “null fitness” (L 121). In the previous version of the MS, we wrote “low fitness” because in 3 other wasp species, diploid males can sire offspring (Cowan et al, PNAS, 2004 and Elias et al, BMC Evol Biol, 2010, Zaviato et al, Evol App, 2017), but we agree that this notation was confusing since it is not the case in *Venturia*.

7. *The introduction shows that a very large diversity of behavioural strategies related to inbreeding risk exists and that the explanation may be that there are different associated costs and benefits for each species. At the moment, paragraphs 2 and 3 of the introduction show case studies of varying strategies limiting (or not) inbreeding risk in various species, but the actual associated cost/benefit of inbreeding in each system is not provided. It would be nice to better link what is known in each investigated species*

*regarding a) the patterns observed in inbreeding avoidance (or not) and, b) the associated known costs/benefits to inbreeding; that is, to know if, indeed, inbreeding is avoided when it leads to larger costs relative to benefits, and vice-versa, for each investigated species. In practise I would like to know whether, for example, the fitness advantage of related matings in the *Marmosa flavipentris* (if known?) or the observed costs of related mating in the ring-tailed lemurs and mole rats?*

When known, the patterns of costs and benefits of inbreeding and inbreeding avoidance have now been added for the case studies we referred to (L 46-59). We have also added more literature on insect inbreeding avoidance as suggested in the general point 3 (L 96-107) (see also the response to the reviewer 2, point 24). These references illustrate the diversity of inbreeding avoidance patterns observed in insects, although mainly in the lab.

### ***Material and methods***

*\* The authors write the methods in a way that gives a bit the wrong feeling that they have not thought that inbreeding avoidance may be obtained by dispersal from natal habitat before actual matings occur. In fact the authors test this option as well (and validate it), and the authors also explore the cost of mating with related individuals given the current population structure (and show it is very low). But these pieces of information come at odd places in the manuscript.*

We have now included our result on sex-biased dispersal in the abstract (L 23-24). There is also a sentence about this phenomenon in the paragraph dedicated to the aim of the study (L 142-143) and we have developed the discussion on this alternative explanation for inbreeding (L 432-437).

8. *“Inference of mate’s genotype : When alternative genotypes were proposed for a given female’s mate, we selected the only compatible one on a case-by-case basis” How exactly was this done (there is information in the suppl data but it is not 100% clear to me).*

We selected the genotype of the father that was compatible with the genotype of a daughter and a mother. When there were several alternatives (e.g. if mother and daughters are heterozygous with the same alleles at a given locus), the right allele cannot be assigned without uncertainty on the father. In this case we assigned to the father the allele with the highest probability of occurrence (this probability is given by COLONY software). We have added this information in the revised version (L 267-270).

9. *The whole section “Estimation of relatedness between potential versus mated partners in the field” is not clear to me and in particular the sentences ““Simulations allowed to keep track of pedigrees, yielding a perfect knowledge of kinship for all potential mates. These were classified in three categories: full-sibs, half-sibs, or unrelated. Besides, we calculated the relatedness coefficient (r) for all female-male pairs from these simulated populations (software COANCESTRY;(Wang, 2011). This allowed to estimate the optimal relatedness threshold minimizing the number of wrong assignments (i.e., unrelated pairs assigned related based on the threshold, or vice-versa). We found  $r_{crit} = 0.16$  (SEM-D, Fig. S2). Logically, this threshold is lower than*

*the theoretical relatedness between a brother and a sister ( $r$  sister-brother = 0.25;  $r$  brother-sister = 0.5) and in the interval of relatedness for 1st degree cousins ( $r = 0.125$  or  $r = 0.1875$  depending on the cross). With this threshold, we expect to wrongly assign 11.4% of the relationships”. In fact, this notion of “relatedness threshold” that the authors say is the threshold « above which individuals were considered related. »: would it be better to define it as the “threshold at which matings occurs among related individuals more (often?) than at random” ? Also, perhaps because I did not understand the procedure, I wonder whether this threshold of  $r=0.16$  is related to any detrimental documented effect of inbreeding in these populations/species? – I suppose it is not, that is why I propose another definition of threshold above. And what does the “ the optimal relatedness threshold” is ? And how can you have « wrong assignments (i.e., unrelated pairs assigned related based on the threshold, or vice-versa) » if the full pedigrees of the simulated populations are known ?*

In the M&M section, we wanted to take into account the bias in the calculation of relatedness coefficients according to the number of markers. Therefore, we could not just use the theoretical relatedness coefficient  $r=0.125$  or  $r=0.1875$  between non-inbred 1<sup>st</sup> degree cousins to determine which individuals were highly related (i.e. from inbred sister-brother to non-inbred 1<sup>st</sup> degree cousins). That is why we estimated the relatedness threshold  $r_{crit}$  to discriminate between highly related individuals and weakly-related or non-related individuals. There is no detrimental effect associated with this particular  $r_{crit}=0.16$ , but we know that sib-mating avoidance decreases steeply when  $r$  increases from  $r=0.1875$  onwards (1<sup>st</sup> degree cousins, Anna Chuine PhD thesis, pp 132-133, 2014). “optimal threshold” was maybe an inappropriate expression and in the revised version we removed this term and kept only the word “threshold” when referring to  $r_{crit}$ .

About the “wrong assignments”. They came from our simulation procedure for matings and formation of families. In one hand, we kept information on the pedigree of each individual to assess the actual relatedness between individuals. In the other hand, we used the same simulated data to calculate, a posteriori, relatedness coefficients between female-male dyads and to test different relatedness thresholds to classify our dyads either in the “related” category or in the “unrelated” category. We then compared these posterior estimations with actual relatedness coefficients for each dyad. Mismatches between the blind and the real relatedness categories were called “wrong assignments”. We have added more details in the M&M (L 278-286) and in SEM-C (last sentence of the first paragraph) to better explain this use of the simulated data.

All simulations assumed random encounter, and this hypothesis was confirmed by our results (see also reviewer 1, point 17 and 20 and reviewer 2 point 43).

We do not clearly understand the comments about the definition of  $r_{crit}$ . This parameter corresponds to the threshold value that allows the assignment, under the hypothesis of random encounters, of each individual to a given category, related or unrelated.

*10. More questions: how balanced is the sex ratio in nature - As this affects the simulations of threshold it may be worth investigating how variation in sex ratio (often biased towards more males than females in terms of effective population available for mating), affects estimate of random mating in the wild- ?*

Unfortunately, there is no publication or data on sex ratio in natural conditions. In the lab, the sex ratio is 50:50 or slightly female biased, and as explained earlier and in the manuscript, we have no evidence for local mate competition (which can produce female-biased sex ratios); we therefore assumed a balanced sex ratio. We have added this explanation in the manuscript (in the Materials et methods part): “Depending on studies, sex-ratio is considered balanced (Beukeboom, 2001) or weakly biased toward females (Metzger et al., 2008). Balanced sex ratio is congruent with the absence of aggregation (Driessen and Bernstein, 1999), the absence of local mate competition, and mate-finding via volatile cues. The proportion of diploid males corresponds to that expected under the hypothesis of a balanced sex ratio (Fauvergue et al., 2015).” (L 159-164). That is why, we used a balanced sex ratio in our simulations.

11. \* *The authors write: “We also estimated the probability that a female encountered a related male in the field the day of her capture.” How did they do exactly – As far as I understand, you use for this estimation the date of recapture (only males captures the same day as the female had a chance to mate with her, according to you) and not, for example, some distance between individuals above which encounter as a mate is no longer likely (which I would assume to be more relevant if individuals are fertile more than one day).*

Mating occurs a few hours after female emergence. Moreover, the sampling areas (where we caught females, Valence: 80m x 70m, Nice: 830m x 560m) are smaller than the distance wasps can disperse (in Desouhant et al. 2003, we estimated that wasps can fly more than 200m in 2h). Therefore, partners present in the same day in the same area have a chance of encountering each other and mate. We added this information in the M&M to clarify our analysis (L300-301).

12. *The authors write: “We recorded mating latency, copulation duration, status (colour) of the successful male, number and timing of male rejections by females and status (colour) of rejected males. “ I do not follow fully the statistical treatment of these data: mating outcome is the results of both male and female behaviours, and in other insects it is clear that male courtship activity is a prerequisite for females to accept or refuse the mating, that is, without male courtship there is no chance of mating. I think that the authors do correct mating success of related versus unrelated males by their relative courtship activity , at least in the treatment of the “latency to mate” but what for the actual number of matings? It would be good to provide the exact GEE-GLM best model structures.*

Thank you for this suggestion. We have added the complete statistical models showing response and explanatory variables (L225-232 and L234-239). We have suppressed what we called “rejection” that was misleading: it corresponded to a male courtship that was not followed by a mating due to rejection by the female (L222-223).

Since male courtship is a prerequisite for mating in *V. canescens*, we have performed additional analyses to test whether male courtship propensity was affected by the treatments (*i.e.* different population densities and percentage of related individuals in the population) (L234-236). Results showed no effect of the treatments (L325-329), which suggest that the absence of sib-avoidance is not a consequence of differential male courtship.

13. \* *Why no males captured in Valencia? (table 1)*



We caught males in Valence also. However, these males were discarded during the sampling period due to an error of manipulation and therefore were not available for the genetic analysis.

14. \* *I do not understand the sentence “As sexual and asexual strains coexist in *V. canescens* ((Beukeboom et al., 1999; Schneider et al., 2002), we used the presence of males among offspring as evidence for arrhenotoky. Mothers and their offspring were killed and preserved individually in 96% ethanol. Thelytokous individuals were discarded. » : can individuals produce only daughters, or only sons, in this species (I thought that the sex of the offspring was dependent on the fertilization of the eggs, hence all mated individuals can produce both sexes) and why discard some individuals?*

In *V. canescens*, two strains coexist in sympatry: one sexual (arrhenotokous, the one used in our study, in which mated females produce both sons and daughters) and one asexual (thelytokous parthenogenetic strain in which females produce only daughters, and do not mate). As both strains co-occur in the field, we needed to check that the females captured were sexual. We used the presence of males in the offspring of each female as a conservative procedure to discard all asexual females from our data set. This sentence has been clarified in the revised version (L 188-191).

## Results

15. *The authors write: “We nonetheless treated the two populations separately because the probability that a male captured in one population had inseminated a female captured in the other is negligible. “: why is that? Dispersal distance is much shorter than 200 m for this species the distance between your two populations)?*

We think there is a misunderstanding. The distance between the two populations, *i.e.* Nice and Valence, is about 300km. The lifetime of adults in this species is about three weeks when well fed in the lab, and therefore probably much shorter in the wild. Three weeks would mean that an individual has to cover more than 10km per day to travel between the two populations, which is unlikely. This is why we considered this possibility as “negligible”. The two patches used in the sex-biased dispersal analysis are 200m away, but are part of the same population (Nice). We already indicated the distance between the two populations (L169) and we have now specified that the sex-biased dispersal analysis concerned individuals between two patches within a single population (L307).

16. \* *In the section about “diploid males: Please start by explaining the relevance of this part, that you aim to assess the cost of inbreeding in this species from your field data. And why work with the Nice population only – evidence of random mating is not correlated to genetic diversity -?*

We have started this section by the main aim (L365-367). Diploid males were identified by genotyping when they were heterozygous at least on 1 locus (Materials and Methods L259).

As no males were captured in Valence (see point 13), we could not estimate the proportion of diploid males in this population, that is why it was done only in Nice.

17. \* *In the section about “dispersal”: there is male-biased dispersal in the species, thus how do you take this into account to your quantify the “randomness” of the field matings*

*patterns in your microsatellite data? I would start by showing sex-specific dispersal and next estimate of the resulting mixed populations may still gain, or not, to increase inbreeding avoidance by other mechanisms.*

We probably did not emphasize sex-biased dispersal and its potential effect on the randomness of mate encounters. We found a male-biased dispersal by comparing relatedness of dyads within and between patches (in the population of Nice). However the estimates of relatedness in males within and between patches are very low (between 0.042 and 0.065), and almost three times lower than our criterion of 0.16 that determined relatives. Therefore, even if we detect a significant difference between relatedness coefficients in males, the biological effect (effect size) is low. This implies that the weak sex-biased dispersal should not induce a strong genetic structuration which could change our assumption of random encounters. We have mitigated our conclusions on sex-biased dispersal in the discussion (L432-437).

*18. Section on the « effect of density and relatedness on mating success » : What is used to quantify mate choice? Mate choice is a complex concept that includes both the expression of mate selectivity (females for example usually become less selective when ageing or when density of partners is low - such as in beetles see Moore and Moore PNAS 2001 “Reproductive aging and mating: The ticking of the biological clock in female cockroaches”) and of mate preference (females exert sexual selection on certain traits, but not on others hence they have preferences for some but not all aspects of males). As explained above, mate choice may also, depending on what trait is used to quantify it, include both male and female behaviours as there is mate choice (assumed to be the fact of females) only if male actually court the female in many insects. Mate choice is a complex trait and it is unclear whether male or / and female sexual behaviours are involved in the variation of this traits in your treatments. Your results suggest that there is no mate choice going on, such that females would not avoid related individuals. However your results also suggest that female can recognize kin from non kin males because latency to mate is reduced after females have rejected a kin male.*

Please note that we have separated this comment in three parts to improve our responses and facilitate the reading.

Mate choice was quantified by the acceptance of the female to mate after a male courtship. As suggested, we have also analysed the occurrence of courtship behaviours (see previous points).

*To try and improve clarity it would be good that: 1) The statistical models are more clearly described, including what is the Y response variables and what are the X variables for all models used.*

We have clarified our statistical models as suggested (see referee 1 point 12).

*2) The biology of the individuals in the experiments is provided: what was the age of the females/males ? ...*

We have added this missing information (L208). Males and females tested were two-day old.

*3) the authors write « confirmed this ability of kin recognition in our choice test, the rejection of brothers (but not that of unrelated males) decreasing the female copulation latency. ». Please clarify how you expect latency to mate to reflect female mate choice (female preference, or female selectivity) for this species ? We may well expect that latency decreases because female selectivity decreases and/or because female preference increases.... It is hard to exclude, again, male willingness to mate (through variation in courtship activity) from this pattern and shorter latency to mate may well highlight variation in male courtship effort rather than variation in aspects of female mate choice.*

Female *V. canescens* were shown to reject males (van Santen and Schneider 2002), which suggests that they are the choosy sex. Nonetheless, we have improved our analysis of mate choice experiment by testing male willingness to court. We found no departure from random expectation (see point 12): when 2/3 of the males were brothers, 2/3 of the courtship events were done by brothers, and conversely with unrelated males.

The referee suggests that we specify the expectations concerning latency before mating. If females assess the male quality during its courtship (as we assume), an increasing number of encounters with sibs should lead the female to decrease its selectivity and, as a consequence, its latency before mating. In the other hand, an increasing number of encounters with unrelated males should not affect selectivity and latency to mate. We have added a sentence to explain these hypotheses in the revised version of the MS (L 240).

## *Discussion*

*19. The authors write « Sib mating avoidance in *Venturia canescens* had been evidenced from behavioural experiments in laboratory conditions (Metzger et al., 2010a). In contrast, the genetic approach developed in this study shows that in field populations, the observed frequency of matings between genetically related males and females is similar to the probability of sib mating expected under an assumption of completely random encounters. Our study thus suggests that sib mating tolerance occurs in the wild. » I do not agree with the conclusions because it may well be that there is no tolerance for sibmating in the species but that this has not been detected in this study as: 1) other mechanisms are in action to avoid sibmating in the wild, such as dispersal, so that there is no need for having in addition mate choice against related individuals to evolve; 2) there is no information of the potential detrimental effects of a threshold of 0.16 which was fixed by simulations or of having 10 (?)% chance of producing diploid males. We lack here evidence of the cost potentially associated or not to the actual level of relatedness experienced in the wild*

The referee is right; dispersal could render mate choice unnecessary. However, despite a significant male-biased dispersal in Nice population, its effect on population structure is only weak. It is therefore unlikely that dispersal affects sib-mating probability. The same pattern was observed in the ant *Formica exsecta*, where male-biased dispersal failed to reduce the worker homozygosity (Vitikainen et al, Am Nat, 2015). What we stated in the manuscript was that females did not avoid related males when encountering them. Evidence is that we observed a proportion of sib-mating in field populations similar to the proportion expected if there were no sib-mating avoidance. We interpreted this result as sib-mating

tolerance. At this stage, we made no hypothesis on the mechanism (active, as mate choice or passive, as dispersal) underlying this result but, in the MS, we discuss the apparent discrepancy between the capacity of females to discriminate their relatives and our population genetic evidence against mate choice in the wild.

Concerning the detrimental effect of the diploid males: as indicated in the MS, with 7.6% diploid males and if mating is random, the number of fertile offspring should suffer a 5.8% reduction (L483). We argue that such a cost of inbreeding is low enough to explain the absence of sib-mating avoidance.

*20. The authors write “in the rest of the discussion, we propose an evolutionary scenario to explain the apparent discrepancy between patterns observed in field and in the laboratory. » As far as I understand, there is no discrepancy between your field and lab datasets, as you write below “Mating rates between relatives, be they inferred from genetic analyses or from a simulation model assuming random encounter between males and females, were both equal to 11%. Consistently, in laboratory cages, the proportion of sib mating perfectly matched the proportion of sib present. Both results are congruent with an absence of sib mating avoidance in *Venturia canescens* ". There is rather, if any, a discrepancy is between your datasets and other published data o inbreeding avoidance based on kin recognition in the lab. Such discrepancies may be explained by differences in sex ratio density, effective size, population structure,.... under which those other populations used in other publications, evolved. So it is more important to show how your multi-level approach make sense of the pattern rather than try and highlight a so called discrepancy.*

The referee is right, there is a discrepancy between evidence for sib-mating avoidance with no-choice bioassays published by Metzger et al. (2010a) and our laboratory and field results, which suggests a tolerance to sib-mating. We have changed the concluding sentence of the first paragraph in the discussion. “In the remainder of the discussion, we propose an evolutionary scenario to explain the maintenance of kin recognition system and its apparent absence of use in the mate choice context, in the lab or in the field.” (L399-401). We hope that this change will also clarify the previous point on tolerance to sib-mating (point 21).

*21. The authors write « This was shown in a small isolated population of moose, with females accepting higher levels of inbreeding in years where males are less available (Herfindal et al., 2014). A similar reasoning may also hold for highly inbred populations ». Do you mean with the word « inbreeding « in fact « inbreeding for their offspring », or ‘ higher relatedness with their mate »? Or “inbreeding level in their mate” : the fact is that in social wasps males cannot be inbred as they are haploid so it is a comparison that is a bit confusing.*

The sentence was actually confusing; we thus have rephrased it (L 442). We meant higher relatedness with their mate, which, by definition, also corresponds to inbreeding of the offspring.

*22. in the section « Tolerance to sib mating, as observed in *Venturia canescens*, is often explained by low inbreeding depression or high cost of outbreeding » : this section*

*would be better placed in the introduction: what is known in Venturia regarding cost and benefit of mating with related individuals?*

We have added details on inbreeding depression in *V. canescens* in our introduction (L123-126). “The parasitoid wasp *Venturia canescens* Gravenhost (Hymenoptera: Ichneumonidae) has a single locus complementary sex determination (first demonstrated by Beukeboom, 2001) and inbreeding reduces the fitness of both males and females via the production of sterile diploid offspring and a negative impact on egg load and hatching rate (Vayssade et al., 2014; Chuine et al., 2015).”

*Orthograph* - Abstract « that sare ... » - Introduction « habitat selection following natal dispersal is negatively correlated...” rather use the words “ habitat selectivity”? - Introduction” Responses to inbreeding... » add « response to inbreeding risk »... » ?

We have corrected all these mistakes.

## Reviewer 2- Reviewed by anonymous reviewer

*This paper is concerned with sib-mating avoidance in natural populations of a parasitic wasp. The authors collected males (dead) and females (live) from the field at two different locations and analyzed microsatellites of daughters to determine the level of relatedness between the parents. The estimated rate of sib-mating was then compared to a threshold that was calculated based on encounter rates under random mating. The main finding is that this parasitic wasp species does not avoid sib-mating in the field, despite costs associated with sibmating. The authors further performed an experiment to determine whether sex-biased dispersal takes place and found that males were dispersing more compared to females. In a laboratory experiment the authors then looked at the effect of male density and proportion of related males on sib-mating avoidance. Overall, density or a higher proportion of sibs did not lead to sib-mating avoidance. Several hypotheses to explain this finding are discussed. I think this is a very interesting study. The authors have performed experiments both in the laboratory and in the field and have further designed an experiment (i.e. concerned with sexspecific dispersal) aimed to support their findings that sib-mating is not avoided in this species. The study of sib-mating avoidance in the field really is rather unique. One issue that I have with the MS, however, is that the authors claim that virtually no research has been done on sib-mating avoidance in insects. I do not think this is true, because in the laboratory several studies have been done on sib-mating (see my comment below for relevant references). Moreover, I find that the authors are not clearly describing their own previous findings on this topic (Metzger et al 2010 PLoS one). This is important, because in the current version of the MS the order of experiments does not make sense to me. If I would have been in your position I would first have performed laboratory experiments to confirm/extend previous findings on sib-mating avoidance in *V. canescens*. I would then have gone into the field to collect samples, followed by the experiment on sex-specific dispersal. I think the latter is indeed a nice addition that offers a potential explanation, but it would have been better if this experiment had been repeated at the different locations. Overall, I suggest you include a more detailed description of the work that has been done on sib-mating (and avoidance thereof) in insects, including your own work. Otherwise, very well done. I did not have access to the supplementary files for this MS, so I have not been able to review those.*

Thank you for this detailed review. Please note that the sex-biased dispersal “experiment” was not a capture-recapture experiment, but that we used the genotype of the wild females and males captured to assess if relatedness differed within and between two distant patches. We have slightly changed sentences in the introduction (L142), the M&M (L303) and the result part (L378-381) to clarify our experimental design.

### *Minor comments:*

- 1. I personally like the title, but it is not very informative. I suggest keeping the first part (i.e. Insects and incest), but making the second part of the title more informative (i.e. no avoidance of sib-mating in natural populations of a parasitoid wasp).*

We have changed the title of the manuscript as “Insects and incest: sib-mating tolerance in natural populations of a parasitoid wasp”

2. *Sib-mating should be written with a hyphen in between sib and mating throughout the MS.*

We have corrected the missing hyphen throughout the MS.

*Abstract:*

3. *You are not mentioning your results on sex-biased dispersal in the abstract. I think that should be included somewhere.*

We have added the result on male-biased dispersal in the abstract (L23-24).

4. *bullet point 1: ... likely evolves in species that are... (remove the s before are).*

Done (L5).

*bullet point 2:*

5. *Remove the comma after species*

Done (L11).

6. *Replace 'congruently' by ', and as a consequence'.*

Done (L12).

*bullet point 3:*

7. *Rewrite to: 'Our study consisted of genotyping...'*

As we have changed the order of the presentation of the experiments, the sentence has been modified: "Our study consisted of a mate choice experiment in laboratory cages and a field study based on the genotyping[...]" (L14).

8. *Rewrite to: 'With these data we were able to reconstruct the genotypic of all females' mates and estimate the relatedness of each mating pair'.*

The sentence has been modified to "With these field data, we reconstructed the genotype of each female's mate and estimated the relatedness of each mating pair." (L16-17).

*bullet point 5:*

9. *Rewrite to: 'These results suggest that V. canescens tolerates sib-mating in the field...'*

Corrected (L25-26).

10. *It is either 'the common belief' or 'common beliefs'.*

We have corrected as "common beliefs" (L28).

11. Rewrite to: *'This inbreeding tolerance also opens up the question as to why kin discrimination is maintained in this species'*.

Rewritten as "The observed sib-mating tolerance raises the question as to why kin discrimination is maintained in this species." (L27-28).

12. *Does this really call into question whether inbreeding depression affects species with sl-CSD? You know that inbreeding has negative effects in this species (and others), so the real question is why sib-mating is not avoided in nature despite those costs (and indeed why discriminatory behaviors are maintained).*

We agreed that species with sl-CSD are not the only species that suffer from inbreeding depression. Species with sl-CSD are however often referred to as relevant models to test inbreeding depression, due to the production of diploid males; this particular form of inbreeding depression is generally considered as highly costly. Therefore, our findings on tolerance to sib-mating in a sl-CSD species lead us to mitigate widespread conclusions on the force of inbreeding depression in these particular species. We demonstrated in the discussion that these costs are probably not as strong as what is commonly thought and thus that the absence of sib-mating avoidance could be the result of these low costs of inbreeding even in a sl-CSD species (L476).

13. *I would remove parasitoid wasp from your key words (it is already in the title and abstract of your paper). You could replace it with parasitic wasp to cover more ground.*

We have changed the keywords as suggested.

*Introduction:*

*paragraph 1:*

14. Rewrite to: *'Various strategies to avoid inbreeding have been described in animals, ....'*

Done. (L82)

15. *Put a comma in front of 'such as'*

Corrected (L 83)

16. Rewrite to: *'strategies are associated with...'*

Suppressed after introduction rewriting.

17. Rewrite to: *'gene associations'*

Suppressed after introduction rewriting.

18. *You write that 'selection on behaviors underlying inbreeding avoidance should depend on inbreeding load, which scales the advantage of inbreeding avoidance and the costs of implementing adapted behavioural responses', but to what does selection scale up to? I suggest rewriting this sentence for clarification.*



We have clarified this sentence “Selection on behaviours underlying inbreeding avoidance should thus depend on inbreeding load, which balances the advantage of inbreeding avoidance with the costs of implementing adapted behavioural responses” (L38-40).

*19. For the final sentence I suggest including a sub-sentence to explain what you mean with inclusive benefits. For instance, ‘, i.e. advantages for an individual’s inclusive fitness’.*

We have followed the suggestion (L41).

*paragraph 2:*

*20. It is not population density itself that constrains mate availability, it is a low population density that constrains mate availability. This should be written clearly in the MS.*

The referee is right. We have added “low” in the sentence (L64).

*21. This paragraph needs a concluding statement. For instance you could summarize by saying that when mates are scarce, it might be beneficial to mate despite high relatedness.*

We have reorganized and completed this paragraph and added a conclusive statement as suggested: “This interplay between environment and inbreeding avoidance patterns requires field data to be able to quantify cost and benefits of each strategy.” (L77-78)

*paragraphs 3 and 4:*

*22. Both of these paragraphs also end a bit abruptly. I would suggest including a concluding sentence to each of these paragraphs to clarify the point that you are trying to make.*

We have included a concluding sentence for the second paragraph. Please note that with the reorganization of the introduction, both paragraphs have a new location.

Ex-paragraph 4: “The diversity of strategies unveiled in the laboratory renders more striking our lack of knowledge on insect inbreeding avoidance patterns in natura, and calls for field studies.” (L106-107).

*paragraph 4:*

*23. It is either ‘an inbreeding avoidance pattern’ or ‘inbreeding avoidance patterns’.*

Corrected (L 77)

*24. You mention here that inbreeding avoidance has rarely been documented in insects, especially in the wild. I agree that not much work has been done in the wild, but in the laboratory plenty of studies have by now been done on inbreeding avoidance in insects, particularly in parasitoids (e.g. Bourdais & Hance 2009 Behav Proces; Lihoreau et al 2007 Behav Ecol; Ode et al 1995 Anim Behav; Visser et al 2014 Behav Ecol Sociobiol). Not all of these studies may actually find that there is sib-mating avoidance, but the absence of positive findings does not mean that no work has been done on the topic.*

*Moreover, your own previous work (Metzger et al 2010 PLoS one) directly addresses sib-mating avoidance, but this aspect of the paper (i.e. choice assays) is only mentioned in the discussion. These findings should already be put forth in the introduction, because it is a critical finding for your species and it puts the work into context. I suggest that you discuss more thoroughly the work that has been done so far on kin avoidance (or the absence thereof) in haplodiploids/parasitoids, including your own work. I think that this would actually strengthen your argument that only little research has been done on inbreeding avoidance in the field.*

We thank the referee for the references. We have added references on laboratory studies focusing on inbreeding avoidance in insects. “Laboratory studies nonetheless suggest various strategies of inbreeding avoidance in different insect genus, from active choices such as precopulatory avoidance of related males, which represent low quality partners with a high genetic load, in the butterfly *Bicyclus anynana* (Fischer et al., 2015) or postcopulatory choice with the preferential use of the sperm stored from unrelated males in *Gryllus bimaculatus* (Bretman et al., 2004, 2009). In a social species of termite, *Neotermes chilensis*, the dispersal of individuals founding new colonies is the main mechanism to avoid inbreeding risks (Aguilera-Olivares et al., 2015). Absence of inbreeding avoidance has also been evidenced in insects such as parasitoid wasps (Bourdais and Hance, 2009) or *Drosophila melanogaster* (Tan et al., 2012).” (L98-106). (see also point 7 of referee 1).

Moreover, we have also completed the discussion on the article of Metzger et al. (2010a), as suggested. “Consistently, in no-choice bioassays, mating success decreases with increasing genetic relatedness between mates (Metzger et al., 2010a; Chuine, 2014). Circumstantial evidence suggests that females are the choosy sex, which makes sense in a species with a monoandrous/polygenous mating system (Metzger et al., 2010a). However, when confronting females to choice between a brother and an unrelated male, inbreeding avoidance vanishes, likely because the mixture of volatiles in small test tubes confounds females (Metzger et al., 2010a).” (L127-132).

*paragraph 5:*

*25. This is an excellent explanation of haplodiploidy and sl-CSD.*

*26. paragraph 6: ... ‘monoandrous/polygenous’*

Corrected (L129).

*paragraph 7:*

*27. At the end of this paragraph you refer to earlier findings and prior expectations, but what were these earlier findings and prior expectations? Please briefly repeat here.*

We have completed our sentence with our findings and expectations as you suggested. “We show that sib-mating tolerance occurs in the wild, and propose an evolutionary scenario to explain why this result contrasts with earlier findings of inbreeding avoidance in this species as well as prior expectations about inbreeding depression in species with sl-CSD (van Wilgenburg et al., 2006).” (L143-146).

*Materials and methods*

*paragraph 1:*

28. *Could you include what kind of insect Ectomyelois ceratoniae? For instance, the moth....*

*I think it is a bit misleading to only focus on E. ceratoniae as a host. As you know V.canescens is a generalist (it has been documented to parasitize more than 20 hosts) and some host species may not be solitary like E. ceratoniae (for instance, E. kueniella or P. interpunctella). The fact that Driessen and Bernstein found about one E. ceratoniae per fruit is not really a good argument that patches for all hosts are small. Their result that V. canescens cannot really distinguish between the presence of 4 or more (up to 20) Ephestia larvae (compared to 1 or 2) is, however, supporting the idea that V. canescens does not often encounter locally aggregated hosts. If this is indeed the case in nature, it is not likely that this species experiences local mate competition (as sib-mating is not expected at the natal patch).*

We agree with the suggestion and therefore have removed the sentence about *Ectomyelois* and replaced it by a sentence explaining the absence of *Venturia* recognition between 4 and more hosts. “Females do not discriminate between host patches with more than four host larvae, suggesting that, in the field, they rarely encounter aggregated host (Driessen and Bernstein, 1999). Therefore, it is unlikely that this species experiences local mate competition.” (L 153-156).

29. *‘Males search for females...’*

Done (L 156)

30. *... ‘with a flight velocity estimated...’*

Done (L 165)

*paragraph 3:*

31. *‘Female traps constituted of an....’*

Corrected (L 173)

32. *‘Saliva secreted by host larvae when...’*

Corrected (L 175)

33. *‘Traps were hung...’*

Corrected (L 176)

*‘Traps consisted of a 125mm...’*

Suppressed when the sentence was rewritten (L 177)

*paragraph 4:*

34. *‘... where they were handled in a climatic chamber...’*

Done (L 184)

*35. Should DL be LD here? In other words 16 hours light and 8 hours dark?*

The referee is right, we have corrected by LD (L185).

*36. I think you need to include a definition here for arrhenotokous and thelytokous (say sexual and asexual).*

We have added the definition for the two modes of reproductions as suggested. “As sexual (arrhenotokous) and asexual (thelytokous) strains coexist in *V. canescens* (Beukeboom et al., 1999; Schneider et al., 2002), we used the presence of males...” (L188) (see also point 14 referee 1)

*paragraph 5:*

*37. ‘..., hereafter referred to as wild females)’*

Done (L 248)

*38. Fewer than 3 daughters per female seems very little to me and suggests that the mothers did not have enough time to lay eggs, that the mothers did not have enough hosts or that mothers produced an extremely male-biased sex ratio. Otherwise it might be because the females caught in the field are already quite old? Why are these numbers so low? You need to explain this in the MS.*

We could not control for the age and egg-laying experience females captured in the field. This, and a possible stress resulting from the capture and translocation, could explain the low numbers of offspring obtained once in the lab. We have added this explanation in the insect rearing section. “As we could not control for the age and previous egg-laying experience of captured females, the number of offspring was highly variable (1-10 daughters and 1-7 sons).” (L187-188).

*39. ‘... at each microsatellite locus...’*

Corrected (L 251)

*Paragraph 12:*

*40. I think you should clearly state in the sub-title of this paragraph that these experiments were performed in the laboratory.*

We have made the suggested change in the subtitle (L 209).

*41. Actually, it is not clear to me which individuals are used for these experiments? Is this a standardized laboratory rearing? If so, then information needs to be added about this rearing earlier in the m&m. If this is indeed another rearing, you should also pay some attention to this in the discussion as results might have been different for wild-caught rather than laboratory individuals.*

It is right; wasps used for experiments came from standardized laboratory rearing. We have therefore added complementary information in the rearing section of M&M. “For laboratory experiments, we used a standardized laboratory rearing. The wasp culture had been established with

about 60 females collected in the field near Valence (same location as the field capture, N 44°58'21" E 4°55'39", INRA, Gotheron), southern France, on several occasions during the summer 2015. *Ephestia kuehniella* was reared in the laboratory on organic wheat semolina medium for three weeks before being exposed to parasitoids (eggs were obtained from the Biotop rearing facility located in Livron sur Drôme, France). Parasitoid development took place in a controlled environment (24±1°C; 60±5% RH; LD regime 12 h:12 h)." (L193-199).

## *Results*

### *paragraph 2:*

42. *'Effective population sizes were estimated to be 79 for...'*

Corrected (L347)

### *Paragraph 5:*

43. *You say that random mating is suggested by your 'previous results'. I think it would be clearer if you mention here again briefly what that result was.*

Done. "...where random mating is suggested by the result on related pairs number in the wild (see previous section)" (L373-374).

### *Paragraph 6:*

44. *Should read 'referred to as ...' throughout this paragraph.*

Corrected (L380-381)

45. *I think paragraph 6 and 7 should be only one paragraph.*

We have merged the two paragraphs concerning males and females (L 383).

## *Discussion*

### *Paragraph 1:*

46. *'Sib-mating avoidance in *Venturia canescens* has been observed in behavioural experiments performed in the laboratory...'*

Slightly modified: "Sib-mating avoidance in *Venturia canescens* had been observed in no-choice behavioural experiments conducted in laboratory conditions" (L389-390).

47. *Remove comma between field populations and the observed...*

Rewritten: "We showed that the observed frequency of sib-mating corresponds to the probability of sib-mating expected from random encounters." (L396).

48. *'In the remainder of the discussion...'*

Corrected (L399).

49. *'...patterns observed in the field...'*

We rewrote the sentence and suppressed this expression: "In the remainder of the discussion, we propose an evolutionary scenario to explain the maintenance of kin recognition system and its apparent absence of use in the mate choice context, in the lab or in the field." (L399)

*Paragraph 2:*

50. *'... assuming random encounters between...'*

Corrected (L 404)

51. *'... the proportion of sibs present.'*

Corrected. (L402)

52. *You mention here again that Metzger et al 2010a only performed no-choice tests, but this is not the case. These authors did perform choice tests and that should be stated throughout the MS. I don't think that leaving out these details adds more novelty to your work, it only obscures the reasoning behind investigating sib-avoidance in the field. It makes more sense to me that first laboratory experiments were done to see whether sib-mating avoidance takes place, in line (or not) with previous findings of Metzger et al 2010a and then to argue that lab experiments may not reflect what is actually happening in nature, thus calling for an investigation of sib-avoidance under natural conditions. I would propose to more openly discuss the findings of Metzger et al 2010a and to introduce your own lab experiments before presenting the results on field individuals.*

We agree that Metzger et al. did also perform choice tests where they did not detect sib-mating avoidance. It was not a deliberate strategy to elude these results and add novelty to our work, and we are sorry if it feels like this. We have now added this information in the introduction (L127-132) to better justify our scientific approach as suggested in point 24 (referee 2). We have moreover modified the order of presentation of the experiment by presenting the lab experiment first as suggested.

*Paragraph 3:*

53. *The first sentence of this paragraph refers to 'these results'. State here briefly what results you are referring to.*

We have changed for "We further discuss eight hypotheses to explain the differences between our findings and those of Metzger et al., (2010a)." (L414).

*Paragraph 4:*

54. *'The second hypothesis is that female choosiness in V. canescens is density-dependent, ...'*

Corrected, and we have slightly rewritten the sentence due to the reorganization of the discussion part: "The fourth hypothesis is that female choosiness in *V. canescens* is density-dependent" (L 438)

55. *'A similar reasoning may hold true for...'*

Done (L 443)

*Paragraph 7:*

56. *' , the probability of matched mating increases to 50%'*

Done (L 481)

*Paragraph 8:*

57. *'threatened' should read 'threatened'*

Done (L 468)

*Paragraph 9:*

58. *'In Venturia canescens the evolution of kin recognition in a superparasitism context....'*

Done (L 496)

59. *'... has a high cost for female fitness'*

Done (L 499)

60. *The sentence 'Under the hypothesis of a primary evolution of kin recognition in the parasitism context and a secondary use in mate choice, the molecular pathways of sib recognition in the two ecological contexts should therefore be close' is not correct grammatically, nor do I understand what you mean here. This needs clarification (or removal).*

We have rewritten this sentence "If kin recognition evolved in this context, kin discrimination in a situation of mate choice could be a by-product of this primary selection on kin recognition. We could therefore expect a relatively general molecular pathway,[...]" (L501-504).

61. *'... unique pathway for kin recognition...'*

Done (L 508)

62. *Remove 'The' before superparasitism avoidance.*

Done (L 508)

63. *'that sib-mating avoidance...'*

Done (L 511)

*References:*

*64. Is the reference of Chuine A. 2014 a PhD thesis or a MSc thesis? This should be mentioned here.*

It is a PhD thesis, this is now clearly mentioned it in the reference list.