Dear Dr Blide

Thanks for your decision on our manuscript formerly entitled "**Sperm production and allocation respond to perceived risk of sperm competition in the black soldier fly Hermetia illucens**" and now entitled "**Sperm production and allocation <u>in response</u> to risks of sperm competition in the black soldier fly *Hermetia illucens***".

We have followed most of the suggestions and addressed all comments. We put a particular effort into revising the materials and methods. In this section, we have addressed the confusions about the treatments by adding a figure explaining the treatments and sample sizes of the mating experiments. We added some nuances in our conclusion by mentioning the male mating rate hypothesis explaining our first result about sperm production. You will find that we've also developed the conceptual context a little further by discussing models of intensity of sperm competition. Finally, because we had a poor sample size for a combination of the two treatment, we increased our sample size for the second experiment to test an interaction suggested by the reviewers.

Overall, we would like to thank the three reviewers for the time they spent reviewing our manuscript and providing such insightful inputs. Thanks to them, our manuscript is now of much better clarity.

You can find a point-by-point reply to the comments of each reviewers below, as well as a track-change version of our manuscript attached to this submission.

Sincerely,

Frédéric Manas, Carole Labrousse, Christophe Bressac

R1: Review by Isabel Smallegange, 01 Sep 2023 14:26

Line 50 : What is the optimal strategy of males when they cannot assess risk of sperm competition? Do they show e.g. bet-hedging strategies?

> There is no general rule so that the optimal strategy in absence of cues would be highly dependent of the context. For example, the general level of sperm competition of each species, the nature of the raffle – i.e. if the competition follows a loaded or a fair raffle – or if there is sperm displacement (see Parker & Pizzari 2010 - Sperm competition and ejaculate economics for review of different predictions).

Line 55 : Please italicize Latin species names

> Done.

Line 67 : Can you be more specific here ?

> We modified the manuscript .

Line 83 : How many per cage?

The rearing cage contains all the individuals not used for experiments; they were not counted.

Line 86 : Collection from where? From the stock cultures or from the experimental mating pairs?

Eggs were collected from the rearing cage.

Line 88 : Individually or in groups?

Pupae were kept in groups before emergence

Line 93 : Were these males virgin? If not, &could this affect your results?

These males were virgin, they were collected just after their emergence so we could ensure that they were virgin.

Line 94 : Is this one group with 19 males, or 19 groups of males?

This sample size (n = 19) corresponds to 19 males maintained under low risks of sperm competition (kept alone). We corrected this confusion.

Line 97 : Are these 24 groups of 10 males each? I hope. If you have 1 group of 10 males each, you have no replication (or only pseudoreplication).

This sample size (n = 24) corresponds to 24 individuals that were collected from groups kept in high risks of sperm competition (10 males together).

Line 99 : Personnal

Done

Line 101 : Again, it is not clear how many cages there are. Is there only 1 cage where matings occur?

One mating cage was considered as one replicate of the allocation experiment. In total we did n = 17 replicates of this experiment, with 17 independent mating cages

Line 102 : If you have 14 replicates (mating pairs) of each treatment (single, grouped); there would be 28 males at least originally? 14 single males and 14 males from groups. But this is not what you write. Could you please clarify?

The former manuscript was not so clear. We clarified the groups and sample sizes used in this experiment with a figure.

Line 103 : How did you identify males that came from single cages, and males that came from groups?

We modified the manuscript: Males from the singled treatments and from the grouped treatment were differentiated by marking a different coloured spot on their thorax.

Lines 104-105 : If I understand correctly, you have a 2 x 3 = 6 treatment combinations. male history: housed singly or in groups

male mating condition: alone, with 10 males or with 10 females.
It would help if you explain the experimental design clearly, including how many replicates you have per treatment combination.

As you suggested, we added a figure including the replicates used per treatment combination.

Line 110 : How long were males in the mating experiment?
When and how did you kill the males?
How long after did you do the dissections?

These informations were in the former manuscript in line 102, lines 114-115, and line 123 respectively.

Line 113 : How did you assess age?

Age was assessed by couting the number of days after emergence.

Line 124 : to what accuracy? Did you take photos through a microscope, if so, at what magnification?

The two individuals of a pair were photographed under a Nikon SMZ745T stereomicroscope (x3.35 magnification) (Nikon, Japan) with a Leica IC 80 HD camera (Leica, Germany).

Line 131 : It would improve clarity if you first list the explanatory variables and then the response variables. Line 141 : Can you please clearly list your fixed treatment effects (explanatory variables) and their interactions so the reader can see what the full statistical model is? Also, did you have any missing values?

We modified the manuscript to firstly list the explanatory variables and then the responds variables as you suggested. We had no missing values.

Line 155 : So the same male mated multiple times? Does that not affect the interpretation of your results?
Line 178 : I just don't understand the numbers. Are you treating multiple copulations by the same individual as independent observations? This is pseudoreplication

There was a misunderstanding. Males mated only once. We hope that all the modifications in the materials and methods will help to clarify that.

Line 210 : Do you know what the cost of increased sperm production is for the males?

We don’t know these costs in the BSF specifically but a lot of physiological costs known to be associated with sperm competition reviewed for example in doi: 10.1111/ele.13593

Line 223 : But males mate multiply in your experiment, right? If so, then how can you know the total sperm amount a male has transferred?

Again we clarified this point.

R2: Review by Rebecca Boulton, 24 Aug 2023 10:43

General comments

The experiments seem appropriate to test the question of interest and are appropriately replicated. I did find it hard to follow what was done at times and some of the methods and results don't match up with each other or with the code. There is also a lot of important detail missing about theory (i.e. sperm predence/intensity vs risk models of sperm comp) as well as life history/ecology of the BSF.

When it comes to the ecology/life history of the BSF, we don't know much. We think that we put as much relevant informations as possible – i.e. polyandry, the supposed leks, the same sex sexual behaviors. Beside these, we don't know how they behave in nature and informations about their behavior in captivity are also scarce as producers got more interested by larvae stage.

Title – suggest changing to 'Sperm production and allocation in response to perceived risk of sperm competition in the black soldier fly Hermetia illucens' for clarity

Done, thanks for the suggestion.

Abstract - In the abstract (and elsewhere) clarify when both manipulations were applied (sperm allocation) or just one was applied (sperm production).

Done.

Introduction – provide a more detailed and nuanced description of Parkers risk and intensity models of sperm competition, including some discussion about strategic ejaculate allocation when competition is almost guaranteed (i.e. when it's better to partition out lots of small ejaculates to many females).

We added some details about intensity models as you suggested.

Methods – the main issue I had with the methods was following when the treatments were applied together, or just one (i.e. depending on whether sperm production or allocation was measured). I also had some questions about how single vs grouped males were differentiated in the sperm allocation test. It would also be very useful either in the methods or introduction to provide a more comprehensive description of ecology of the BSF (i.e. longevity, fecundity, oviposition resources, are high population densities common in mating aggregations, differences between mass culture and field conditions etc).

We tried to clarify the problems that you pointed out in the methods. When it comes to the ecology of the BSF, as we answered sooner, informations are scarce.

I have some more specific comments about the methods below:

L89-90 – did they have ad-lib access to food/water before experiments started?

All individuals had ad-lib access to water before experiments. BSF don't eat at adult.

L92-97 – how long were males kept like this? Did they have access to food? This is relevant when thinking about possible life-history trade-offs (i.e. was there competition over food or other resources in the grouped treatment).

We added these informations in the revised manuscript. Males from both treatments were kept in these conditions from 5 to 8 days with access to a cotton ball saturated with water. No signs of competition for access to water were observed in the grouped-treatment.

L92 – 97 – so in total you had 240 grouped males but only 19 single males? Or was 19 a group of 10 but each male was kept separately?

We clarified this point; we dissected 19 individuals from the singled treatment and n = 24 individuals from the grouped treatment (24 individuals that were kept in groups of 10). Not all the individuals were dissected because dissection of the seminal vesicules of the male is really tough.

L93-94 – were these males age matched? If not, how did you

Their age matched.

L98-109 – this appears to be a factorial design, where mated females could either mate with a grouped male or a single male and then either alone or with 10 males or 10 females (i.e. 6 combinations of the 2 treatments in total) but it's hard to follow. A schematic showing the treatments and how they were set up (including sample sizes) would be helpful, as would a more comprehensive description of the factorial nature of the experimental design.

As you suggested, we included a figure to explain how the allocation experiment was set-up.

L102 – were they watched constantly to ensure that only first matings for each individual were recorded?

Exactly, we specified it in the revised manuscript.

L102 – was this long enough for all pairs to copulate (report that it wasn't, L153 here). Did you record time they started copulation – this could be important if some individuals were kept in these mating groups for longer than others and this should be included in the statistical model.

Thanks for this very relevant suggestion. We added the number of individual present in the mating cage before the beginning of mating in the model for the immediate risks experiment.

L103 – how many mating pairs per replicate were used and how can you be sure females hadn't already mated with another male? And for pairs put in with 10 males did you make sure females didn't mate again before sampling?

We specified it in the revised manuscript that the pairs were removed from the cage immediately after separation to ensure that females did not remate.

L113 – for the grouped males were males age matched? If not, how did you distinguish the age of each male? Was age balanced across the 2 treatments?

Age was matching between the grouped males. Because age was not balanced we controled it in the models.

L118 – was this section a measured grid on the slide or was it arbitrarily determined and then measured using a graticule? Did you take photographs of the preparations? If possible these measures should be validated by counting all the sperm in some of the replicates (for instance using imageJ) and comparing them to estimates.

The section was measured directly with the camera of the microscope and counted. Because spermatozoa could not be separated, counting them requires to adjust the focus continuously to ensure that all levels of the preparation were observed. At our knowledge, no automatic method exist to count entangled and crossed fluorescent lines, see pictures on Munsch-Masset et al, 2023, entomol exp appl.

L123 – why the day after copulation? When do they start laying/using sperm?

They start laying eggs 3 to 7 days after mating, and it was showed that sperm storage is finished after one day (Munsch-Masset et al., 2023)

L124/134 – say that the head measured and taken into account for the sperm production measures of males as well as the sperm allocation.

Done.

L129-151 – state which independent variables were fixed effects/factors and covariates (continuous).

Done.

L133-134 – why wasn't the interaction effect between mean and immediate sperm competition treatment included in the model?

As we had a poor sample size for a treatments combination in particular (Low mean risks x 10 Females treatment: n = 7), we did 3 more replicates to get n = 17 for this combination in particular. We included the interaction in the model and it wasn't significant.

L133 – define treatments more clearly in methods (repeat where necessary what the treatment is) – i.e. mean risk of sperm competition (males alone or groups of 10).

We hope that the figure will be clear enough.

L134-137 – include copulation start time in models as this affects how long pairs were in high density conditions prior to mating (for sperm allocation and mating duration), this

might influence allocation strategy. It would also be worth testing whether males from the grouped or isolated treatments started copulating earlier.

As you suggested we added the number of individuals present in the mating cage before the beginning of mating in the model for the immediate risks experiment. Also, we tested if the males from the grouped treatment started copulation earlier.

Results

L153-156 – it's still not clear how these males were differentiated when they were put in a cage together with females.

We added this information in the materials and methods.

L154-155 – seems likely that grouped males do copulate earlier so it would be good to take into account in models.

Thanks for the suggestion, we added the timing of mating in the model.

L163-164 – report model coefficients to support this statement.

Done.

L170-179 – in the code and in the methods it

Sorry, we don't understand this sentence.

L170 – again – why was the interaction effect between mean and immediate sperm competition intensity not fitted? The main effect of mean sperm competition is included in the model but not mentioned in the results.

Because the sample size for one combination of treatments was low (singled-males x 10 Females, n = 7), we sampled more individuals to test the interaction between the two treatments as you suggested (now singled-males x 10 Females, n = 17). Obviously, we added the Fstats and p-value of the interaction in the results

L178 – report coefficients to support this statement

Done.

L196-201 – for age say that these stats are for the model with all individuals included (and this pattern was more pronounced when the long duration pair was removed).

We changed the results.

Figure 3 – this is confusing because there were actually 3 levels in the 'immediate sperm competition' treatment, split up 'with conspecifics' into 'with males' and 'with females' to show that there is no difference and to make it clearer what this result refers to.

Done.

Figure 4 – where in the results does it say that you categorised the males and females as 'large' and 'small' or how you chose these categories? In the model that these results represent size is numeric so instead of surv plots scatter plots might be a more appropriate way of presenting the data (with one plot, points representing males and females coloured differently; similar for age and duration plot).

Thanks for the suggestion. This information was in the legend of the survplots. Anyway, those effects were not significant anymore after adding the series of sampling we did.

Discussion

A better overview of BSF mating system and life history would help to contextualise the results here, i.e. why it same-sex sexual behaviour makes sense (high densities of individuals make it hard to distinguish).

We tried to discuss more the same-sex sexual behaviour and to clarify what we know about BSF.

L222-223 – the results don't actually say this– for the sperm allocation models only the effect of immediate sperm comp is reported, not the null effect of mean sperm comp or the interaction effect (which isn't tested in the models).

It was a mistake in the former manuscript. We added the lacking results.

L224-230 – this explanation could be clearer – males use social environment as a general cue to produce more sperm perhaps because they anticipate higher population densities which means both more matings and a higher risk of sperm competition, but it doesn't influence their sperm allocation strategy – only the immediate environment affects that.

Thanks for the suggestion, as the other reviewer did, you refer to the male mating rate hypothesis that we developed in the discussion.

L233-234 – what are these 'co mplex physiological mechanisms'? Males might transfer sperm in one go and then stay mounted while the female is processing sperm or as a mate guarding strategy.

Sorry, the phrase was not clear. We meant that the transfer of sperm was not simply a linear transfer of spermatozoa during copulation.

L260 – could also be adaptive explanation – males are primed to produce more sperm when around females, this could be a general response to population density, it may be worth repeating the 'mean sperm competition' experiment with females which can be sensed but not mated with (i.e. enclosed in a mesh container) to see if the presence of female conspecifics also influences sperm production. Sperm production might reflect a life history trade-off between likely sperm use and longevity possible. Again it would be useful to have some more information about BSF life history (i.e. do males produce sperm throughout life or just as larvae, do they emerge with their full complement of sperm – pro vs synspermatogeny).

We didn't repeat the mean sperm competition experiment with females. As it would demand too much individuals given that it would be necessary to place 1 male with 9 females and mainly because failing these dissections is usual (seminal vesicles are coiled-up and really easy to break) it would require too much females to get a proper sample size.

L266-267 – this should come earlier and more detail about mating system needed.

Unfortunately, these details are scarces, in the introduction we listed almost all the informations published about the BSF mating system.

L276-278 – consider the possibility of life-history trade-offs (plasticity in investment)

L279 – this is the first time this complexity is mentioned, more detail needed to understand how the spermatheca is complex and compare it to other species where more is known about the dynamics of sperm competition – for instance there is nothing on sperm precedence in this species, but this can be predicted from the shape of the spermatheca (i.e. https://doi.org/10.1016/j.zool.2016.12.001)

We firstly mentioned the complexity of the spermathecae in the introduction.

L281 and more generally – rearing conditions are important for productivity – do mass rearing conditions differ a lot in nature vs mass rearing and how might this influence results and efficacy of mass rearing (i.e. if not well adapted to higher sperm comp/pop density in nature – or not a problem?)

We tried to discuss these points in the last paragraph.

References

L344-345/L355-358 /L368-370/L397-399– formatting not consistent with other references

Thanks, we changed that.

Code

No code to calculate descriptive statistics (L153-154; L176-179; 193-194; 198-201)

We added that.

Can’t find code to get the R squared value for with fig 2 (L181)

We changed that.

No code for producing figures

Sorry, we changed that.

Read.Me would be useful to explain the codes for all the variable and what they refer to or at least some annotation in the code.

We added that.

I get different values for anova of mod_Production and mod_Allocation – are there any data processing steps missing from the code? I.e. outlier removal etc or are any arguments missing from the anova function (to specify sums of squares etc).

We changed the code and hope that everything will be coherent now.

It seems like age is being treated as numeric in the mod_Allocation code but in your results it looks like it’s been input as a factor.

We considered age as a numeric variable and changed the text accordingly.

Consider using DHARMa to check model specifications

Thanks for the suggestion, we added simulations plots realised with Dharma to check the model specifications.

R3: Review by anonymous reviewer 1, 30 Aug 2023 10:08

This ms reports on a study of male reproductive traits in the economically importnat Black Soldier Fly (BSF). The paper studies male sperm priduction and sperm transference under different treatments to simulate 'sperm competition risks'. Clear responses were found for several traits as a reponse to the manipulation.

The study is interestingand relevant, but could be improved on a number of parameters.

The introduction introduces sperm competition as a novel aspect of sexual selection. This can easily be toned down as sperm competition is a very well reconised and established in evolutionary biology (so a bit much to claim a paradigm shift at this point).

Sorry for the confusion, we didn't want to pass this concept off as something brand new. Although sperm competition has been widely studied since it first appeared, it is

a paradigm shift that took place 50 years ago considering Khun's definition of these events: "a fundamental change in the basic concepts of a scientific discipline". It was the considerable impact of this discovery that we were trying to highlight while defining it.

The referee is not convinced that the responses detected necessarily is in response to sperm competition risk and the the treatments necessarily confer sperm competition. They likely could but there might be other explanations. An increased sperm production in the presence of multiple conspecifics could be interpreted as an increased male investment in response to the potential for more maitings. This nuance could be better represented.

We completely agree on that. You're quite right about production. The mechanisms making a male investing more in sperm production in response to the potential for more matings is called the *male mating rate hypothesis.* We developed it a little further in the discussion and tried to add more nuances in our conclusions in the discussion, as you suggested.

The test of responses in male reproductive investment is partly validated by the statement that it is known from many species but not investigated in BSF (yet). I hope there are much specific learning and knowledge to achieve and suggest that a stronger rationale and a stronger set of hypotheses would strengthen the whole paper. This would also allow strengthen the conclusion / perspectives of the reserach. It is currently claimed that the species and its fertility is important, but not discussed exactly why and how this will advance the field (as far as I known, BSF breeders do have plenty of eggs if they have enough flies). The final sentence states that the results should be integrated (where?) to control genetics (what does it mean to control genetics?).

It is true that the manuscript lacked clarity on this point. In fact, the majority of flies do not mate under rearing conditions (based on unpublished results). In addition, the presence of multiple mating and post-copulatory sexual selection raises the question of which individuals actually manage to fertilise the females.

When it comes to the breeding, genetic selection is a particularly useful and sought-after activity. For example, breeders are looking for bigger and bigger larvae. However, if the individuals you are trying to select for a certain phenotype are not reproducing because of barriers linked to sexual selection (pre- or post-copulatory), it is vital to understand why in order to resolve this problem. Especially when the individuals we are trying to select are precisely those that reproduce the most.

The matrials and methods are somewhat hard to follow as several experiment with several co-variables are studied. A better description of the methods section and a figure on the experimental design will help the readers understand what is studied where - and align the experiments to the questions with the answers/results.

We did a thorough proof reading of the materials and methods to better describe the experiments. In addition, as you suggested, we included a figure to explain the immediate risks of sperm competition experiment which can be particularly hard to follow.

The text should be proof read to improve clarity and remove odd phrasing. The terminology of the two main treatments coul be developed to become even more clear and logic. "Immediate competition risk" makes sense (I left out "sperm, as I am not convinced that it can be assumed that it truely is sperm competition risk). However, "Mean risk" is not similarly logic. Would "Historic risk" / "Prior risk" or similar be better to logically distinguish between the two?

I understand that the terminology may not seem clear at first. Nonetheless, we wrote the terms widely used in the literature, taken from Parker's models, and quoted on several occasions, for example in the great paper of Engqvist & Reinhold about testing prediction from sperm competition theory.

The mean risk of sperm competition is effectively modified because, in an environment full of males, " the probability that the male's sperm will compete against the sperm from other males for a given set of ova " increases. The question we can ask ourselves, however, is whether the **perceived** risks of sperm competition are modified by our treatments. This seems to be the case (for allocation and the immediate risks of sperm competition) and is likely to be the case for production (although the male mating hypothesis cannot be ruled out as you suggested).

Apart from methodologically details, some facts about BSF reproduction are stated and in cases a foundation of assumptions. These might to some extent be anecdotal facts. One is whether the species rely on leks (which the paper did not observem and which could mean that the available area was nadequate or that no leks are required). Another is whether remaiting can be ruled out due to small space, as some personal observations suggest that mating can take place in ver small room. Please consider being clear (and provide references) for established facts, and feel free to share speculations, ideas and personal observations (but be clear about this as well).

We tried to clarify when the manuscript was referencing personal observations and unpublished data. However, considering the fact that we don't know much about the reproduction of BSF, discussing the established data on it in light of what have been found in this manuscript seems important to us.