

Dear Dr Bonte,

Thank you for allowing us to submit a revised version of our manuscript ‘Transgenerational cues about local mate competition affect offspring sex ratios in the spider mite *Tetranychus urticae*’ to *PCI Evolutionary Biology*. We would like to thank you and both of the reviewers for their helpful comments, which we hope have improved the manuscript. Please find below responses to your and each of the Reviewers’ comments.

Kind regards,

Alison Duncan (on behalf of all the authors).

Round #1

Decision

by Dries Bonte, 2018-02-09 08:48

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recommendation of your preprint: revision needed

Alison Duncan and colleagues investigated their role of maternal effects and sib mating on offspring sex-ratios. They conducted several laboratory experiments in which maternal LMC was manipulated by changing the number of founders, while maintaining density. The paper is to my opinion well written, data well analysed and collected.

The reviewers agree with my evaluation but came up with several issues that the authors want to take into account to improve their paper. While I found the reasoning regarding predictability well explained (I am also of opinion that the perspective of local information not matching cross-generationally integrated population-level information is indeed key to understand many demographic dynamics), the reviewers want to see some aspects of the reasoning better worked out. Reviewer one questions the validity of the experiments because of putative problems with the experimental setup and the used patch sizes. As a recommender of your manuscript, I believe issues on this can be avoided by providing a schematic presentation of your breeding design. Especially the descriptions on the different sample sizes (patches) in the quarters of the boxes are hard (not to say impossible) to understand since no reasoning for this setup is given. I recommend the authors to make this part clearer to the audience, as this information seems central to understand the potential drivers of the recorded maternal effects on offspring sex ratio.

Boxes were divided into quarters so that the number of replicates contributing to estimates of sex ratio and fecundity were similar between treatments. Indeed, 1 patch in low LMC is composed of 15 females, while 1 patch in high LMC is composed of a single

female. To avoid having many more replicates under high LMC than under low LMC, composed of different number of females, we considered replicates to be quarters of boxes, which contained similar number of females. There is now a schematic presentation of the experiment shown in Figure S1 and this is now better described lines 176 – 179.

In addition, the authors should provide the raw data (e.g. in supplementary material) or the link to the raw data if they are deposited on an open repository. Similarly, the authors should give more details about their statistical analysis (a SAS script would be particularly welcome). These requirements are indicated in the PCI ethical code of conduct (<https://evolbiol.peercommunityin.org/about/ethics>).

We have now uploaded the raw data as supplementary files and provide SAS script in the supplementary materials (Appendix 2).

Reviews

Reviewed by anonymous reviewer, 2018-01-10 08:57

In general, this study aims to experimentally test potential cues that the spider mite, *T. urticae*, uses to adjust sex ratio of its offspring. I have a number of concerns or questions related to interpretation of their results. One of the important questions I have is related to patch size as actually experienced by the females under the experimental design and how that relates to typical patch size in nature. Having multiple small experimental patches located in the same box could be a problem if odor is a cue the female uses to assess presence of other females in a patch. I am also not completely convinced that inter-generational information exchange is the most parsimonious explanation for the results in Fig. 1B, especially given contradictory results in earlier studies. Detailed comments along these lines are presented below, mixed in with more minor comments.

L42: "Furthermore ... should also select..."? compared to what? In the previous sentence, the authors simply state that sons are of less value than females in a LMC situation. It does not say why that should be. Only the sentence starting at L42 gives a reason, and that is only for haplodiploid species (i.e., mothers more related to daughters than to sons). The authors say (138-39) that LMC "is expected to select for female-biased offspring sex ratios". It would be helpful to the reader to explain what underlies that expectation; Why is it expected? Is it expected only for haplodiploid species? If not, why is it also expected in diploid species?

We have written a new first paragraph to clarify under what conditions female-biased offspring sex ratios are expected (lines 38– 49). We first state that in structured populations, when females are the dispersing sex, related males can compete for mates. In such a scenario female biased sex ratios are selected to reduce this competition. We then add that for haplodiploid species, in addition to LMC, female-biased sex ratios are expected when females mate with kin because of an asymmetry in relatedness between mothers and their sons and daughters.

L65: change "whole" to "wide"

This has been changed (line 60).

L72: I cannot make sense of this sentence. How can under-study of anything result in biological variation? Something is missing that would clarify the logic. Do the authors mean variation in adjustment may be related to variation in cues? Or do they mean lack of experimental control of cues results in different outcomes of adjustment between studies? Or something else?

Here we mean that the fact that variation in sex allocation is not always observed may be because certain stimuli or cues may have been overlooked when designing experiments, due to our ignorance of such cues. We have changed this sentence (lines 76 – 77).

L75: change "in the immediate whilst" to "while"

This has been changed (line 79).

L79: why "In contrast"? These just look like more examples of types of cues, not something that contrasts with the previous sentence.

The 'In contrast' refers to immediate cues in the environment as opposed to cues experienced at different life-stages. We have modified the text to make this clear (lines 83 – 86).

L82: delete comma after "cues"

This has been deleted.

L90: change "this" to "that"; otherwise it reads as if the authors are talking about their own current study, rather than the 2011 study.

This has been changed.

L113: missing a close-parenthesis

This has been added.

L123: 2011a or b?

2011a, this has been added.

M&M: It would be helpful to have schematic figures illustrating the experimental design; it is difficult for me as a reader to keep it all straight with only a textual description.

A schematic figure of the experiment is provided in the supplementary materials (Figure S1).

L158: change "haphazardly" to "arbitrarily"

This has been changed (line 163).

L168-173: Is there any information on how a female may "count" other females or eggs? Odors (e.g., L78-79)? Direct encounters? If LMC is sensed by females via odor, having 4 patches in

the same box could essentially be sensed as a single patch by the females in that box. Have the authors considered this possibility, and how would that affect interpretation of results?

We do not know how females count one another, and therefore do not know if they use odour. However, we believe this is unlikely for the following reasons. A number of studies indicate that *Tetranychus urticae* detect conspecifics using mechanical and chemosensory processes via web and faeces rather than volatiles (Royalty, 1992 and Clotuche et al, 2014). Volatiles released by infected host plants can indicate the presence of conspecifics (Pallini et al, 1997). In this latter study, the experiment was done using entire plants (and not leaves like in our experiment) and there were many more mites (several hundred to over a thousand) than in our experiment. Thus this latter mechanism is also unlikely in our experiment. This information has been added to the Discussion (lines 322 – 331).

Similarly, in the single 60-cm² patch per box, are the authors assuming that each mite somehow "experiences" conditions across the full patch? Maybe odors can be sensed that far away, but if the cue requires contact (e.g. encounters with other mites or eggs), is it possible the patch is too big? This possibility is alluded to at the end of the paper, but not really discussed [L347-349]. Do the authors have information on how big a "patch" in nature is?

Again, and according to the literature, we do not believe that mite odour plays a role in detecting conspecifics. One study estimated that single females lay their eggs on leaves in 1cm² and that juveniles do not move more than 5 mm from this area (Mitchell, 1973); this is now mentioned in the Discussion (lines 396 – 39). The patch sizes we used controlled for density at 0.25 females per cm², which is indeed lower than that used in other studies (Wrench & Young, 1978 and Roeder, 1982). We now discuss that mites in our experiment may not detect one another (see response to the previous comment and lines 322 – 331 in the main text).

L214: change to "The sex ratio only of individual females could be obtained..."

This sentence has been removed.

L222-223: Is there support in the literature that can be cited for this method of determining significance? Requiring only > 5% of runs to have p<5% seems not very stringent for declaring significance.

We needed to find a statistic to test for the effect of LMC treatment on sex ratio while controlling for the number of females (sampling variance), i.e. for groups of similar numbers of females. So we resampled on single females from the LMC treatment to form these groups and produced statistics for the test of LMC on sex ratio. We agree that our bootstrap strategy was not conventional and now have changed it for a two-tailed standard permutation test (under the null hypothesis of no effect of LMC). The observed statistics were generated by using the experimental design: we used the number of males (that follows a binomial distribution) produced in quarters (= replicates) of boxes by 12 females for the high LMC treatment and by 15 females for the low LMC treatment. We agree that this is not ideal as we should have set up the same number of females per quarter independently of the LMC treatment. The statistics under H0 were generated using the same design (12 vs 15 females for high vs low LMC). The p-value for the test is 0.938 and conforms to the previous conclusion of no effect of LMC. This is described in the text (lines 230 – 238).

Table 1 is confusing. The caption for part A indicates that the values in the table are number of offspring per female; but the "title" for part A in the table indicates the data are some sort of value for sex allocation. I think the authors are using the part A title to indicate their name for the experiments that these offspring number data came from, but this should not be done because of the confusion it can cause for the reader about what they are looking at in the table. When column headings differ, as they do for parts A and B of the table, this is an indication it needs to be split into two tables. In this case, it also means that the data in part B should be reported in the text, not in a table, because there is only one line of entries. Also, please include the p values in the table. L370: part of what is hard to keep straight is the use of "females" for Gen. 1 and "mothers" for Gen. 2. Could the authors say, "and generation 2 when the grandmothers of the offspring were exposed to..." ?

We agree that the previous Table was confusing. We have removed Table 1 from the manuscript and have placed this information in the main text (lines 315 - 323).

The number of eggs laid by the females seems surprisingly low. I could be mistaken, but I thought *T. urticae* typically lay 100-200 eggs per female. If true, this suggests the mites are not being reared under optimal conditions; do the authors agree, and if so, how might this affect the results of their experiments and their interpretation?

The fecundity measured in our experiment is the number of eggs laid by single or groups of females during days 1 to 4 of the experiment. We now refer to offspring production and state in the methods that this refers to ~4 days of egg laying (lines 240 - 241). Lifetime fecundity can indeed be much bigger and reach 100 – 200 eggs.

Fig. 1: There are no A, B, or C panels labeled in the figure (although I can guess which they apply to).

Figure 1 now refers to panels A, B and C.

L254-257: Relative to the baseline ratios in Generation 1 where level of LMC had no effect, the effect of maternal environment (gen 1) on sex ratio produced by the daughters (gen 2) was to increase the proportion of males at low LMC, not to increase the proportion of females at high LMC. So the wording of this sentence should be changed accordingly.

This sentence now states that females whose mothers experienced low LMC produced a less female biased offspring sex ratio, compared to those whose mothers experienced high LMC (lines 267 - 271). This point is interesting. Unfortunately it is very difficult to compare sex allocation between generations due to uncontrolled environmental variation which may affect results.

L265-266: In the Intro (L96-98), the authors cite Wrensch & Young 1975 and Roeder 1992 where females produced female-biased sex ratios under high LMC conditions. But this result was not seen in the current study where level of LMC had no effect (Fig 1A). What do the authors think may account for these conflicting results? I see the authors admit this in L290-291, but they do not address the reasons why their results differ, which should be examined.

These studies did not control for density and so in their low LMC treatments a higher number of females were placed on a small leaf patch (6.25 cm² in Roeder and 1.8 cm² in Wrensch & Young). As stated by the reviewer, it is possible that females on our low LMC

patches did not detect each other, or they may respond to density. Both these possibilities are now in the Discussion (lines 322 – 331).

L291-292: The number of females in the current patch may or may not provide reliable information (which would be independent of the population tested); all that can be said is that this population did not use that information as a cue under the conditions of the study.

We now state that this result may be unique to the experimental conditions used in our study (lines 315 – 318).

L288: change "for" to "with"

This has been changed.

L320-326: Rather than an alternative explanation, this seems the most likely (most parsimonious) explanation for the results, because it does not require cross-generational communication. The potential cue (juvenile crowding and/or frequency of encounters with siblings) is a direct experience of the individual female that will control sex allocation of her offspring.

As stated we cannot rule this out as a possible explanation. As juvenile density does not change between treatments, interactions between siblings versus unrelated juveniles or the absolute number of juveniles on a patch could be explanatory factors. However, interactions with conspecifics during the juvenile period occur prior to female dispersal. Thus our result that cues prior to female dispersal, resulting from maternal laying decisions, impact a female's sex allocation remains. This is stated in the Discussion (lines 372 – 384).

L339-340: This is the right question, but only if the opposing results of previous studies on *T. urticae* (Wrensch & Young 1975; Roeder 1992) can be discounted in favor of the present study.

As we do not know what the maternal environment was in Wrensch & Young, 1978 and Roeder, 1992 we cannot say anything about the validity of our results versus theirs. However, our results do show that factors due to the maternal laying environment, either the number of foundresses or the juvenile environment, impact a female's sex allocation. We have added that the juvenile environment may be the cue to this question (lines 372 - 384).

L347-349: The appropriate size of a patch is a serious question that the authors must consider in the experimental design, as I indicated above. It is related to the question of what form the actual cues take. It would seem important to discuss what is known about patch size and how that relates to the experimental design in the current study and perhaps in previous studies.

We now describe what constitutes a patch for *T. urticae* (lines 396 - 398). As stated, patches are quite small relative to the size of a leaf. Thus the idea that patches may be subdivided into smaller ones may be relevant for *T. urticae*.

L349-350: It is not clear how "maternal cues [may] provide an integrative measure of population structure", nor how that would somehow be superior to knowledge of immediate conditions experienced directly by the female who is to make the sex allocation decision. I am

not saying the authors are wrong, just that this argument needs to make the logic clearer so as to be more convincing.

Females may lay their eggs in a small area on a large leaf/patch. Accordingly, local information about population structure in the immediate vicinity of a patch may be combined with information obtained from the mother about population structure at larger scales. We have expanded this paragraph and discuss this (lines 389 - 398).

Reviewed by anonymous reviewer, 2018-01-11 15:12

Main comments: Hamilton (1967) predicted that, in a structured population, females would bias to produce more female offsprings to reduce Local Mate Competition (LMC) among sons and thus maximize her reproductive success. The spider mite (*Tetranychus urticae*) appears to be an ideal subject for experiments to test LMC as the species has a short turnaround generation time and the females can adjust the sex ratio of offsprings through manipulating the fertility rate of the eggs. The ability of adjusting offspring sex ratio seems to be heritable (i.e., females from the lines produce female-biased sex ratio usually produce more daughters than sons; Mitchell 1972), though it may be terminated if the females have experienced generations of high LMC (Macke et al. 2011). This Duncan et al. (2017) study further shows that, neither the kinship between the mating males and female, nor the presence/absence of other adult females in the same patch, but the LMC condition in the maternal environment was the cue influencing the daughters' offspring sex ratio.

The finding is interesting, although there are some drawbacks about the study. For instance, the observations were limited among two generations, and therefore it was unable to verify how many generations would be affected by the LMC cue experienced in a predecessor generation, and at what point the adjustment ability to be lost.

Our experimental design does only test for the persistence of LMC cues over 2 generations. This was the point of the experiment, investigating plastic not evolved responses. We were interested to see if cues from the maternal environment could impact a daughter's sex allocation. Having now found that maternal effects exist, one could verify for how long they persist. This was partially addressed in a previous study that showed that plasticity can be lost, but only for mites experiencing high LMC (see Macke et al. 2011a).

The authors proposed some biological mechanisms to explain such maternal inference, but that does not sound very convincing: If the mothers can control the offspring sex ratio through manipulating the egg conditions in their daughters' ovaries, why can't they just adjust their own egg condition? Even if the living environment is indeed stable enough, what is the benefit to use maternal cues instead of immediate, social environment cues to adjust offspring sex ratio?

A maternal effect on sex allocation may not be adaptive, which we now state in the discussion (lines 387-389). We also state that our result may be due to conditions experienced by the female herself in the juvenile environment (lines 372 - 384). However, if it is a true maternal effect we offer a possible mechanism by which it may occur and why it may be adaptive.

The result is certainly novel and worth publishing; however, I would probably recommend it as a reading material only for a journal club specialising in *T. urticae* behaviour.

Other comments: 1. Need references for the statement “Other studies have shown that spider mites can respond to both maternal cues and those in the immediate environment.” (Line 94-96).

This sentence referred to the references cited in the following two sentences. We have now changed these sentences so that the relevant references are cited in each (lines 97 - 103)

2. Check the citation for the statement “This study uses the same base population as Macke et al. 2011” (Line 123). There are two Macke et al. 2011 papers in the reference list.

2011a, this has been added.

3. How was the genetic relatedness in the 200 adult females in the new base population? (in Material and Methods)

We expect that the new base population should be more genetically diverse than the previous population since two different populations were mixed. This is now in the text (lines 135 – 137)

4. There is no A, B, C marked in the Figure.

Figure 1 now refers to panels A, B and C.

References: Hamilton, W. D. (1967). Extraordinary sex ratios. *Science*, 156(3774), 477-488. Macke, E., Magalhães, S., Bach, F., & Olivieri, I. (2011). Experimental evolution of reduced sex ratio adjustment under local mate competition. *Science*, 334(6059), 1127-1129. Mitchell, R. (1972). The sex ratio of the spider mite *Tetranychus urticae*. *Entomologia experimentalis et applicata*, 15(3), 299-304.

Author's Reply: