

Round #1

by Trine Bilde, 21 Jun 2022 07:58

Manuscript: <https://www.biorxiv.org/content/10.1101/2022.03.25.485836v2>

minor revision

Dear Authors

I have read this interesting manuscript, and have also obtained reviews from three experts.

The reviewers and I are excited about your paper, and they have provided very constructive comments aimed to improve clarity and presentation.

I invite you to revise the manuscript by addressing the comments provided.

Reviews

Reviewed by anonymous reviewer, 16 Jun 2022 22:39

This preprint assesses variation in reproductive modes in a species of stick insects, so far believed to reproduce by obligate asexuals. The authors demonstrate through multiple lines of evidence that the two investigated locations are in fact inhabited by three lineages of facultative asexuals with one lineage (one location) being much closer to obligate asexuality than the other two lineages (both from the other location). The findings further show that asexuality occurs by mode that leads to complete or almost complete autozygosity in one generation (gamete fusion or terminal fusion without recombination or similar mechanisms; see a new paper by Archetti for the possibility of inverted meiosis, where complete homozygosity may also occur through suppression of meiosis I in absence of recombination). In addition, there is some evidence for rare interbreeding with a related sexual species (mostly from discordance of nuclear and mitochondrial phylogenies), as well as for a potential trade-off between sexual and asexual reproduction (individuals of the more “obligate” parthenogenetic lineage have poor hatching success after fertilization). Taken together, this is a detailed investigation into facultative parthenogenesis in an insect species, whose relatives are known to show a variety of reproductive modes including “obligate sexuality” with rare asexual reproduction and obligate asexuality (even though in the light of this study one wonders whether evidence for “obligate” reproductive modes shouldn’t be questioned also in other species). The results are discussed in light of the hypothesis that facultative parthenogenesis might be an intermediate state between obligate sex and obligate asex (but the results themselves cannot resolve this question neither whether these facultative asexual evolved from sexual or asexual ancestors).

Overall, the manuscript is very well written and clear, and the experimental, genetic, and statistical approaches are sound. I have almost never seen a manuscript on which I had as few specific, minor comments for possible improvements as for this one. However, there are two issues that diminish, in my opinion, the suitability for PCI recommendation in the current version (I feel that both these points would be relatively straightforward to address in a revision). First, parts of the manuscript, especially large parts of introduction and discussion are too system-specific, and it may be good to present the manuscript from the beginning from a somewhat larger, more general perspective to increase the overall scope of the study. Second, and related to the previous point, parts of the discussion are not very well referenced and comparisons with other study systems could be strengthened. Neither of the points, however, requires extensive changes.

We thank the reviewer for their overall positive evaluation of the manuscript and associated work. We now highlight more clearly that the general question addressed refers to transitions between reproductive modes and have overall reduced the system-specific focus (see notably L52-57).

We have also extended comparisons to other systems as suggested. We were already citing known examples of asexual species with recently described cryptic sex. We now also cite examples of sexual species with rare parthenogenesis. (L562 – 563).

We have also addressed all the specific points listed below.

Specific points:

The sentence on L. 69-71 is cryptic (unless one has already read the study)

clarified

L. 162: How was this DNA obtained? From collaborators? From authors of a previous study?

We have specified that this DNA was available from previously published studies in our lab (L198).

L. 230: hemizygous rather than homozygous

changed

L. 457: Or perhaps just variation in the rate of recombination (not two distinct parthenogenesis mechanisms).

We suggest that genome-wide homozygosity is a consequence of “specific forms of automixis, notably gamete duplication or fusion of non-recombined sister chromatids”. Given the formulation of our text, a fusion of recombined sister chromatids would be included under “different parthenogenesis mechanisms”. We prefer not going into further detail in the text given our data do not allow us to distinguish different possibilities.

I may have missed it, but I don't remember that the previous evidence for “obligate” parthenogenesis in the species is discussed in any detail nor where (geographically) these putatively obligate populations occur.

***Timema douglasi* was originally described as an obligate asexual species. The purpose of our manuscript was not to confirm this, but we cited the paper that showed it (L62; L195). We also added an additional figure (Figure 2B) to further clarify this point. Because the “Orr” and “Eastern Manchester” lineages are found along both transects, there does not seem to be a strong geographic pattern (see Figure 1B,D).**

Reviewed by anonymous reviewer, 16 Jun 2022 11:22

This paper presents new results regarding the reproductive modes of the North American stick insect *Timema douglasi*. It is based on field sampling on two transects, experiments to investigate reproductive modes and extensive genomic data. The key result is that this taxon, originally thought to be exclusively parthenogenetic, is in fact able to reproduce sexually, although to a different extent depending on the genotype.

I read this manuscript with interest. This is yet another case study showing that asexuals are not reproducing in the way initially envisioned. The demonstration is convincing and the data supports the main finding without ambiguity.

I have however some reservations regarding the interpretation and some of the analyses. I think they can be addressed in a revision.

Main comments

1- Overall, the authors present and discuss their work by asking whether it represents a case of vestigial sex or ‘re-evolution’ of sex, even invoking “Dollo’s law of irreversibility” for the latter. I think that this is casting the problem in an unnecessarily complicated way. I would suggest to cut the part on Dollo’s law. This “law” is of very little interest as it is based on no clear process. It is just a mere label for disparate observations without real content. More fundamentally, the results might be interpreted differently, by simply saying that these timemas are all facultative parthenogens, although to a different extent across genotypes. I think it is possible to make this case much more strongly. In fact, there is some inconsistency in the reasoning that pre-date this paper. The term “tycho-parthenogenesis” has been introduced to depict situations where sexual females, could lay viable unfertilized eggs. This occurs for instance in absence of mates. The observation that these females are capable of reproducing by parthenogenesis and sexually is basically the same as the observations made in this paper. The only difference is quantitative, not qualitative. Often, the term tychoparthenogenesis is used for low rates of parthenogenesis, but it must involve a mechanism of producing diploid unfertilized eggs. So, apart from the rate of sex vs asex, is there really a difference between the mixed reproductive mode observed in the paper and the already well-known fact that these timema are capable of both sex and “tychoparthenogenesis”. It is very likely that the underlying mechanism are the same and that only sex-asex rates differ across different genotypes. Hence, one could make the relatively strong argument that the use of the label ‘tychoparthenogenesis’ has been misleading from the start, and that the current paper is simply documenting the fact that the rate of sex-asex varies more importantly than was previously considered. In this view, this is not a question of vestigial sex or ‘re-evolved’ sex. All *T. douglasi* have a mixed reproductive mode. It varies quantitatively across genotypes, but sex (and asex) were never ever really either lost or regained. The capacity for sex and asex may simply have been there, all along, in all lineages.

We certainly agree that the parthenogenesis mechanism in facultative parthenogens and spontaneous parthenogenesis in sexual species (“tychoparthenogenesis”) is very likely the same (this is, after all, one of the points we are making in the paper based on our new findings). However, the variation in parthenogenesis frequencies are not merely “quantitative” as suggested by this reviewer. There is a clear distinction between sexual females, asexual females, and the facultative parthenogenetic females we describe here. Specifically, the vast majority of sexual *Timema* females are unable to produce diploid eggs that hatch and develop into new adults (except in some marginal population which apparently experience recurrent selection for reproductive assurance, and in which females are characterized by a better capacity for parthenogenesis; Schwander et al. 2010). In contrast, all females belonging to the newly described facultative lineages can reproduce via parthenogenesis with a decent efficiency. We have now added an additional figure with extensive data from other sexual and asexual species to the manuscript to illustrate this point (Figure 2B; L 354 – 356).

Regarding the comments on Dollo’s law, we have removed reference to this “law” and reformulated the relevant sections (L531 - 538) as we largely concur with the reviewer that it is not particularly useful in the absence of a specific process.

2- The paper does not clearly explain why the sex ratio varies strongly in Manchester transect between populations 7 and 8. Regarding the sampling and genotyping results, there are very few individual tested in populations 8-12 in Manchester transect (and none in population 8 and 9 where the sex ratio abruptly varies), which makes it hard to well understand what is going on. However, the transition in

sex ratio is not mirrored by a genotypic transition, as one would expect. The main geographical pattern is therefore left unexplained in the paper, and this is certainly a missed opportunity. I think that it is possible to make the case that the yellow clade can be split between two subgroups with distinct reproductive modes. I have tried to look at this based on the Fig 1E and Fig 3, and it does seem to exist such a difference, which may really help to understand why the sex-ratio varies so abruptly after population 7 : the yellow individuals seem to belong to different subgroups before and after this transition. I think it is necessary to better analyze what is going on. I understand that automatically separating clusters yields to these red/yellow/blue subgroups. However, I find it very surprising that the low heterozygosity individuals tend to cluster together in the phylogeny, suggesting that different yellow subgroups may actually reproduce differently, perhaps explaining the geographic transition in Manchester transect. Even if this suggestion is not correct (it is only based on my limited ability to investigate this using the figure), it is necessary to better analyze and discuss it. In the end, the reader expects to see an explanation for the extraordinary sex-ratio pattern seen on Manchester transect. This should be a primary concern of the paper.

Our intuition was also that polymorphism within the yellow lineage could explain the sharp transition in sex ratio. However, we found no evidence for this hypothesis in our data. Out of the five individuals from the yellow lineage that form a separate cluster on Figure 1E, three come from Manchester 12 (female-only), one from Manchester 10 (female-biased) and one from Manchester 6 (balanced sex ratio). Surprisingly, the only individual that was not capable of parthenogenesis comes from Manchester 12 (none of the 13 eggs laid prior to mating hatched). It is true that three of these five individuals have low heterozygosity (Figure 3), but this is most likely a consequence rather than a cause of the absence of males in their population (although genotyping of a larger panel of the field collected individuals and their offspring would be required to properly investigate this). We have added explicitly that, within the currently available data, the two clades did not explain the observed patterns (L425 – 426).

3- Regarding the results of the experiments illustrated on Fig 5, it would be important to clarify whether the hatching success varies between fertilized and non-fertilized eggs, especially for the yellow group. This information is not directly available, but a correspondence might be doable across females. This has to be clarified. Maybe splitting the yellow group in two subgroups as suggested above will also clarify the pattern shown on Fig. 5. Similarly for the blue group, there are individuals with or without heterozygosity and it would be important to know if they represent different subgroups with different reproductive modes. These considerations are not sufficiently detailed and thoroughly investigated.

The test for significant differences in hatching success between fertilized and unfertilized eggs was shown in L257 – 260 (methods) and 410 – 415 (results). In the yellow lineage, hatching success was higher for fertilized than for unfertilized eggs. Splitting this lineage into subgroups would require higher sample sizes.

4- In the discussion, the authors insist on the idea that sex and parthenogenesis are traded-off against each other (line 518). Can they produce a figure illustrating this directly? I do not see very well how the results can be used to directly support this claim. An important clarification is needed here.

We suggest the trade-off between sex and parthenogenesis as a possible explanation for the observation that many lineages of *Timema* (and other organisms) use obligate strategies. Testing it directly was not possible with our data as it would require more females per genotype, but this is certainly an exciting future direction.

Other, more specific comments

1184-188. Explain better the reason behind this hypothesis involving paralogues and missing data.

We have expanded the explanation.

1245. Was it possible to remove PCR duplicates without paired-end sequencing information?

These libraries were sequenced paired-end. This was not explicit in the first version and we have clarified it.

1261-264. Looking whether a value falls in an interval is not a proper test. Many tests are also performed here.

We performed Fisher's exact tests as a complement to address this comment.

1501-504. This is, I think, an important observation indeed, supporting the view that these "lineages" may not be really lineages given that sex is relatively frequent. Hence a phylogenetic representation is probably misleading. Some discussion about this would be useful.

Our phylogeny based on nuclear markers and our clustering analysis showed that the lineages were good lineages indeed, and that the putative rare gene flow that could account for the mito-nuclear discordance was not sufficient to disrupt the topology of the nuclear tree. Our RADseq data provides a relatively low marker density, which is not ideal to perform classical tests of gene flow. We were therefore very careful with wording and we believe that expanding further is not appropriate in the absence of stronger evidence. We have added one sentence as perspective (L552 – 553).

Reviewed by anonymous reviewer, 02 Jun 2022 15:13

This is a fascinating paper about the new discovery of mixed-sex populations in a species of *Timema* stick insect that was formerly thought to reproduce only by parthenogenesis. The authors perform a series of field surveys, laboratory experiments, and genetic analyses to answer questions about the incidence of and capacity for parthenogenesis versus sex in these newly discovered populations. The study offers very compelling evidence that the species is not an obligate parthenogen, but is in fact facultatively parthenogenetic, with variation in reproductive mode observed across different populations, resulting in varying sex ratios. This lends support to the idea that facultative parthenogenesis is an important stepping-stone in evolutionary transitions from obligate sex to obligate parthenogenesis—an important question in evolutionary biology.

The paper is very nicely framed and beautifully communicated (both in the writing and the

figures). My suggestions for changes are all rather minor, requiring only additional clarification, rewording, or elaboration. This is a very exciting paper! I can't wait to see it in its published form!

We thank the reviewer for the very kind and useful feedback.

L48-49: "When mated, sexual females fertilize all their eggs, and population sex ratios are close to 50:50". Please provide a citation for this statement of fact.

We have now added a citation.

L59-62: "Such variable population sex ratios could, for example, result from a mix between sexual and parthenogenetic females, or from facultative parthenogenesis ". Please reword this for clarity. Do you mean that female-only populations might be obligately parthenogenetic with no capacity for sex and mixed-sex populations might be obligately sexual with no capacity for parthenogenesis, or, alternatively, all populations might be facultatively parthenogenetic?

Rephrased.

61-62: "Alternatively, if the newly discovered populations were sexual, variable sex ratios could also stem from genetic drive". Please reword to something like: "Alternatively, all the newly discovered populations could be obligately sexual with sex-ratio distortion determined by mechanisms other than parthenogenesis". Distortions in sex ratio can be mediated by any number of factors; genetic drive is only one of them. Other mechanisms include male-killing endosymbionts, sex-specific zygote mortality, sex-specific sperm mortality, temperaturedependent sex determination, etc. (see Krackow BiolRev. 1995). Some of these may be more likely in *Timema* than others. But given that you only measured the sex ratio and didn't attempt to assess the presence or absence of any of these competing mechanisms, I don't think it's possible to say anything about the mechanism involved.

We were assuming that X-chromosome drive was the most likely mechanism of sex ratio distortion in *Timema*, but we agree with this comment. We have changed "X-chromosome drive" to "sex ratio distortion" throughout the manuscript.

62-64: Please cut this final sentence. You didn't assess whether X chromosomes exhibited genetic drive, at least I can't see it in your methods. It seems as though you only assessed

what the sex ratio was.

Now that we changed “X chromosome drive” to “sex ratio distortion” throughout the manuscript, we kept this sentence as one example.

90-91: “We aimed to characterise the reproductive mode of approximately 10 females from female-only populations and of approximately 20 females from populations with both sexes”.

Do you mean 10 females from each mixed-sex population, and 20 females from each femaleonly population? Or 10 and 20 females in total?

Clarified.

L97: Females were isolated in petri dishes?? How big were these petri dishes?

We added the diameter info.

L98: Please add some brief words to explain why soil and cottonwool were included in the petri dishes.

Added.

L108-114: I think these sentences can be reworded to avoid repetition, something like: “To test whether the individuals collected in the field belonged to different genetic lineages, we genotyped 32 females and their mates (1 - 3 per female, 42 males in total). We also tested whether eggs produced before and after mating were fertilized or not. To do this, we genotyped 3 hatchlings from eggs laid before mating (when available) for each of the 32 genotyped females (77 hatchlings from 27 females in total; for the 5 remaining females, none of the eggs laid prior to mating hatched), and 7 hatchlings from eggs laid after mating (when available) for 24 of the 32 females.”

Rephrased accordingly.

L225-226: Measuring distortions in sex-ratio can't tell you what the mechanism for it is. As mentioned above, there are numerous possible mechanisms. What criteria did you use to assess that any possible distortion you observed was due to X chromosome drive and not some other mechanism?

Changed to “sex ratio distortion”.

L266: Because you calculated sex ratios in both parental and offspring generations, it would be good to orient readers as to which sex ratio you're talking about here.

Clarified.

L277-281: “The second lineage (yellow in Figure 1B) was found mostly in the eastern section of Manchester and is hereafter referred to as the “eastern Manchester lineage”. Finally, the third lineage (red in Figure 1B) was mainly found in the western section of Manchester and is hereafter referred to as the “western Manchester lineage””. Perhaps it would make sense to justify the names by saying that there was a gradient of genotype incidences from east to west, with the red genotype common at the far west of the Manchester transect, and the yellow genotype common at the far east of the transect.

We added one sentence.

L334: Figure 3: Is it possible in this figure to also indicate the sex-ratio of the population from which each female originated? Some of the blue and yellow mothers have low relative heterozygosity indicating that they were produced parthenogenetically, but it would be interesting to know if they came from an all-female population or a mixed-sex population. It would be interesting to know whether the sexually produced mothers with higher relative heterozygosity always came from mixed-sex populations.

We had population sex ratio included in previous versions of this figure, but there was no clear pattern and we removed it for the sake of clarity.

L361 (and throughout the results): Please provide test-statistics, model coefficients, and SEs for all models. It isn't sufficient to just cite p-values.

Added.

L393-394: “Finally, we tested whether X chromosome drive could be contributing to femalebiased sex ratios by looking at the sex ratio of sexually produced offspring”. I don't think merely testing for a skew in the sex-ratio is evidence for or against X chromosome drive. Sex-ratios of sexually produced offspring can be skewed by mothers (or fathers) in any number of ways. It might be worthwhile just reporting the sex ratio analysis here without mentioning X chromosome drive at all.

Changed to “sex ratio distortion”.

L405-406: "...and not by X chromosome drive". I would say "and not through sex-ratio distortion by other means".

Changed.

L409: Add a comma after "strictly".

Added.

L416-417: "However, lower hatching success of post-mating eggs could also be due to females being older". But wouldn't you then expect to see a decline in emergence in eggs produced by ALL females after mating? Or do you mean that females from the Orr lineage might suffer reproductive ageing to a greater degree than females from the other lineages?

This seems unlikely. I'd cut this sentence.

Added "This effect would be compensated by the positive effect of fertilization in both Manchester lineages, but not in the Orr lineage where fertilization does not seem to improve hatching success."

L419: "Females of the two facultatively parthenogenetic lineages". Are only two of the lineages facultatively parthenogenetic?? I thought you found that all populations showed a capacity to reproduce parthenogenetically, and mating resulted in at least some sexually produced offspring in all lineages. That would seem to suggest that all three lineages are, by definition, facultatively parthenogenetic but vary in their ability to reproduce sexually versus parthenogenetically. I suggest rewording this for clarity and accuracy.

We have clarified in the previous paragraph instead.

L420: "...the largely obligate one...". Characterising the third population this way doesn't seem correct either, based on your results. Yes, the Orr lineage mostly *reproduced* parthenogenetically, but females of this lineage also produced some offspring sexually when they mated (which, by definition, means the lineage cannot be *obligately* parthenogenetic—it's a facultative lineage. It's just that females show variation in their facultative capacity). The wording should be revised accordingly.

Idem.

L425: “Five females reproduced via obligate parthenogenesis...”. Just because these females *reproduced* only parthenogenetically doesn't mean they are necessarily obligately parthenogenetic. You don't know whether these females would have produced offspring sexually if they had mated more times with more males. You don't even know whether the matings that these females received resulted in sperm transfer. They may have received poor quality sperm, or incompatible sperm. I think it makes more sense to just say that these females only reproduced parthenogenetically, even after mating. Given that sex and parthenogenesis occur in all three lineages, it makes more sense to characterise all three of them as being facultatively parthenogenetic, but that there is population and individual variation in the incidence of sex and parthenogenesis.

Changed to “only via parthenogenesis”.

L425: What was the phenotypic similarity? Just that they reproduced only parthenogenetically? Or was there some other aspect of the phenotypes that was similar?

Removed.

L426: “...”obligately” parthenogenetic females of the Orr lineage”. Again, I would avoid this kind of characterisation.

Removed.

L430-431: “...(facultative and largely obligate parthenogenesis) ...”. Again, I don't think it makes sense to make this distinction. Your results suggest that all populations are facultatively parthenogenetic, but the capacity of individual females to reproduce sexually versus parthenogenetically differs.

Removed.

L432: Delete “as”.

Done.

L439-440: “However, variable selection pressures along the transect could also play a role, favouring respectively sexual or parthenogenetic reproduction”. One such pressure that's expected to generate these kinds of spatial mosaics of sex-ratio in facultatively

parthenogenetic species is sexual conflict (see Burke & Bonduriansky, PhilTrans, 2018). It might be a good idea to mention that here.

Added.

L485-486: “Repeated transitions towards parthenogenesis are not surprising if the ancestor of these species was already capable of reproducing via facultative or spontaneous parthenogenesis”. I would add: "..., and if selection has the opportunity to increase parthenogenetic capacity".

Added.

L491-492: “Widespread tycho parthenogenesis capacity could thus serve as a stepping stone for the repeated evolution of more successful obligate parthenogenesis”. More successful than what? Tycho parthenogenesis is just a rare capacity for parthenogenesis in organisms that otherwise and typically reproduce sexually. Sex with a capacity for tycho parthenogenesis may in fact be more successful, especially depending on the environmental or ecological conditions. I'd cut "more successful".

Hatching success of unfertilized eggs was higher in the “obligate” parthenogenetic lineage (Orr) and in the asexual species than in the two “true facultative” parthenogenetic lineages (Manchester). This is what we meant by “more successful”. This information is now added explicitly in the manuscript (L354 – 356; Figure 2B).

L492-493: “... which could help explain why obligate parthenogenesis evolves so frequently in the *Timema* genus”. In an ultimate sense, I don't think widespread tycho parthenogenesis explains why obligate parthenogenesis evolves so frequently in *Timema*.

Tycho parthenogenesis is only a pre-adaptation. What's required for obligate parthenogenesis to evolve from tycho parthenogenesis is some selective pressure (such as persistent mate scarcity). Greater clarity here would be welcome.

This is now explained more clearly (L525 – 529).

L514-515: “However, the loss of sex in facultative parthenogens could in theory be driven by sexual conflict (if mating always reduces female fitness; Burke & Bonduriansky, 2017), but this has never been demonstrated in nature”. But your results provide some support for this, no? Females from the most parthenogenetic lineage (the Orr lineage) showed a significant

reduction in hatching success after they mated, and very few of their eggs were actually fertilised, suggesting that mating and sex might be more costly for these females. This contrasts with the other two lineages which both increased in hatching success following mating. Obviously, comprehensive fitness estimates would be required to properly assess this sexual conflict hypothesis. But still, there seems to be some quite suggestive evidence for it in your results. It might be worthwhile highlighting that here.

Our results are compatible with this hypothesis, but they could also be explained by, for example, a tradeoff between sex and parthenogenesis or an age effect (whereby hatching success would decrease as a female ages; a discussed in L467 – 468). We have removed “but this has never been demonstrated in nature”.

L518-521: “In this case, whether obligate strategies are likely to replace facultative ones will depend on local ecological conditions favouring sex or parthenogenesis, and on the fluctuations of such conditions”. I think it would be good to provide an assessment of the likelihood of this explanation, given your results. I'm not sure this explanation makes a lot of sense given that you found sharp differences in the incidence of and capacity for sex vs. parthenogenesis over just a few hundred meters. Are ecological conditions likely to fluctuate so greatly across such a short distance that the selective advantage of one reproductive mode over the other would switch so completely? Seems unlikely. I suggest adding something at the end of this paragraph like: “This explanation is unlikely in our case, since populations showed large differences in sex ratio over very short distances but did not differ greatly in ecology”.

Estimating the likelihood of this explanation would require at least some insights into which ecological conditions matter. One factor that could play a role is parasite prevalence, which could vary greatly between patches given the very limited dispersal of their hosts. We do know that *Timema* can be infected by some fungi and nematodes, yet their mode of infection is unknown. We also know that feeding niche breadth tend to be narrower in asexual *Timema*, but *T. douglasi* was the exception (see Larose et al. 2018 Proc B). Assessing which factors could influence relative habitat suitability for sexuals and asexuals was beyond the scope of the present study and we are afraid that discussing them would be a bit off topic.

L528: Delete “other” and “would”.

Done.