Supplementary material for the manuscript:

Facultative parthenogenesis: a transient state in transitions between sex and obligate asexuality in stick insects?

By Chloé Larose*, Guillaume Lavanchy*, Susana Freitas, Darren J. Parker, Tanja Schwander

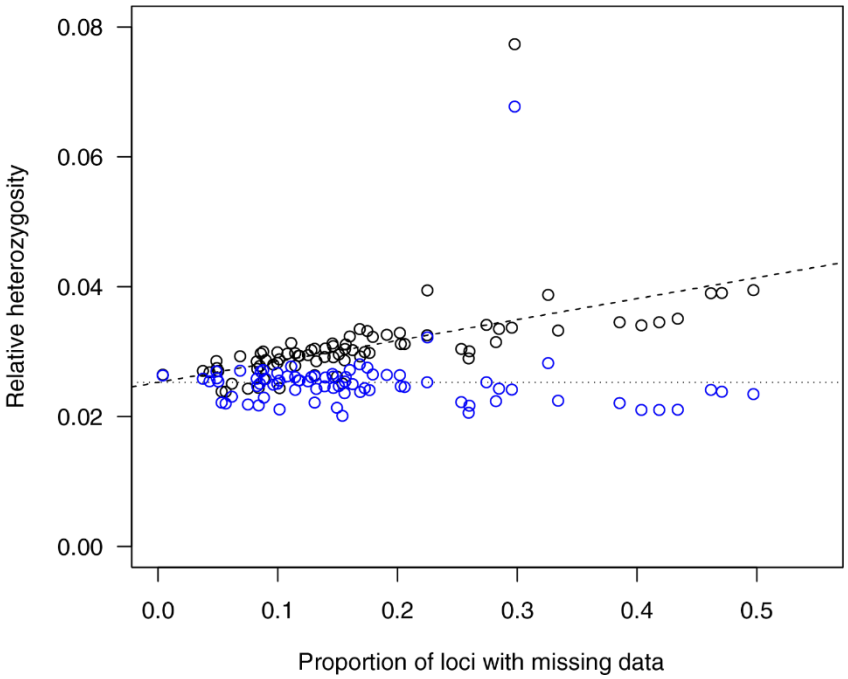

Figure S1 Relative heterozygosity (based solely on polymorphic loci) in hatchlings from eggs laid before mating. Black dots: raw heterozygosity value, measured as the proportion of heterozygous SNPs for each individual, as a function of the proportion of missing data. Blue dots: corrected heterozygosity value for the same individuals, obtained by summing the residuals of a linear model predicting heterozygosity with the proportion of missing data (dashed line) and the intercept of that model (dotted line). This correction removes the variance in heterozygosity explained by missing data. The model is built on hatchlings from unfertilized eggs laid prior to mating only because of their extremely low heterozygosity. The effect of missing data is masked in normally heterozygous individuals because the effect size of the missing data on heterozygosity is smaller than the natural variation in heterozygosity levels (Figures 3 and 4). The model was then used to correct heterozygosity for all individuals.

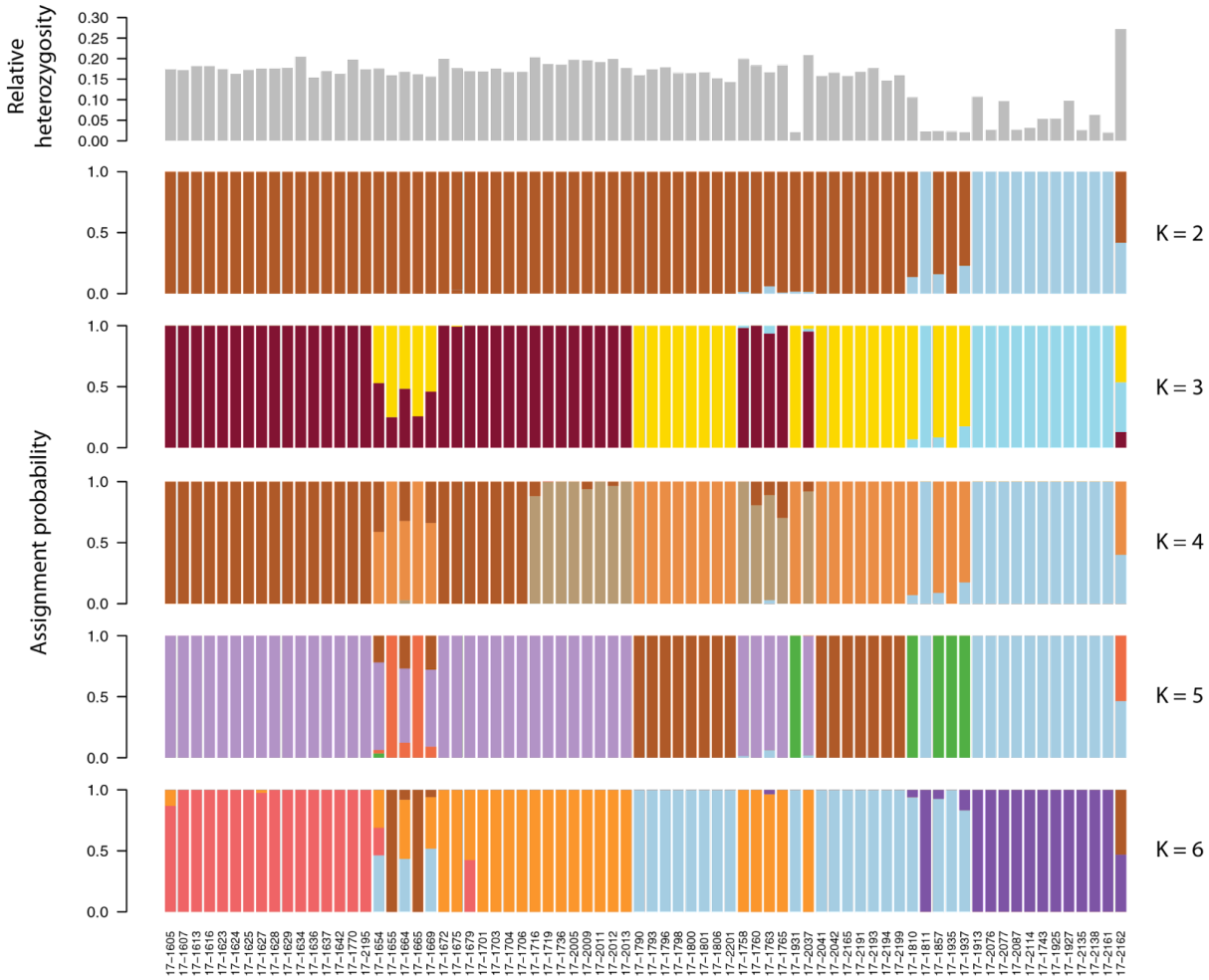

Figure S2 Relative heterozygosity and genetic structure of all field-collected individuals. Top row: corrected relative heterozygosity. Following rows: fastSTRUCTURE results with different levels of K. For K = 3, colours as in main figures. Individuals ordered by location, as in Figures 1B and 1D. Note the high heterozygosity of individual 17-2162, which has a genetic contribution from all three inferred genetic lineages (k=3). This may indicate that this individual is a triploid hybrid between these lineages, but additional data would be needed to confirm (see Figure S5). Note also that all genotyped individuals (all males) from the Manchester 2 population are hybrids between the eastern and western Manchester lineages, with approximately equal contributions from the two. They are likely the result of past hybridization rather than F_1 hybrids, as their heterozygosity levels are similar to non-hybrid individuals. Surprisingly they have maintained a high frequency of alleles from the eastern Manchester lineage while neighbouring populations are fixed for alleles from the western Manchester lineage. This further supports the very limited dispersal in these wingless insects.

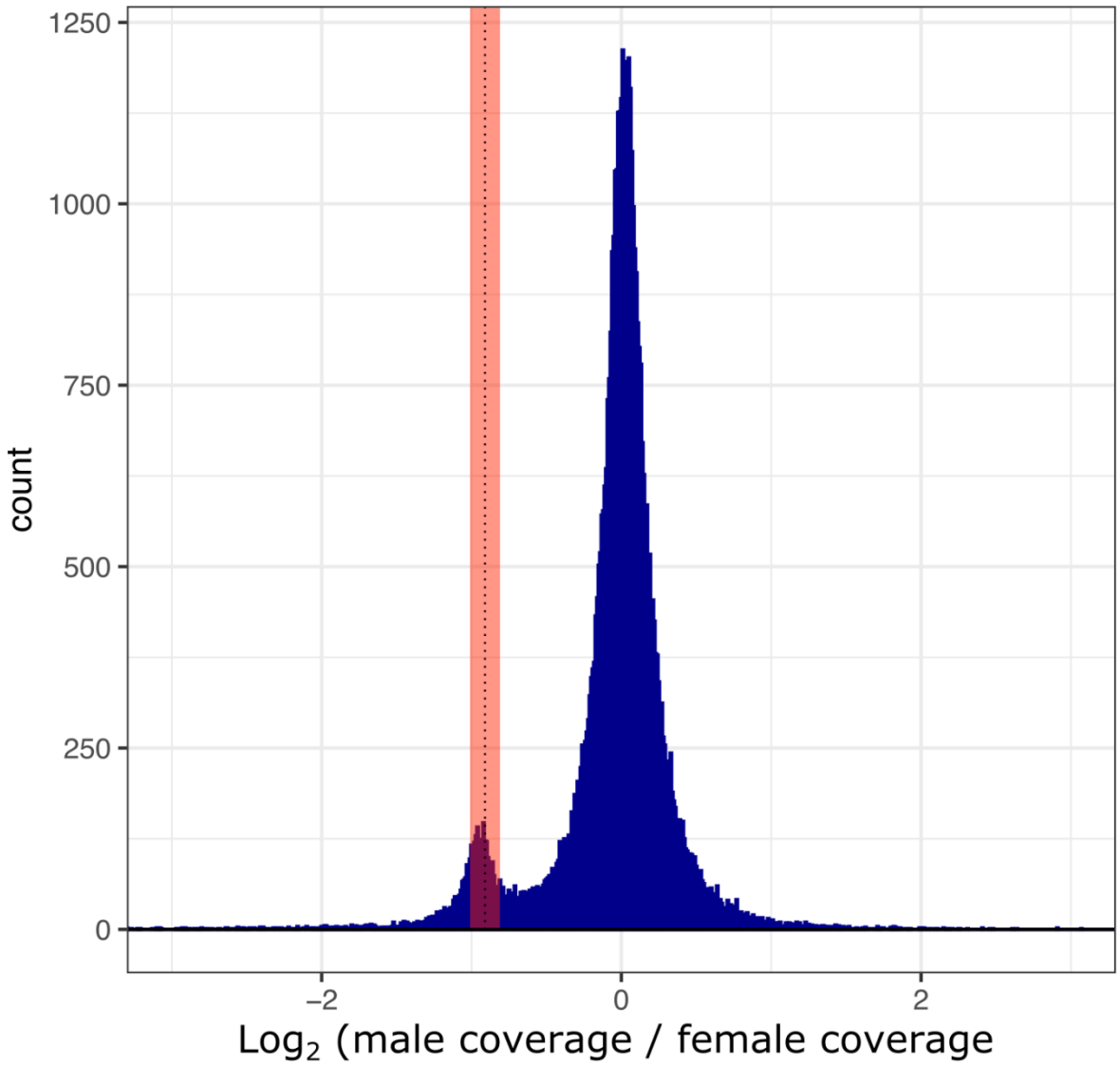

Figure S3 Frequency distribution of $\log_2$ ratio of male to female coverage for all contigs >= 1000 bp in length. Autosomal contigs should have equal coverage in males and females ($\log_2$ ratio of male to female coverage = 0). X-linked contigs should have half the coverage in males than in females ($\log_2$ ratio of male to female ratio coverage = -1). X-linked scaffolds were classed as contigs with a $\log_2$ ratio of male to female coverage within 0.1 of the X linked peak (black dotted line).

Assessing the amount of false-positive heterozygosity

We found that all but one offspring produced from unfertilized eggs laid prior to mating had similar and very low relative heterozygosity (~ 2-3%), similar to the heterozygosity measured in obligately parthenogenetic reference individuals, and independently of the heterozygosity of their mother (Figure 3). Previous work revealed that the extremely low heterozygosity measured in obligately parthenogenetic individuals is largely due to divergence between paralogs and structural variation between individuals (Jaron et al., 2022), i.e., represents mostly false-positive heterozygosity. False-positive heterozygosity calls generated by structural variation among individuals is increasingly recognized as a general problem in sequencing data sets, even in model organisms with very well resolved genomes such as *Arabidopsis* (Jaegle et al., 2021). For all these reasons we suspected that the 2-3% relative heterozygosity measured in parthenogenetically produced offspring was also largely generated by repeated regions and structural variation, and not due to heterozygosity from allele divergence.

We investigated whether this was indeed the case by looking at heterozygosity on the X chromosome. Because the X chromosome is monosomic in males (*Timema* have XX:XO sex determination), all heterozygosity measured on the X in males can be classified as false positives. Thus, by comparing heterozygosity on the X chromosome of males with the heterozygosity of the X chromosome of parthenogenetic (diploid) offspring, we can assess whether the heterozygosity in parthenogenetic offspring is also generated by false positives. This would be the case if heterozygosity on the X was similar in the two groups.

We found no difference in heterozygosity levels on the X chromosome of males and parthenogenetic offspring (t-test: $t = 0.296$, $df = 39.741$, $p = 0.77$; Figure S4A). This means that parthenogenetic hatchlings are fully homozygous on their X chromosome. Interestingly, heterozygosity in parthenogenetic offspring was higher on the X chromosome than genome-wide, indicating that repeated regions and other structural variants that are generating false-positive heterozygosity are enriched on the X.

To further confirm that heterozygosity on the male X was largely due to repeated regions, we compared average depth at homozygous vs heterozygous loci. We standardised depth per individual and tested whether there was a significant difference using a paired t-test. We found that depth at heterozygous X-linked loci was higher than at homozygous X-linked loci in all 40 males (average: 2.8x higher; $t = -21.1$, $df = 39$, $p < 2.2^{-16}$; Figure S4B).

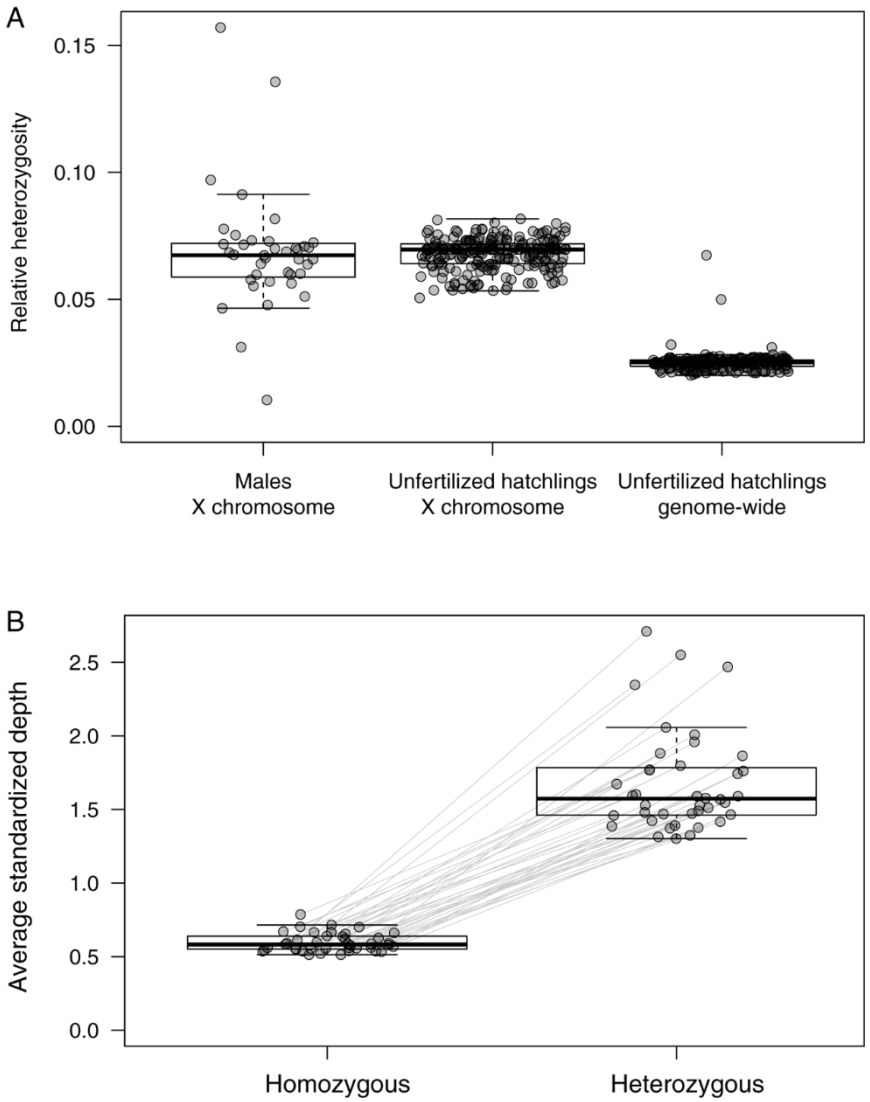

Figure S4 Repetitive regions generate false-positive heterozygosity on the X chromosome of males and in parthenogenetically-produced offspring. **A** Relative heterozygosity (based solely on polymorphic loci, see main text) on the X chromosome of males, on the X chromosome of parthenogenetically-produced offspring and on the whole genome of parthenogenetically-produced offspring. One point represents average heterozygosity across loci for one individual. Note that the heterozygosity estimates are corrected for the effect of missing data as in Figure S1 (separately for males and parthenogenetic offspring as the effect of missing data is expected to differ for monosomic vs. diploid chromosomes). **B** Average depth (standardised per individual) of homozygous and heterozygous loci on the X chromosome of males. Each dot is a male, depth values for homozygous and heterozygous loci of the same individual are linked with a line.

Sexing sexually produced offspring

In order to sex the sexually produced hatchlings, we looked at heterozygosity on the X chromosome and depth ratio between the X chromosome and autosomes. Because they have only one copy of the X chromosome, males are expected to be homozygous at all X-linked loci. In addition, X-linked loci should have half the coverage of autosomes. However, we observed a higher amount of false-positive heterozygosity on the X than on autosomes, which was largely generated by repetitive regions (see previous section and Figure S4A). In addition to generating false-positive heterozygosity, these replicated regions also bias the X/autosome depth ratio. Both these effects are enhanced in individuals with large amounts of missing data because of low sequencing coverage (because loci with too low coverage are filtered out in all individuals). For this reason, we used a stricter cutoff for this analysis, retaining only individuals with fewer than 20% of missing data. 176 out of the 211 sexually produced offspring passed this cutoff, of which 95 were inferred to be males and 81 inferred to be females (Figure S5).

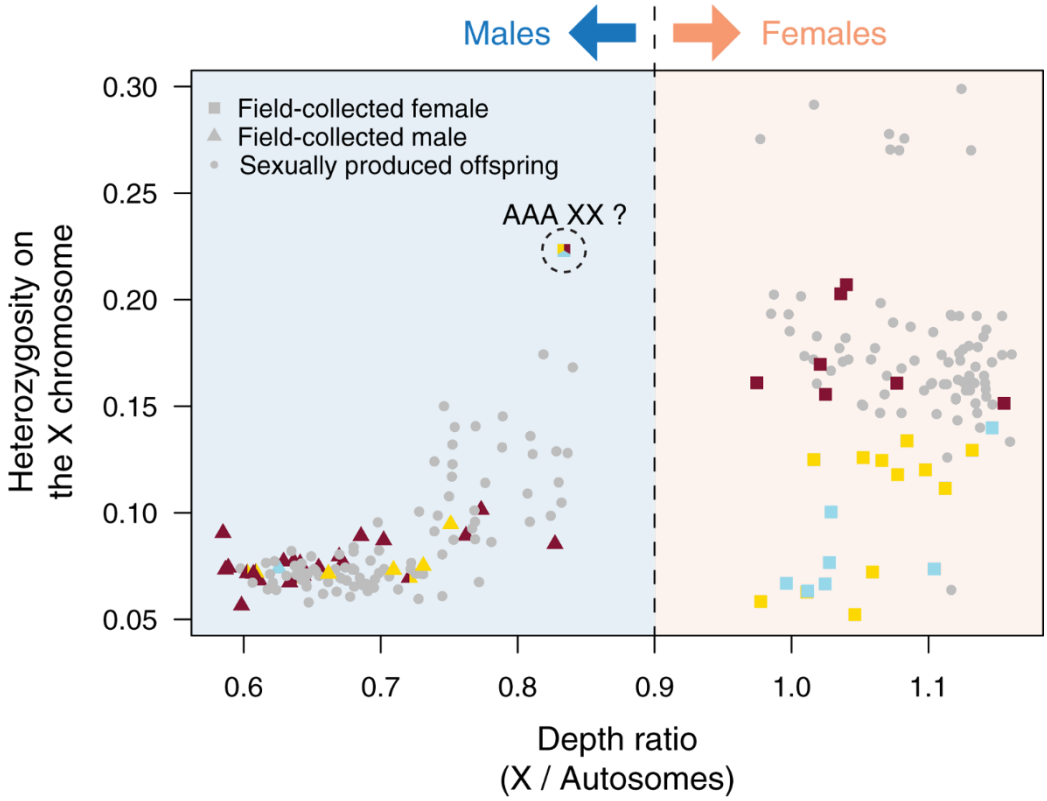

Figure S5 X / autosomes depth ratio and heterozygosity on the X, used to sex the sexually produced hatchlings (hatchlings cannot be sexed by morphology). Males and females collected in the field (and sexed by morphology) are indicated for comparison. Colours as in Figure 1. Hatchlings were considered males if they had an X / autosomes depth ratio lower than 0.9 (dotted line), and female otherwise. Note that the X/autosome depth ratios are higher than expected for both males and females, consistent with the X chromosome being enriched for repetitive content. Female 17-2162, the hybrid with a contribution from each of the three genetic lineages, stood out for both measures. Its X chromosome was more heterozygous than that of all field-collected adults, but its X/autosome depth ratio was lower than that of other females. While further research is required to explain this pattern, it could stem from a triploid constitution with only two X chromosomes.

Hatchling sex ratio

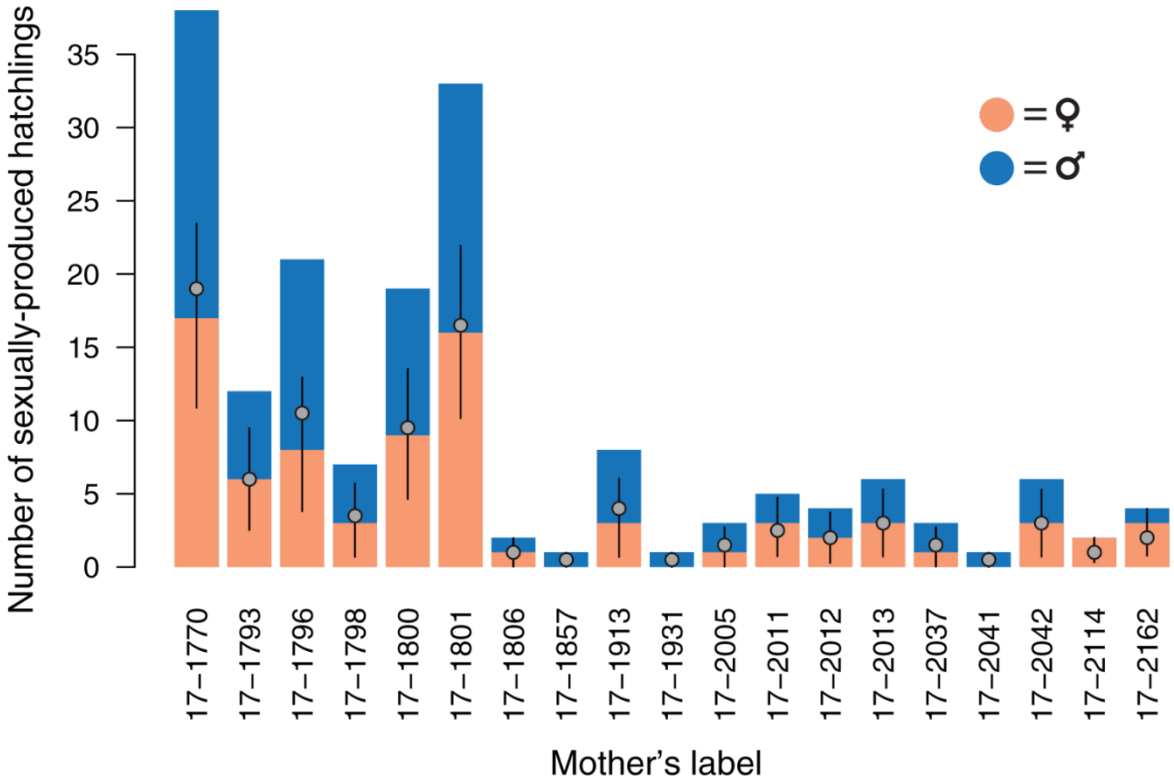

Figure S6: Sex ratio of sexually produced hatchlings per family. The grey dots represent a 50:50 sex ratio (the null hypothesis). The black lines are 95% binomial proportion confidence intervals around the observed sex ratio. All confidence intervals overlap with the expected value under mendelian inheritance, and the p-value of Fisher's exact tests for each family were higher than 0.5, providing no evidence for X-drive underlying biased sex ratios.