Facultative parthenogenesis in an asexual stick insect: re-evolution of sex or vestigial sexual capacity?

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Abstract

Transitions from obligate sex to obligate parthenogenesis have occurred repeatedly across the tree of life. Whether these transitions occur abruptly or via a transient phase of facultative parthenogenesis is rarely known. Here, we discovered and characterised facultatively parthenogenetic populations of the North American stick insect *Timema douglasi*, a species in which only obligately parthenogenetic populations were known so far. These populations comprised three genetic lineages. Females from all lineages were capable of parthenogenesis (with variable efficiency) but their propensity to reproduce sexually after mating varied extensively. In all three lineages, parthenogenesis resulted in the complete loss of heterozygosity in a single generation. Obligately parthenogenetic *Timema* have also lost all heterozygosity, suggesting that parthenogenesis evolved via a gradual capacity increase while conserving the proximate mechanism. It is unclear whether the populations we discovered have re-evolved sex (via re-expression of existing pathways), or whether they are vestigial sexual populations in a species where all other lineages have evolved obligate parthenogenesis already. We speculate that facultative parthenogenesis may often be transient and be replaced by obligate strategies (either sex or parthenogenesis) because of a trade-off between the efficiency of the two reproductive modes.

Introduction

The way organisms reproduce varies extensively across the tree of life. While most animals engage in some form of sex to produce offspring (Bell, 1982; The Tree of Sex Consortium, 2014), many species take alternative routes to reproduction. The most widespread alternative is obligate parthenogenesis, whereby females always produce daughters without genetic contribution from males (Normark, 2003; Suomalainen et al., 1987). Obligate parthenogenesis and sex are two extremes of what is in fact a continuum: in some cases, a female can reproduce via parthenogenesis when virgin, but can switch to sex upon mating. This reproductive mode is called facultative parthenogenesis and is thought to combine the long-term advantages of recombination and segregation that come along with sex, together with the short-term advantages of asexuality (D'Souza & Michiels, 2010; Otto, 2009). However, such facultative strategies are rather scarce in most animal groups (Bell, 1982), and most extant parthenogenetic species are largely unable to reproduce sexually (but see Boyer et al., 2021 for an exception). Yet taxa which reproduce via facultative strategies could offer ideal opportunities to study the transitions between reproductive modes and the generation of the diversity we observe (Liegeois et al., 2021).

The stick insect genus *Timema,* native to the western USA and Mexico, has been studied extensively for its repeated transitions from sex to obligate parthenogenesis. Five of the 21 described species reproduce via female-producing parthenogenesis, while the others are sexual (Law & Crespi, 2002). In parthenogenetic species, fertilization of oocytes does not occur even when females are mated with males of related sexual species in the laboratory (Schwander et al., 2013). Conversely, females of the sexual species are largely incapable of parthenogenesis, although spontaneous parthenogenesis ("tychoparthenogenesis", the hatching of up to 1-2% unfertilized eggs laid by virgin sexual females) via automixis is well documented (Schwander et al., 2010; Schwander & Crespi, 2009). When mated, sexual females fertilize all their eggs, and population sex ratios are close to 50:50.

Closely related parthenogenetic and sexual *Timema* species have distinct geographic distributions, and the northernmost populations (*T. douglasi* and *T. shepardi*) are exclusively parthenogenetic (Law & Crespi, 2002). Yet during standard sampling of well-known, female-only *T. douglasi* populations, we unexpectedly discovered populations with large numbers of males. *T. douglasi* cannot be morphologically distinguished from females of its sexual sister species *T. poppensis* (Vickery & Sandoval, 1999), so it was not possible to assess phenotypically whether the collected individuals were *T. douglasi* or belonged to a geographically isolated *T. poppensis* population. The male-containing populations were geographically close to female-only populations, with an apparently abrupt transition from female-only populations to populations with relatively even sex ratios. Such variable population sex ratios could, for example, result from a mix between sexual and parthenogenetic females, or from

facultative parthenogenesis. Alternatively, if the newly discovered populations were sexual, variable sex ratios could also stem from genetic drive. For example, a driving X chromosome, which would be present in more than 50% of transmitted sperm cells, could also generate female-biased sex ratios (Helleu et al., 2015; Jaenike, 2001).

In order to investigate these different hypotheses, we sampled populations along two separate transects and quantified the local sex ratios. We then characterized the reproductive modes of females from populations with different sex ratios by measuring the capacity for parthenogenesis of virgin females and by testing for sexually produced offspring in controlled crosses. Sexually produced offspring were identified via genotyping based on Restriction-Associated DNA Sequencing (RADseq) and screened for deviations from the expected 50:50 sex ratios. Finally, we also used the RADseq genotypes to assess whether females with different reproductive modes belonged to different genetic lineages, and to study the phylogenetic relatedness between these lineages and previously described lineages of *T. douglasi* and the sexual species *T. poppensis*.

Methods

Population sampling and reproductive mode characterisation

We characterized the population sex ratios at 29 sampling locations along two transects located in northern California, respectively referred to as "Manchester" and "Orr" transect (Fig. 1). For both transects, we chose the sampling locations according to the host plant distribution along the main road, with isolated patches of redwood (*Sequoia sempervirens*) or douglas fir (*Pseudotsuga menziesii*) considered as distinct locations. We sampled each location during 2 hours with approximately constant sampling intensity and collected up to 270 individuals (all juvenile) per location. We sexed the individuals based on external morphology. In total, we collected 1195 individuals for the Manchester transect (864 females and 331 males in 12 different populations, hereafter called Manchester 1 [westernmost] to Manchester 12 [easternmost]; see Figure 1) and 1074 individuals for the Orr transect (1029 females and 45 males in 12 populations, hereafter called Orr 1 [easternmost] to Orr 17 [westernmost]; no *Timema* were found at 5 locations).

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 (when available). However, the final numbers varied considerably because of mortality, and because females sometimes laid too few eggs prior to mating to determine hatching success (see below). Individuals of each population were separated by sex and maintained in cubic mesh cages (30 x 30 x 30 cm) on douglas fir,

in a climatic chamber at 23°C, 55% humidity and 12:12h day:night cycle. Since all individuals were collected as juveniles and only adults mate, this allowed us to obtain virgin females for all populations. We isolated 352 females in Petri dishes to obtain unfertilized eggs, with fresh food (a small branch of douglas fir), soil, and a moistened piece of cotton wool added every two days. Once a female had laid approximately 20 unfertilized eggs, we allowed her to mate with one to three males from sampling locations with high male frequencies (Manchester 1 - 4, 6 - 7, Orr 5). For each female, the eggs laid before and after mating were kept separately until hatching, which occurs after approximately 5-6 months of diapause. The eggs began to hatch on October, 27th of the same year. The eggs were then checked every other day and the number of hatchings recorded. Hatchlings were stored in 100% EtOH until further use. No eggs hatched after December 27th.

Genotyping

To test whether the individuals collected in the field belonged to different genetic lineages and whether eggs produced after mating were fertilized or not, we genotyped 32 females and their mates (1 - 3 per female, 42 males in total), as well as hatchlings from eggs laid before and after mating, when available. When available, we genotyped 3 hatchlings from eggs laid before mating 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). We further genotyped seven hatchlings from eggs laid after mating (when available) for 24 of the 32 females. For the eight remaining families, we genotyped all available hatchlings from eggs laid after mating (17 - 51), initially for a different project. In total, we genotyped 365 post-mating hatchlings. Details on the number of hatchlings and males per family are provided in Table S1.

We extracted the DNA of a leg (adults) or the whole body (hatchlings). We flash-froze the tissue in liquid nitrogen and ground it via sonication with ceramic beads. We then performed the extraction using the Qiagen DNA Blood and Tissue kit on a BioSprint 96 workstation, following manufacturer protocols.

We built double-digested RADseq libraries following the protocol by Brelsford et al. (2016). In short, we digested genomic DNA with EcoRI and MseI and ligated Illumina TruSeq adapters to the cut site. The EcoRI adapter included an individual 8-base barcode. We amplified the ligated fragments in 20 PCR cycles. We then multiplexed the libraries and size-selected fragments of 300 - 500 bp on an Agarose gel. The resulting libraries were then sequenced on 10 Illumina HiSeq 2500 lanes (100 - 125 bp, single end) at the Lausanne Genomics Technology Facility.

We built loci using Stacks version 2.3e (Catchen et al., 2013). We first demultiplexed the raw reads with the process_radtags module with parameters -c -q -r and --filter-illumina and trimmed all the reads to 92 bases. We then mapped the reads to the reference genome of *T. douglasi* (Bioproject accession

number: PRJEB31411; Jaron, Parker et al., 2022) using the mem algorithm of BWA version 0.7.17 (Li & Durbin, 2010) . We built loci using the gstacks module, enabling the --phasing-dont-prune-hets option. Finally, we output a vcf file with a single SNP per locus using the --write-single-snp option in populations. We then filtered genotypes in Vcftools version 0.1.15 (Danecek et al., 2011) as follows: we retained genotype calls if they had a minimum coverage depth of 11, and discarded loci that had a minor allele count lower than 3 or a genotype call in fewer than 75% of all individuals. We further discarded five individuals which had more than 60% missing genotypes. Following this filtering approach, we obtained 8764 SNPs in 517 individuals for further analyses. Because the adults had on average more missing data than the hatchlings, we generated a second dataset comprising only the 82 wild-collected adults, filtering with the same criteria as above. This second dataset contained 4943 SNPs and was used to infer the number of genetic lineages present. All downstream analyses were conducted in R 4.0.3 (R Core Team, 2021). We read the vcf file using the R package vcfR v1.8.0 (Knaus & Grünwald, 2017).

We amplified and sequenced an 814 bp fragment of the COI mitochondrial gene for all adults in our dataset, plus reference *T. douglasi* and *T. poppensis* from other populations (Table S2) using a nested PCR. We first amplified DNA with primers 3'-TCCAATGCACTAATCTGCCATATTA-5' and 3'-GGAACNGGATGAACAGTTTACCCNCC-5'. We then amplified a subset of this fragment using the former and 3'-CAACATTTATTTTGATTTTTGG-5'. All primer sequences were obtained from Simon et al. (1994) . All reactions were conducted in 20 µL of Promega Buffer 1X containing 0.3 µM of each primer, 0.2 mM of each dNTP and 0.02 U/µL G2 Taq polymerase (Promega). All cycles consisted of 5mn at 94°C followed by 20 (first PCR) and 35 (nested PCR) cycles of 30 s at 95°C, 1 mn at 50°C, 1 mn at 72°C, and a final extension at 72°C for 10 mn. The PCR products were sequenced both ways at Microsynth (Switzerland), and we generated the consensus from the forward and reverse sequences using BioEdit.

Population structure and phylogeny

To identify the number of genetic lineages present across the two transects, we conducted a clustering analysis on the adults using faststructure v1.0 (Raj et al., 2014), with K ranging from 1 to 10. We then used the chooseK.py script to select the value of K that maximises marginal likelihood.

We also wanted to assess whether individuals collected along the two transects belonged to known lineages of the parthenogenetic species *T. douglasi* or the sexual species *T. poppensis.* Because these two species cannot be distinguished morphologically (Vickery & Sandoval, 1999), we used DNA from previously identified reference individuals of both species from different populations, five of *T. douglasi* and 26 of *T. poppensis* (Table S2). We used these 31 reference individuals to study their phylogenetic relationship with the genetic lineages identified in our newly sampled populations for mitochondrial and

nuclear (RADseq) markers. We generated RADseq and COI data for these individuals with the same protocols as described above. For the RADseq data, we retained genotype calls with a minimum depth of 8 instead of 11, as allelic dropout would cause less bias for phylogenetic reconstruction than for inference of reproductive modes (largely based on heterozygosity estimations, see below). We discarded loci that had a minor allele count lower than three and were genotyped in less than 80% of all individuals. This dataset contained 16391 sites. The COI dataset contained 159 polymorphic sites. Note that some individuals had multiple COI copies (as indicated by double peaks in the sequencing trace), as expected for the presence of nuclear copies of mitochondrial genes or heteroplasmy. In these cases, we called the allele corresponding to the largest peak, and used an ambiguous genotype following the IUPAC code when both peaks were equally large. We added the COI sequence from one individual of *T. cristinae* as an outgroup (EU251511.1; Schwander et al., 2011). We then aligned the sequences using the MUSCLE algorithm (Edgar, 2004). We reconstructed separate maximum likelihood phylogenies for the nuclear and mitochondrial data, with iqtree2 (Minh et al., 2020), using ModelFinder Plus to select the best substitution model in each case. We used the cophylo function of the R package phytools (Revell, 2012) to plot them as a cophylogeny.

Reproductive mode analyses

For individuals of the Manchester and Orr transects, we calculated heterozygosity as the proportion of the SNPs that were heterozygous. Note that because we only retained polymorphic positions in our dataset, this measure overestimates genomic heterozygosity and is hereafter referred to as "relative heterozygosity". We observed that relative heterozygosity increased linearly with the proportion of missing data (Figure S1), likely because heterozygosity stemming from merged paralogs is enriched in individuals with a large fraction of missing data (Supplementary Material). We therefore corrected relative heterozygosity to remove the effect of missing data by using the residuals of the linear regression and adding the intercept value (Figure S1).

To assess whether hatchlings from eggs laid after mating were produced via sex or parthenogenesis, we used two complementary measures. For the first measure, we looked for genotypes in hatchlings that cannot be produced via parthenogenesis. At loci where the mother is homozygous, offspring can only be heterozygous if they were produced from a fertilized egg (in the absence of genotyping errors; see also Brown et al., 2021). For each offspring, we therefore computed the proportion of heterozygous loci that had a homozygous genotype in the mother. Loci at which either the mother or the offspring were not genotyped were excluded.

For the second measure to assess whether hatchlings were produced via sex or parthenogenesis, we compared relative heterozygosity between mothers and their offspring. We expected heterozygosity levels of hatchlings produced via sex to be similar to that of the mother, or higher in hatchlings sired by

a male originating from a different lineage than the mother. On the other hand, we expected heterozygosity levels of parthenogenetically produced hatchlings to be lower than that of their mother. A decrease is expected because facultative parthenogenesis generally occurs via automixis (Suomalainen et al., 1987). Under automixis, meiotic divisions occur and diploidy is restored secondarily via fusion or duplication of meiotic products. Similar to selfing, automixis can result in a loss of heterozygosity between mothers and offspring (Pearcy et al., 2006; Suomalainen et al., 1987). However, heterozygosity losses can only be observed at sites where the mother was heterozygous, and where heterozygosity losses are not mechanistically constrained or removed by selection (Jaron, Parker et al. 2022). The extent of the heterozygosity loss then depends on the type of automixis and the amount of recombination (Pearcy et al 2006, Suomalainen et al 1987). In the most extreme case (gamete duplication) a haploid gamete duplicates to produce a fully homozygous zygote. Other forms of automixis (e.g., terminal or central fusion) result in a partial loss of heterozygosity. In these cases, offspring heterozygosity depends on the heterozygosity of the mother, and on the position and frequency of recombination events. By combining the two heterozygosity-based measures, we were able to unambiguously assign each of the 365 genotyped hatchlings from eggs produced after mating to either sexual or parthenogenetic offspring (see Results).

We then asked whether females from different genetic lineages used different reproductive strategies. To answer this question, we tested whether there were significant differences between lineages in (i) hatching success of eggs laid before mating, (ii) hatching success of eggs laid after mating, and (iii) the proportion of post-mating eggs that were fertilized (which measures the propensity to reproduce via sex) using binomial Generalized Linear Models (GLMs), and performed multiple comparisons using the glht function implemented in the R package multcomp v1.4-16 (Hothorn et al., 2008). We also looked for differences in hatching success of eggs before vs after mating within each lineage using binomial GLMMs implemented in the R package lme4 (Bates et al., 2015), using the mother identity as random factor.

In order to test whether variable population sex ratios along the Orr and Manchester transects were generated by X chromosome drive, we quantified the sex ratio in sexually produced offspring. Because female and male hatchlings cannot be distinguished morphologically, we sexed offspring genetically by combining heterozygosity at X-linked loci and the read depth ratio between the X-linked and autosomal loci. Because *Timema* males have only one copy of the X chromosome and no Y (XX:XO sex determination; Schwander & Crespi, 2009), they should be fully homozygous at all X-linked loci. Furthermore, X-linked loci should have half the coverage compared to autosomal loci in males, but similar coverage in females. To sex sexually produced offspring via these approaches, we first had to identify X-linked scaffolds in the *T. douglasi* reference genome (Jaron, Parker et al., 2022).

To identify X-linked scaffolds we used a combination of available genomic data from *T. douglasi* females (Bioproject accession number: PRJNA670663) in addition to whole-genome sequencing of two male *T. douglasi*. DNA extractions were done on adult male carcasses using the Qiagen Mag Attract HMW DNA kit following the manufacturer instructions. Sequencing libraries were generated for each male using a TruSeq DNA nano prep kit (550bp insert size). Libraries were then sequenced using an Illumina HiSeq 4000 at the Lausanne Genomic Technologies Facility. Reads were trimmed before mapping using Trimmomatic v0.36 (Bolger et al., 2014) to remove adapter and low-quality sequences (options: ILLUMINACLIP:3:25:6 LEADING:9 TRAILING:9 SLIDINGWINDOW:4:15 MINLEN:90). Reads from each individual were then mapped to the reference genome using BWA-MEM v0.7.15 (Li & Durbin, 2010). Multi-mapping and poor quality alignments were filtered (removing reads with XA:Z or SA:Z tags or a mapq < 30). PCR duplicates were removed with Picard (v. 2.9.0) (http://broadinstitute.github.io/picard/). Coverage was then estimated for all scaffolds at least 1000 bp in length using BEDTools v2.26.0 (Quinlan & Hall, 2010). To compare coverage between males and females, coverage was first summed up for all male and all female libraries per scaffold. Male and female coverage was then normalised by modal coverage to adjust for differences in overall coverage. X-linked scaffolds were then identified using the log2 ratio of male to female coverage. Scaffolds were classified as X-linked if the $\log_2$ ratio of male to female coverage was within 0.1 of the value of the X-linked peak (Figure S3), and as autosomal otherwise.

By distinguishing between autosomal and X-linked loci, we then sexed sexually produced offspring from our controlled crosses using the RADseq data. We first standardised read depth per individual, dividing the depth value at each locus by the average depth for that individual. We then computed the X to autosomes depth ratio by dividing average standardised depth of loci on the X chromosome by that of autosomal loci (See supplementary material). Using this depth ratio in combination with heterozygosity on the the X chromosome, we were able to sex 176 out of the 211 sexually produced offspring (see Figure S5; the remaining 35 offspring had insufficient sequencing coverage for these analyses). We tested for deviations from a 50:50 sex ratio among sexually produced offspring overall using a chi-squared test. We further investigated whether there were deviations within each family. For this, we looked whether the 95% binomial proportion confidence interval of the observed sex ratio overlapped with the expected 50:50 sex ratio.

Results

We found that geographically close populations differed strongly in their sex ratio, with significant sex ratio variation among populations overall (Figures 1A, 1C; Manchester: Likelihood ratio $\chi^2 = 456.49$, df = 11, $p < 2 \times 10^{-16}$; Orr: : Likelihood ratio $\chi^2 = 106.46$, df = 11, $p < 2 \times 10^{-16}$). The Orr transect consisted

of a mosaic of female-biased and female-only populations. Five populations (Orr 1, 5, 8, 9 and 17) had between 77.8% and 91.7% of females, while the others had only females (Figure 1A). By contrast, we found an abrupt transition from roughly even to strongly female-biased or female-only sex ratios along the Manchester transect. Sex ratios in populations 1 to 7 ranged from 43.2% to 54.7% of females, while populations 8 to 12 consisted of at least 93.5% females (Figure 1C).

We then investigated whether the field-collected individuals belonged to different genetic lineages using a clustering analysis. The best supported number of lineages among all genotyped field-collected individuals was three. The first lineage (blue in Figure 1B) was mostly found along the Orr transect, with only one individual in Manchester 10, and is hereafter referred to as the "Orr lineage". 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". We found little evidence for admixture between lineages in 14 out of the 15 populations tested, with only one female from Orr 11 (17-2162) appearing to be a hybrid, perhaps triploid, between the three lineages (Figure S2). In one population however, Manchester 2, all 5 genotyped individuals appeared to be admixed, with approximately equal contributions of the eastern and western Manchester lineages. These results were robust with varying numbers of clusters (k in faststructure), indicating that these were most likely hybrids and not a fourth lineage (Figure S2).

In our nuclear phylogeny, all individuals collected along the two transects formed a monophyletic clade with the parthenogenetic *T. douglasi* reference individuals, indicating that all three lineages belong to *T. douglasi*. All *T. poppensis* reference individuals formed a second, monophyletic clade (Figure 1E). The mitochondrial phylogeny also showed two monophyletic groups. One contained mostly *T. poppensis*, with five *T. douglasi* individuals (including four from our transects). The second contained mostly *T. douglasi*, with three *T. poppensis* individuals (Figure 1F).

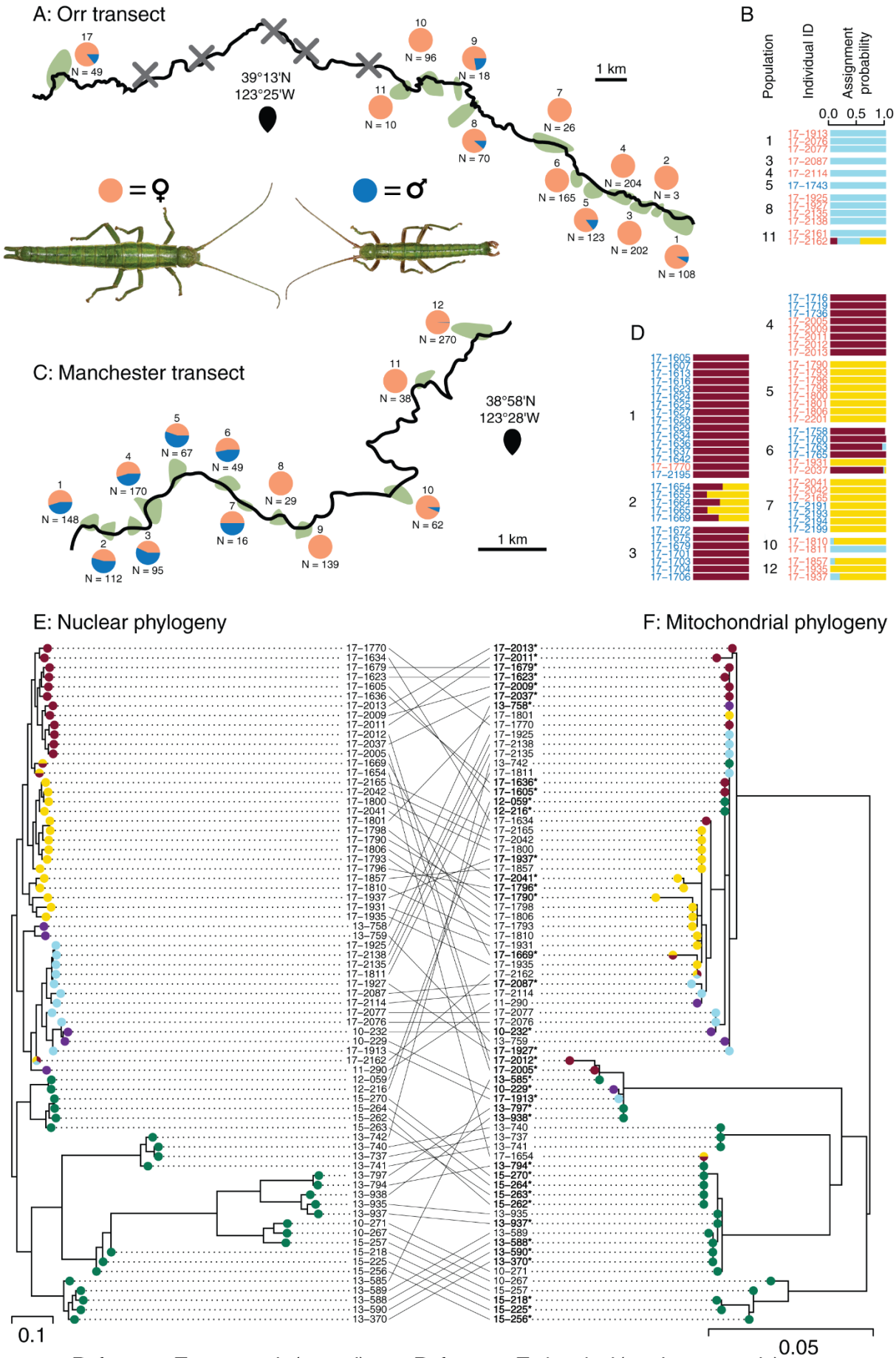

Figure 1 Sex ratios in the sampled populations, genetic structure and species identification of the collected individuals. **A** and **C**: Schematic maps of the Orr and Manchester transects, with host plant patches represented in green along the main road. Pie charts represent the sex ratio of individuals collected in the field. Crosses indicate surveyed host plant patches with no *Timema* present (locations 12-16 along the Orr transect). **B** and **D**: Assignment probabilities to each of the 3 genetic lineages for each genotyped individual, split by transect. **E**: ML nuclear phylogeny (16391 SNPs) of the genotyped field-collected individuals plus 26 *T. poppensis* (sexual) and five *T. douglasi* (parthenogenetic) reference individuals to infer species assignments for individuals from the transects. **F**: ML mitochondrial phylogeny of the same individuals based on 159 polymorphic sites of the COI mitochondrial gene. One individual of *T. cristinae* was used to root the tree (not displayed). Tip colour refers to genetic lineage (individuals from Orr and Manchester transects) or reference individuals used for species assignment. Individuals denoted in bold with a star had double peaks in their COI sequence and the allele corresponding to the highest peak was retained. Timema photos taken by © Bart Zijlstra - www.bartzijlstra.com

We isolated 352 females to characterise their reproductive mode and assess the hatching success of the eggs they laid as virgins. 265 of these females laid at least five eggs as virgins. At least one unfertilized egg hatched from the clutches produced by 234 (88.3%) of them, indicating that they were able to reproduce via parthenogenesis. In addition, females capable of parthenogenesis were found in all populations tested. The hatching success of unfertilized eggs varied between 3.3 and 100% (Figure 2).

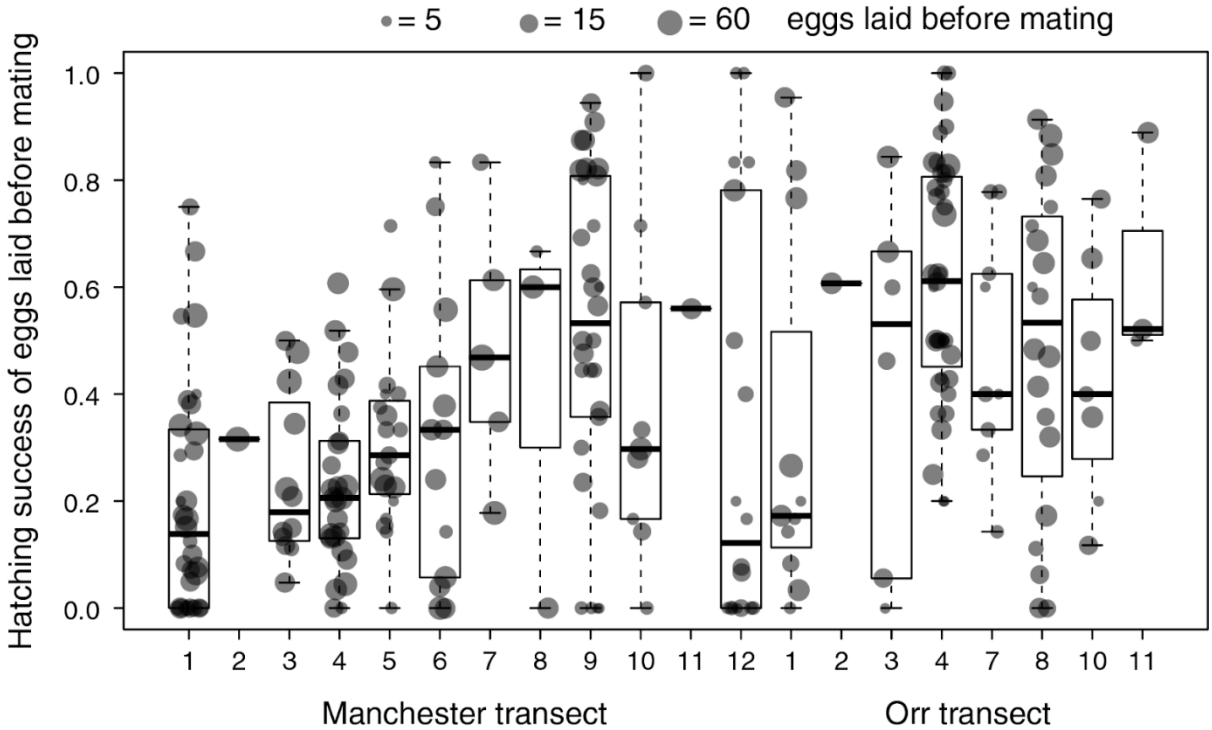

Figure 2 Parthenogenesis capacity in field-collected females from different populations along the Manchester and Orr transects, measured by the hatching success of unfertilized eggs. Dot size is proportional to the number of unfertilized eggs laid by each female.

In order to gain insights into the mechanisms of automictic parthenogenesis in females from the Orr and Manchester transects, we compared relative heterozygosity in mothers and their offspring produced from eggs laid prior to mating. Heterozygosity losses can only be observed at sites where the mother was heterozygous, and where heterozygosity losses are not mechanistically constrained or removed by selection (Jaron, Parker et al. 2022). However, if heterozygosity loss does occur, the extent of it can

inform on the type of automixis (Pearcy et al 2006). We found extensive loss of heterozygosity or extremely low relative heterozygosity in all offspring from eggs laid prior to mating (Figure 3), as would be expected from gamete duplication or terminal fusion without recombination. With one exception, relative heterozygosity in these offspring amounted to 2 - 3% and was not correlated with their mothers' heterozygosity (as would be expected i.e., under central or terminal fusion automixis with recombination). In addition, most, if not all of the heterozygosity observed in hatchlings is caused by false positives (see Supplementary Material), meaning that these hatchlings are most likely completely homozygous. Only one parthenogenetic hatchling from female 17-1793 retained some heterozygosity (Figure 3), which could suggest that it was produced by a different parthenogenesis mechanism than the other individuals.

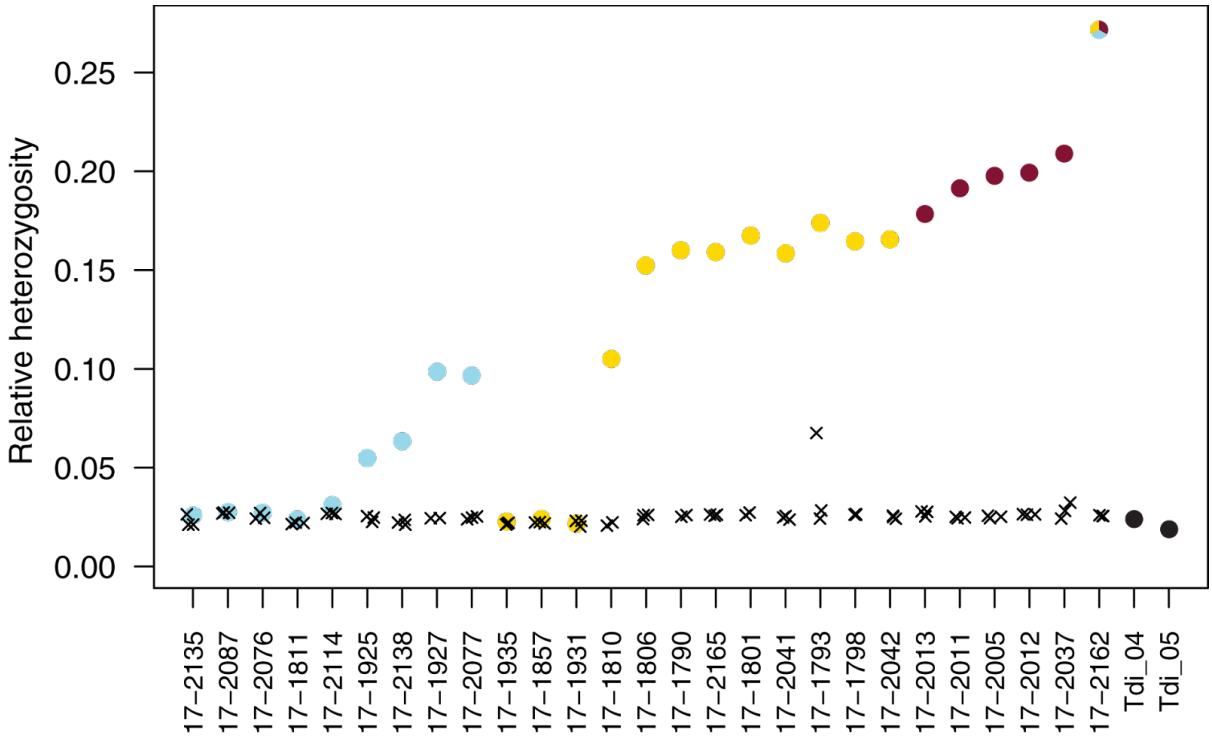

Figure 3 Relative heterozygosity of mothers (circles coloured as in Figure 1) and their offspring produced from unfertilized eggs laid before mating (black crosses, two to three per mother), and of two reference parthenogenetic individuals (Tdi_04 and Tdi_05) from Jaron, Parker et al. (2022). Note the much higher heterozygosity in one hatchling from family 17-1793, indicating that it could have been produced via a different mechanism of parthenogenesis.

We were able to unambiguously infer whether each offspring from eggs laid after mating was produced via sex or parthenogenesis by using a combination of relative heterozygosity and proportion of genotype transitions from homozygous in the mother to heterozygous in the offspring (Figure 4; see also Supplementary Material). These inferences revealed that 211 (58.3%) of offspring were produced by sex, and 151 (41.7%) were produced via parthenogenesis.

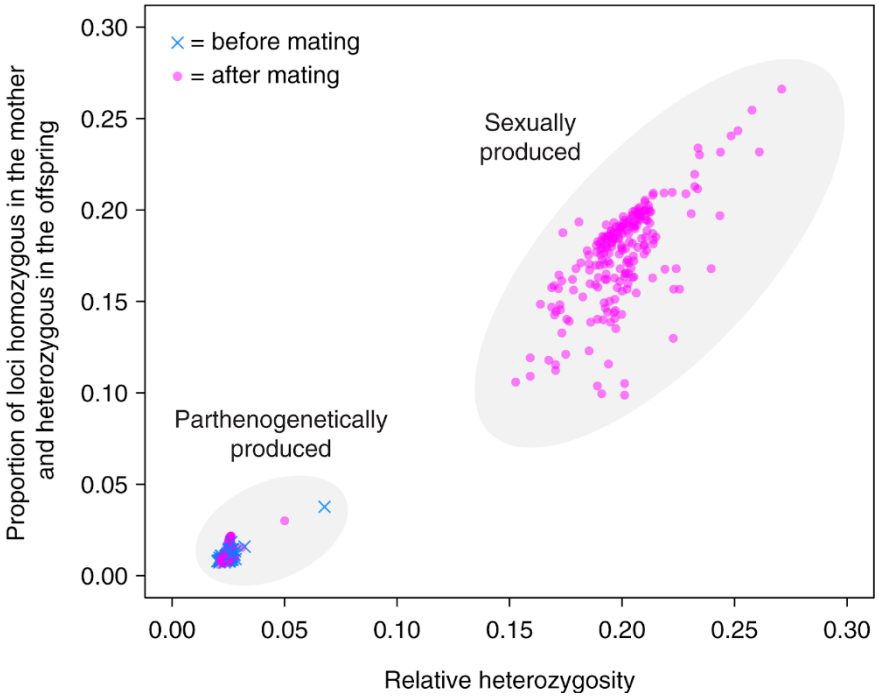

Figure 4 Relative heterozygosity and proportion of transitions from homozygous genotype in the mother to heterozygous in the offspring, in offspring from eggs laid before (blue crosses) and after mating (pink dots). These data were used to infer whether offspring from eggs laid after mating were produced via sex or parthenogenesis.

We then looked at differences in the parthenogenetic capacity or the propensity to use sex following mating between females from the Orr, western and eastern Manchester lineages. All 32 females for which we were able to infer the lineage via genotyping were capable of parthenogenesis, but with varying efficiency (Figure 5A). Note that one of these females (17-1937 from the eastern Manchester lineage) did not produce any offspring from the 13 eggs she laid before mating, but she was capable of parthenogenesis since all three offspring produced from the 14 eggs laid after mating were produced via parthenogenesis. Hatching success of eggs laid before mating was on average higher for females of the Orr lineage (67%) than of the two other lineages (western Manchester: 42%, $p = 0.035$; eastern Manchester: 33%, $p = 0.001$; Figure 5A). There was no significant difference between the lineages in hatching success of eggs laid after mating (Figure 5B). Mating influenced the egg hatching success in all three lineages. In the eastern and western Manchester lineages, eggs laid after mating had a significantly higher hatching success than those laid by virgin females (western Manchester: 55% vs 42%, $p = 0.04$; eastern Manchester: 55% vs 33%, $p = 2.7*10^{-6}$). On the other hand, in the Orr lineage, eggs laid after mating hatched at a lower rate than those laid before mating (56% vs 69%, $p = 2.7*10^{-6}$). We also found that the proportion of eggs laid after mating that were actually fertilized was highly variable among females. Twelve females fertilized between 80% and 100% of their eggs laid after mating, while eleven fertilized none of them. Only five fertilized an intermediate proportion (14% to 50%). There were significant differences in the proportion of fertilized eggs between lineages. Females from the Orr lineage fertilized on average fewer eggs (10%) than those from the eastern and western

Manchester lineages (79%, $p = 0.0002$ and 95%, $p = 0.001$ respectively). All females from the western Manchester lineage fertilized more than 80% of their post-mating eggs, while seven out of 10 females from the Orr lineage fertilized none of them. Finally, the eastern Manchester lineage consisted mostly of females with either a very high or very low fertilisation frequency (Figure 5C). The putatively triploid hybrid female (17-2162) was also capable of facultative parthenogenesis: a large portion of eggs laid prior to mating hatched (24 out of 27), and out of the 17 genotyped hatchlings from the eggs she laid after mating, seven were produced via sex and 10 via parthenogenesis.

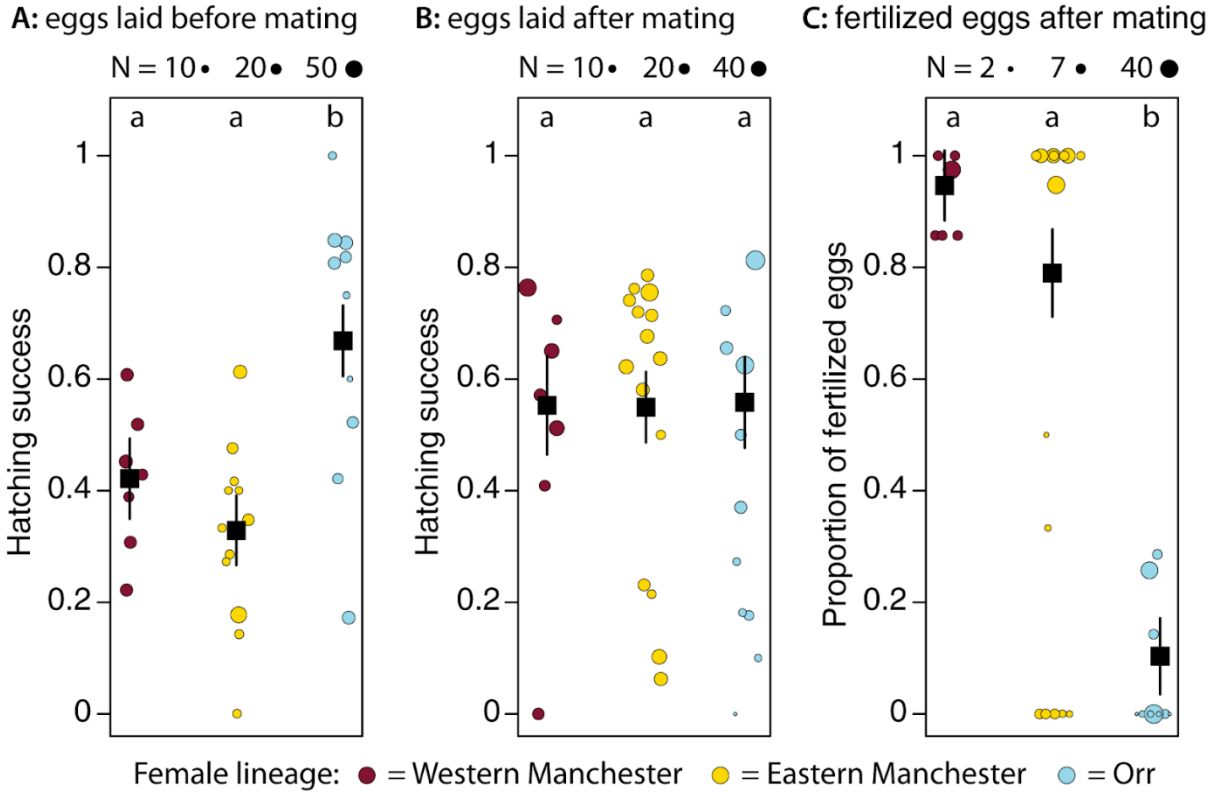

Figure 5: Reproductive mode differences between females of the three genetic lineages (distinguished by colours as in Figure 1). **A**: Hatching success of eggs laid prior to mating, which measures the capacity to do parthenogenesis. Dot size proportional to the number of eggs laid before mating. **B**: Hatching success of eggs laid after mating. Dot size proportional to the number of eggs laid after mating **C**: The proportion of sexually produced hatchlings that originated from eggs laid after mating, which measures the propensity to reproduce via sex. Dot size proportional to the number of hatchlings genotyped to assess whether they were produced via sex or parthenogenesis. In all panels: the black squares and bars represent the weighted average per lineage +/- standard error. Different letters indicate significantly different groups ($p < 0.05$ in multiple comparison tests after binomial GLMs).

Finally, we tested whether X chromosome drive could be contributing to female-biased sex ratios by looking at the sex ratio of sexually produced offspring. A driving X chromosome should lead to an excess of female offspring. Across all families, there were 81 female and 95 male offspring, providing no evidence for biased sex ratios ($\chi^2 = 1.1$, $p = 0.29$). The same was observed within families (Figure S6).

Discussion

How transitions between fundamentally different reproductive modes – such as sex and obligate parthenogenesis – can occur, is unclear. Transitions might be abrupt, with a direct switch from sex to obligate parthenogenesis within one generation. Alternatively, transitions might be gradual and involve a period where individuals are capable of facultative parthenogenesis. In this study, we discovered high frequencies of males in populations of *Timema douglasi*, a species previously believed to reproduce solely via obligate parthenogenesis. These populations were characterized by variable sex ratios, which were caused by different frequencies of sexual versus parthenogenetic reproduction, and not by X chromosome drive. These findings raise the possibility that obligate parthenogenesis, which evolved repeatedly in the genus *Timema*, may generally derive from facultative parthenogenesis, with facultative parthenogenesis being a transient state. Facultatively parthenogenetic *T. douglasi* belonged to two differentiated lineages, and were found geographically close to a third, largely but not strictly obligately parthenogenetic lineage of the same species. Females of the latter lineage (Orr) had a high capacity for parthenogenesis and they mostly did not fertilize their eggs when mated. Furthermore, eggs produced after mating suffered from a lower hatching success, suggesting that fertilisation of eggs that would normally develop into parthenogenetic offspring can sometimes result in developmental failure. These results may point to a tradeoff between sexual and parthenogenetic pathways as was previously suggested to be the case in sexual *Timema* populations: the ability for spontaneous parthenogenesis likely reduces hatching success of fertilized eggs (Schwander et al., 2010). However, lower hatching success of post-mating eggs could also be due to females being older.

Females of the two facultatively parthenogenetic lineages had a lower capacity for parthenogenesis than those of the largely obligate one and featured different propensities to reproduce sexually following mating. Females of the first lineage (western Manchester) generally fertilised all their eggs when mated, which resulted in higher hatching success than under parthenogenesis. Females of the second lineage (eastern Manchester) were more variable. Approximately half (8 out of 15) of them reproduced the same way as the western Manchester lineage: using mainly sex when mated but being able to reproduce via parthenogenesis. Five females reproduced via obligate parthenogenesis, with a phenotype similar to the one described for “obligately” parthenogenetic females of the Orr lineage. The two remaining females seemed to use both sex and parthenogenesis at approximately equal rates (facultative parthenogenesis), but genotyping larger clutches would be needed to assess whether this was indeed the case.

The mechanisms that maintain reproductive polymorphism (facultative and largely obligate parthenogenesis) within populations, and those that cause the sharp transition from equal to strongly female-biased sex ratios over a few hundred meters along the Manchester transect remain as an open

433 question. Since more than half of the females from the eastern Manchester lineage were able to
434 reproduce via sex and fertilized most or all of their eggs following mating, a male immigrating from a
435 nearby sexual population would be expected to have extremely high reproductive success. This should
436 then lead to an increase in male frequency in the next generation. One possible explanation for the
437 maintenance of different sex ratios between neighbouring populations is that dispersal is very limited in
438 these wingless insects (Sandoval, 2000), and that it is just a matter of time until the female-biased
439 Manchester populations shift towards more equal sex ratios. However, variable selection pressures along
440 the transect could also play a role, favouring respectively sexual or parthenogenetic reproduction. This
441 could help explain why males, even though present in small numbers in the strongly female-biased
442 populations (such as Manchester 12 with <1% males), have not triggered sex ratio shifts. Identifying
443 such selection pressures would constitute a major leap towards understanding the costs and benefits of
444 different reproductive modes in natural populations.
445
446 Our comparison of heterozygous genotypes in mothers and their parthenogenetically produced offspring
447 also provides insights into the mechanisms of parthenogenesis and the evolution of obligate
448 parthenogenesis in *Timema*. Relative heterozygosity in all but two parthenogenetic offspring (out of 228
449 produced before or after mating) was around 2.5%, regardless of their mothers' heterozygosity. Given
450 the 2.5% heterozygosity is most likely generated by structural variation between individuals and
451 divergence between paralogs (Supplementary Material), the main mechanism of parthenogenesis in
452 *Timema* thus appears to generate genome-wide homozygosity in a single generation. This would be
453 expected under specific forms of automixis, notably under gamete duplication or fusion of non-
454 recombined sister chromatids (i.e., terminal fusion without recombination). The two remaining out of
455 228 parthenogenetic offspring (one originating from an unfertilized egg laid prior to mating, the other
456 from an unfertilized egg laid after mating; see Figure 4), produced by different females, had somewhat
457 higher heterozygosity. This suggests that some females are able to reproduce via two different
458 parthenogenesis mechanisms in addition to sex, as was described for spontaneous parthenogenesis in
459 sexual *Daphnia* (Svendsen et al., 2015). Automixis with "complete" heterozygosity loss in a single
460 generation is also the proposed mechanism of parthenogenesis in obligate asexual *Timema* species
461 (Jaron, Parker et al., 2022) and of spontaneous parthenogenesis in sexual *Timema* species (Schwander
462 & Crespi, 2009). In *Timema*, obligate parthenogenesis thus likely evolves via a gradual increase of the
463 capacity for parthenogenesis from a tychoparthenogenetic ancestor, with a conserved proximate
464 mechanism.
465
466 Our finding of sexually reproducing *T. douglasi* lineages also raises the question whether these lineages
467 represent vestigial sexual reproduction in a species which is otherwise largely obligately
468 parthenogenetic, or whether they represent a re-evolution of sex. Sex could in theory reappear in all-
469 female lineages via accidental male production. Rare male production in species with XX/X0 sex

determination such as *Timema* can occur via aneuploidy at the X chromosome, because the resulting X0 individuals would develop into males (Pijnacker & Ferwerda, 1980). However, such a re-expression of the sexual pathway would require the sexual functions of both sexes to be maintained. Reversal to sexual reproduction is therefore possible at least shortly after the transition to parthenogenesis, which may also have been the case in the stick insect *Clitarchus hookeri* (Morgan-Richards et al., 2019). Such re-expressions of sexual reproduction do not break "Dollo's law of irreversibility", i.e., the assumption that complex traits cannot re-evolve once lost (Gould, 1970). As long as the molecular bases underlying sexual reproduction still exist, even when not expressed in a parthenogenetic lineage, re-evolution does not equate with independent emergence via convergent pathways.

Alternatively, the facultatively parthenogenetic *T. douglasi* lineages we describe in this paper could constitute vestigial lineages in a species where all other lineages would have transitioned to obligate parthenogenesis already. This would require that obligate parthenogenesis has evolved several times in the *T. poppensis*/*douglasi* species complex. Phylogenetic analyses of the *Timema* genus revealed that *T. douglasi* consisted of multiple lineages that independently derived from the sexual species *T. poppensis* (Schwander et al., 2011). Repeated transitions towards parthenogenesis are not surprising if the ancestor of these species was already capable of reproducing via facultative or spontaneous parthenogenesis. Schwander & Crespi (2009) found that around 30% of females from sexual *Timema* species can produce viable eggs when virgin. The hatching success of such unfertilized eggs is usually low (typically around 2%) but can reach up to 20% in populations with low male availability (Schwander et al., 2010). This roughly corresponds to the lowest pre-mating hatching success observed in the present study. Widespread tychoparthenogenesis capacity could thus serve as a stepping stone for the repeated evolution of more successful obligate parthenogenesis, which could help explain why obligate parthenogenesis evolves so frequently in the *Timema* genus.

Finally, gene flow between sexual and parthenogenetic lineages could contribute to frequent transitions between reproductive modes. One such example is contagious parthenogenesis, whereby rare males from otherwise parthenogenetic lineages transmit the capacity for parthenogenesis to a related sexual lineage via hybridization. This has been documented in aphids (Delmotte et al., 2001), wasps (Sandrock & Vorburger, 2011) and *Daphnia* (Innes & Hebert, 1988). Conversely, gene flow from a sexual to mostly but not completely parthenogenetic species could in theory result in an increase of the frequency of sex in the latter. Occasional gene flow between *T. poppensis* and *T. douglasi* is suggested by the mismatches we observed between nuclear and mitochondrial genomes in about 10% of our individuals. This could be caused by rare hybridization events between the two species in both directions and contribute to the maintenance of different reproductive modes within populations.

More generally, the faculty for occasional and facultative sex in *T. douglasi*, a species previously believed to be obligately parthenogenetic, adds to recent re-evaluation of the obligate status of some parthenogenetic species (Boyer et al., 2021b; Kuhn et al., 2021; Laine et al., 2022). Many such "ancient asexuals" could in fact have retained the capacity for occasional, cryptic sex (Schurko et al., 2009). They would thus represent extremes of a continuum from mostly sexual to mostly parthenogenetic reproduction. Still, the factors that would favour obligate over more facultative strategies remain elusive. Facultative parthenogenesis is believed to be an efficient reproductive mode, combining the "best of both worlds" – the long-term advantages of recombination and segregation along with the short-term advantages of asexuality. 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. Alternatively, the results of the present study as well as interspecific comparisons in facultatively parthenogenetic mayflies (Liegeois et al., 2021) suggest that the efficiency of sex and parthenogenesis are traded-off against each other, consistent with the "jack of all trades, master of none" hypothesis. 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.

In conclusion, we discovered the first case of facultative parthenogenesis in *Timema* stick insects, a genus where obligate parthenogenesis has evolved repeatedly from a sexual ancestor, with an apparently conserved proximate mechanism of parthenogenesis. We found three genetic lineages that differed both in their capacity to reproduce via parthenogenesis and in their propensity to reproduce sexually. It is thus far unclear whether these populations reverted to sex starting from a largely asexual ancestor, or whether they are sexual relicts in the largely asexual *T. douglasi*, where all other populations would have transitioned to obligate parthenogenesis already. The latter would suggest that transitions to asexuality were numerous during the evolution of this species, and that asexual populations almost always prevailed on sexuals. In that case, we could be witnessing an ongoing transition to asexuality via facultative parthenogenesis. Why facultative parthenogenesis would be a transient stage in *Timema* and which factors would favour a transition to obligate parthenogenesis would thus be exciting foci for future research.

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Data and code availability

All demultiplexed RAD-seq reads have been deposited in NCBI's sequence read archive under BioProject ID PRJNA804475 and whole genome sequence reads of *T. douglasi* males under BioProject ID PRJNA808673. The code used to run the analyses presented in this article is available on GitHub under https://github.com/glavanc1/Timema_facultative_partheno.

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