Can sexual conflict drive transitions to asexuality? Female resistance to fertilization in a facultatively parthenogenetic insect

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Abstract

Facultatively parthenogenetic animals could help reveal the role of sexual conflict in the evolution of sex. Although each female can reproduce both sexually (producing sons and daughters from fertilized eggs) and asexually (typically producing only daughters from unfertilized eggs), these animals often form distinct sexual and asexual populations. We hypothesized that asexual populations are maintained through female resistance as well as the decay of male traits. We tested this via experimental crosses between individuals descended from multiple natural sexual and asexual populations of the facultatively parthenogenic stick insect *Megacrania batesii*. We found that male-paired females descended from asexual populations produced strongly female-biased offspring sex ratios resulting from reduced fertilization rates. This effect was not driven by incompatibility between diverged genotypes but, rather, by both genotypic and maternal effects on fertilization rate. Furthermore, when females from asexual populations mated and produced sons, those sons had poor fertilization success when paired with resistant females, consistent with male trait decay. Our results suggest that resistance to fertilization resulting from both maternal and genotypic effects, along with male sexual trait decay, can hinder the invasion of asexual populations by males. Sexual conflict could thus play a role in the establishment and maintenance of asexual populations.

Keywords: facultative parthenogenesis, evolution of sex, reproductive isolation, Phasmatodea, geographical parthenogenesis, Megacrania batesii

Introduction

Theory predicts that facultatively asexual animals should outcompete those that are obligately sexual or obligately asexual (D'Souza & Michiels, 2010), yet facultatively asexual animals are paradoxically rare (Burke & Bonduriansky, 2017). Furthermore, many facultatively parthenogenic species, in which all females are capable of both reproductive modes, form geographic mosaics of sexually reproducing (mixed-sex) populations and parthenogenetically reproducing (all-female) populations-a pattern similar to geographical parthenogenesis, where closely related sexual and asexual animals occur in different geographic areas (Bell, 1982; Burke & Bonduriansky, 2018; Vandel, 1928). Such distinct all-female and mixed-sex populations have been reported in harvestmen (Machado & Burns, 2022), mayflies (Liegeois et al., 2021), stick insects (Larose et al., 2023; Miller et al., 2024a; Morgan-Richards et al., 2010), jumping plant lice (Shapoval et al., 2021), and Chironomid midges (Sæther & Andersen, 2011). While some of these systems include populations with intermediate sex ratios (Burns et al., 2018), others tend to have only two discrete types of populations (Miller et al., 2024a). Since parthenogenetically produced (thelytokous) eggs usually develop into females (Suomalainen, 1950), a mixed strategy

combining sex and parthenogenesis should result in intermediate, female-biased sex ratios. Instead, it seems that facultative animals in each population tend to adopt one of two strategies: sex (producing even sex ratios) or parthenogenesis (producing very few or no males). These geographic mosaics, therefore, suggest that facultative asexuality may be unstable despite its theoretical advantages, perhaps often transitioning to obligate asexuality (Larose et al., 2023). The same processes that tip the balance in the direction of sexual or asexual reproduction in facultatively parthenogenetic populations might lead to the evolution of sexual and asexual species over longer timescales. Yet, these crucial processes remain poorly understood.

In many facultatively parthenogenic species, unfertilized eggs yield only daughters (thelytoky), but when mating occurs, fertilized eggs yield equal numbers of sons and daughters (Suomalainen, 1950). In some species, rare spontaneous males can also be produced from unfertilized eggs (e.g., Carson, 1967; Morgan-Richards et al., 2019; Pijnacker, 1969). Intriguingly, all-female populations often persist over many generations (Burns et al., 2018; Morgan-Richards, 2023; Tsurusaki, 1986), even in close proximity to mixedsex populations (Miller et al., 2024a). Males from nearby

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mixed-sex populations should, in principle, be able to reach all-female populations and mate with the females there, producing sons, and causing the populations to become mixedsex. In some cases, geographical barriers and low population densities can reduce encounter rates, generate mate limitation, and promote isolation between all-female and mixedsex populations (Burke & Bonduriansky, 2018; Schwander et al., 2010). Indeed, patterns of geographical parthenogenesis often involve higher rates of asexuality in isolated or marginal habitats (Burns et al., 2018), but such patterns are far from universal (Tilquin & Kokko, 2016). How do mosaics of mixed-sex and all-female populations persist?

Recent theoretical work suggests that sexual conflict could play a role in the establishment and maintenance of such reproductive mosaics (Burke & Bonduriansky, 2018; Gerber & Kokko, 2016). Sexual conflict often occurs in sexual species because the two sexes tend to have different optimal mating rates (Arnqvist & Rowe, 2005; Bateman, 1948; Parker, 1979), but theory suggests that sexually antagonistic selection on mating rate could be exacerbated in facultatively parthenogenic systems because females can reproduce without mating at all (Burke & Bonduriansky, 2017). Females could be under selection to avoid or resist mating and its associated costs (Daly, 1978; Parker, 2006), and there could also be selection to resist fertilization after mating (see Firman, 2018) if sexually produced offspring are less fit than parthenogenetically produced offspring (e.g., because sex breaks up beneficial allele combinations and can disrupt local adaptation; Maynard Smith, 1978; Peck et al., 1998). Simulation modeling shows that the ensuing evolutionary arms race between persistent males and resistant females could determine whether sex persists in a population (Kawatsu, 2013). Female resistance could involve pre-copulatory mechanisms such as struggling to dislodge males or producing repellent pheromones (Burke et al., 2015), or post-copulatory mechanisms such as changes to the female sperm-storage organs (Schwander et al., 2013). Such resistance could tip the scales in favor of parthenogenesis and cause local male extinction because unfertilized eggs typically yield only daughters in thelytokous animals (Burke & Bonduriansky, 2019). However, strong sexual selection favoring males that can overcome female resistance could lead to the evolution of coercive male strategies that prevent females from reproducing asexually, or impose sufficient costs on female resistance to negate its benefits (Burke & Bonduriansky, 2019; Gerber & Kokko, 2016). The balance could then tip in the direction of sexual reproduction and result in even sex ratios (Burke & Bonduriansky, 2019). The arms race between male coercion and female resistance might have different outcomes in different populations, so that sex prevails in some populations while males go locally extinct in others. Female resistance could also contribute to the persistence of all-female populations by impeding invasions by male dispersers from mixed-sex populations.

In addition, the persistence of all-female populations could be promoted by maternal effects. If males are highly persistent or coercive, female resistance to mating can become too costly and may only be adaptive in patches or times where females rarely encounter males (Gerber & Kokko, 2016). Facultative females should then modulate their resistance based on the local sex ratio (Gerber & Kokko, 2016; similarly to convenience polyandry—Rowe, 1992). In this situation, maternal effects on female resistance may evolve because a mother's reproductive history could predict her daughters' reproductive environment. Impaternate (asexually produced) daughters are more likely to encounter environments with few or no males; whereas paternate (sexually produced) daughters will likely face mixed-sex environments and higher male-encounter rates. A history of alternating sexual and asexual generations could thus drive the evolution of a maternal effect that causes impaternate females to be resistant to sex, as has been reported in the facultatively parthenogenic stick insect *Extatosoma tiaratum* (Burke & Bonduriansky, 2022). If females in mixed-sex populations exhibit such maternal effects, these effects could promote the establishment of all-female populations because any population founded by an unmated female would instantly have a degree of resistance to male invasion.

Nonetheless, even without selection for female resistance, reproductive isolation between all-female and mixed-sex populations could occur simply as the result of incompatibility due to genetic divergence. This could happen if the gene pools remain separate long enough to diverge via genetic drift or local adaptation (such as through geographic isolation; Schluter, 2001), or if there is a sudden change in ploidy that causes instant incompatibility between gametes from different populations (such as through hybridization; Adolfsson et al., 2010). Moreover, female traits involved in mating or fertilization could decay in long-established all-female populations (Schwander et al., 2013; van der Kooi & Schwander, 2014). Such decay could occur passively through the absence of selection against mutations that reduce sexual functionality (Carson et al., 1982), or via selection acting to reduce resource investment in costly structures that are not being used (Schwander et al., 2013; Wiens, 2001). Female resistance to fertilization could thus evolve even in the absence of sexually antagonistic selection.

Likewise, the maintenance of all-female populations in facultative systems could be promoted by the decay of male traits. Once males disappear from a population, male-specific traits in that gene pool are no longer exposed to selection and can deteriorate via genetic drift and the accumulation of mutations (van der Kooi & Schwander, 2014). Moreover, some traits have different optima for each sex, and alleles that benefit males can therefore be subject to negative selection in females (Bonduriansky & Chenoweth, 2009). In an all-female population, selection is expected to shift these traits towards the female optimum and away from the male optimum, as it does in sexual species under experimentally imposed sex-limited selection (e.g., Morrow et al., 2008). Once an all-female population has undergone male trait decay, females that mate with males that disperse from mixedsex populations would produce sons with low reproductive success, hindering the invasion of sexual reproduction. The decay of male traits and reduced fertilization resulting from the evolution of pre- and post-copulatory female resistance, maternal effects, and incompatibility due to divergence are not mutually exclusive alternatives. These processes could all contribute to the maintenance of reproductive mosaics in facultative systems, but their roles remain unknown. While previous studies have reported barriers to fertilization in all-female populations of facultative parthenogens (Larose et al., 2023; Morgan-Richards et al., 2019), no previous study has investigated the contribution of both evolutionary (i.e., genotypic) changes in all-female populations and existing maternal effects in mixed-sex populations to geographical parthenogenesis.

presents a mosaic of mixed-sex and all-female populations (Figure 1B), some of which occur in close proximity and without obvious barriers to dispersal or habitat differences (Miller et al., 2024a, 2024b). We hypothesized that male invasion of long-established all-female populations is prevented by an



Figure 1. Study system. (A) *Megacrania batesii* mating pair on *Pandanus* host plant. The female is the larger insect, eating a leaf. (B) Observed distribution of *Megacrania batesii* in Northern Queensland, Australia. Diamonds represent mixed-sex populations, and circles represent all-female populations. Fill color represents phylogenetic clustering: The Northern (N) genotype is in blue (darker color); the Southern (S) genotype is in gold (lighter color). Populations used in this study are labeled with their population code. Two more all-female populations (including BL) are located approximately 180 km south of the area shown in this map (not shown). The population distribution, sex ratio, genetic relatedness, and codes are based on Miller et al. (2024a). (C) Three processes that could give rise to mosaics of mixed-sex and all-female populations. Starting with ancestrally mixed-sex populations (panel I), dispersal of unmated females (a), stochastic local extinction of males (b), and/or the evolution of female resistance to mating/fertilization (c) (panel II) could give rise to a mosaic of all-female and mixed-sex populations (panel III).

evolved genotypic effect that confers female resistance to fertilization. To test this, we paired females descended from such all-female populations with males from mixed-sex populations and quantified offspring sex ratios as a proxy for fertilization rate (validated by DNA sequence analysis of a subset of offspring). To determine whether fertilization rates reflect the genetic compatibility of parents, we crossed females with males from both genetically similar and genetically distant populations. If reduced fertilization is driven by resistance rather than incompatibility, then it should occur regardless of genetic distance. To test for a maternal effect that confers resistance to fertilization after a single generation of parthenogenesis, we compared fertilization rates and offspring sex ratios in paternate vs. impaternate females descended from mixed-sex populations. In addition, to test whether male trait decay presents a further barrier to male invasion, we compared the fertilization success of males descended solely from mixed-sex populations to that of intraspecific hybrid males produced from crosses between males from mixed-sex populations and females from all-female populations.

Materials and methods

Study system

The peppermint stick insect Megacrania batesii (Figure 1A) is a facultative parthenogen endemic to far-north Queensland, Australia. Parthenogenesis occurs via automixis (Miller, 2024), and fertilized M. batesii eggs yield approximately equal proportions of male and female hatchlings; whereas unfertilized eggs yield only daughters (Miller et al., 2024a). Therefore, any consistent female bias in offspring sex ratios suggests that some daughters were produced parthenogenetically, with higher proportions of daughters indicating a higher rate of parthenogenesis and a lower rate of fertilization. A single generation of parthenogenetic reproduction typically results in complete or near-complete homozygosity in M. batesii females, making it possible to use the level of heterozygosity to differentiate impaternate females from paternate females and males (Miller, 2024; Miller et al., 2024a). Spontaneous (parthenogenetically produced) males can occur in systems with XX/XO and ZZ/ZW sex determination (Boyer et al., 2023; Lampert, 2009; Morgan-Richards et al., 2010; Pijnacker, 1966, 1969; Schwander et al., 2013; van der Kooi & Schwander, 2014). However, spontaneous males have never been observed in M. batesii, suggesting an XX/XY sex-determination system.

Two discrete types of M. batesii populations-all-female and mixed-sex—occur in a geographical mosaic (Figure 1B), sometimes in close proximity and with no obvious barriers to dispersal (Cermak & Hasenpusch, 2000; Miller et al., 2024a). The all-female populations could have arisen via dispersal of unmated females, or via extinction of males (Miller et al., 2024a; Figure 1C). The known species range spans only 1.8° in latitude (with most populations occurring within 0.24° latitude), and there are no appreciable differences in climate, habitat, or density between mixed-sex and all-female populations (Miller et al., 2024a, 2024b). Annual field surveys since 2019 have shown that all-female populations contain only females, and all eggs collected from such populations have hatched into females (see Miller et al., 2024a for a summary of the first 4 years of field data). Mixed-sex populations typically have approximately even or slightly female-biased sex ratios and reproduction is usually sexual, although approximately

10% of females in natural mixed-sex populations were found to have been produced parthenogenetically (Miller et al., 2024a). Males develop more quickly and mature several weeks before females (DW, pers. obs.), and females in mixedsex populations tend to be almost constantly guarded by a male (Boldbaatar et al., 2024).

Populations located north of Noah Creek form one genetic cluster ("Northern genotype" or simply "Northern"); while populations south of Noah Creek form another genetic cluster ("Southern genotype" or simply "Southern"). The two genetic clusters are clearly differentiated, with high intercluster Fst values (Miller et al., 2024a). Both population types (all-female and mixed-sex) occur in each genetic cluster, but most known Southern populations are all-female while most known Northern populations are mixed-sex. Although the ages of these populations are not known, the Southern allfemale populations appear to be relatively long-established and exhibit high genetic differentiation (Miller, 2024; Miller et al., 2024a). We therefore expected the Southern all-female populations to exhibit evolved resistance to fertilization. By contrast, some Northern all-female populations (such as NS; Figure 1B) are genetically very similar to Northern mixed-sex populations (Miller, 2024; Miller et al., 2024a), and might therefore lack evolved resistance to fertilization.

Experiment 1: Are females from long-established all-female populations resistant to fertilization?

To determine the effect of the population type (mixed-sex vs. all-female) from which females originate on resistance to fertilization, we paired females from four all-female populations (Southern genotype) and four mixed-sex populations (Northern genotype) with males (all from the Northern mixed-sex populations), and we compared the resulting off-spring sex ratios from the two types of mothers as an index of fertilization rate (Experiment 1, Figure 2; see Table 1 for sample sizes). We validated the use of offspring sex ratio as an index of fertilization rate by sequencing the DNA of a subset of daughters and using heterozygosity at 260 polymorphic (SNP) loci to differentiate paternate vs. impaternate daughters (Supplementary Material, Heterozygosity; Supplementary Figures S1 and S2; Supplementary Tables S1 and S2).

In addition, we quantified the fecundity and egg-hatching success of these male-paired females and of control (unpaired) females (see Table 1 for sample sizes) to investigate whether sex ratios were biased by deaths of male embryos and to compare reproductive outcomes (Supplementary Material, Fecundity and Viability). If female-biased offspring sex ratios are caused by the death of male embryos (e.g., due to male-killing bacteria, Engelstädter & Hurst, 2009), then mated females with female-biased offspring sex ratios should also have reduced fecundity (if male death occurs before eggs are laid) or reduced hatching success (if male death occurs after eggs are laid).

We first collected eggs from four Northern mixed-sex populations (BK, MB, MK, CO; Figure 1B), and four Southern all-female populations (B1, CB, KR, TB; Figure 1B) in Far-North Queensland, Australia, in early February 2020. We reared the hatchlings inside clear plastic cylindrical containers (200 mm diameter × 400 mm height) with mesh lids, in controlled temperature rooms (~27 °C; 12-h light cycle) at UNSW Sydney. They were sprayed daily with de-ionized water (for drinking and to maintain high humidity) and fed *Pandanus tectorius* leaves ad libitum. Hatchlings were first



Figure 2. Experimental design. Crosses testing for: resistance in females descended from all-female populations of the Southern genotype (Experiment 1), incompatibility between genotypes (Experiment 2), maternal effects (Experiment 3), and male trait decay (Experiment 3). Diamonds = mixed-sex populations; circles = all-female populations. Populations and female insect silhouettes are colored according to genotype and impaternity. Male insects are in black ("N" = fully Northern male; "H" = North-South intraspecific hybrid male). Thick black arrows indicate sexual reproduction of female-male pairs, and thick grey arrows indicate parthenogenic reproduction of unpaired females. Sample sizes (number of female-male pairs or unpaired females) are indicated above each grey arrow (for parthenogenetically reproducing females) or x (cross). Each female was only used once. *One Southern pair in Experiment 2 produced no offspring, leaving only six pairs of that treatment for offspring sex ratio analyses.

 Table 1. Experiment 1 sample sizes. Females were either from Northern mixed-sex populations or Southern all-female populations, and they were either paired with males from Northern mixed-sex populations or kept alone to reproduce parthenogenetically. Offspring sex ratio analyses only included paired females (bolded).

Female type and treatment	Number of females	Number of hatchlings
Paired females from Southern all-female populations	20	354
Unpaired females from South- ern all-female populations	23	243
Paired females from Northern mixed-sex populations	14	246
Unpaired females from North- ern mixed-sex populations	12	115

placed on small potted host plants inside the plastic containers, in groups of two to four same-sex full-sib nymphs (juveniles). As they grew and started to defoliate their plant, larger nymphs were transferred to containers without plants but with a cloth to retain moisture and provided fresh-cut leaves every 2–3 days. Once insects underwent their final molt to the adult stage, they were separated into individual containers. Some of the experimental females were sisters (from 12 mothers from Southern all-female populations, and 10 maleguarded mothers from Northern mixed-sex populations), and some of the experimental males were brothers (from 16 maleguarded mothers from Northern mixed-sex populations). We distributed siblings randomly across treatments, using each individual only once.

We paired 34 newly molted adult females (20 Southern all-female population females, and 14 Northern mixed-sex population females) with 34 adult males from Northern mixed-sex populations. Most mixed-sex population females were paired with a non-sibling male from their same population, but three were paired with males from a different population, and one was paired with her brother. On average, females were paired 1.5 days after their final molt (SD = 1.1 d), and males were paired 28.6 days after their final molt

(SD = 8.3 d). Because *M. batesii* males develop more quickly than females, these age ranges probably mimic natural conditions. We collected eggs from each pair 20 days after the female started laying, keeping the pair together throughout this time (three males died during this time, but all females had access to a male for at least 18 days). We later also paired eight females from an isolated all-female population at the southern edge of the species range (population BL, Southern genotype; Miller et al., 2024a) with Northern mixed-sex population males to test whether this distant population is capable of sexual reproduction (Supplementary Material, BL Crosses; Supplementary Figure S3).

As a control, we kept 35 females (23 from Southern allfemale populations; 12 From Northern mixed-sex populations) in individual containers to allow parthenogenetic reproduction, and collected their eggs 20 days after they started laying. Our experimental vs. control housing was designed to mimic natural conditions: in all-female populations, two adult *M. batesii* females are rarely found on the same small host plant or tree branch (R.B., unpublished data); by contrast, most adult *M. batesii* females in mixed-sex populations are constantly guarded by a male (Boldbaatar et al., 2024). These conditions are also unlikely to have substantially affected food availability because adult *M. batesii* males eat much less than females (Boldbaatar, 2022), and food was provided ad libitum.

Eggs collected from paired (n = 937; mean = 27.6; SE = 0.9) and unpaired (n = 925; mean = 26.4; SE = 0.8) females were checked daily for hatching until 20 weeks after the last female had started laying eggs. Hatchlings (n = 600; mean = 17.6; SE = 1.3 from paired females; and n = 358; mean = 10.2; SE= 1.1 from unpaired females) were sexed based on the morphology of the 8th and 9th abdominal sternites (Miller et al., 2024a). We also quantified the number of eggs, hatching success, and number of hatchlings (Supplementary Material, Fecundity and Viability).

All statistical analyses were done in R 4.2.1 (R Core Team, 2023). We used the MuMIn package (Barton, 2024) to compare models with and without our predictor of interest, using "corrected" AIC (AICc) in all cases to avoid bias associated with small sample sizes (Johnson & Omland, 2004). To test the effect of female population type (Southern all-female, or Northern mixed-sex) on offspring sex ratio, we compared a model with female population type as the only fixed effect to a null (intercept-only) model. Both models were generalized linear mixed models (package glmmTMB, Brooks et al., 2017), using the binomial family and logit link, and including an observation-level random effect (female ID) to account for overdispersion. We did not include population of origin in our models because of small sample sizes from some populations; however, the individual populations within each population type showed similar trends to each other (Supplementary Table S3; Supplementary Figures S4 and S5). We used a similar approach to investigate the effects of female population type and pairing treatment on female fitness measures (Supplementary Material, Fecundity and Viability). The large language model ChatGPT (OpenAI, 2024) was used as an aid in writing the R code for this study.

Experiment 2: Is low fertilization rate explained by genetic incompatibility?

Reduced fertilization rates can be a consequence of incompatibility between male and female genotypes (Dobzhansky, 1937; Howard, 2003; Matute & Cooper, 2021; Mayr, 1963). This could potentially explain the results of Experiment 1 because females from all-female populations were of the Southern genotype whereas all males were of the Northern genotype (i.e., population type was confounded with genotype). To address this, we performed three additional sets of crosses using males and females from the same genetic cluster ("matching genotype") versus differing genetic clusters (see Table 2 for sample sizes). These crosses (Experiment 2, Figure 2) were: "Southern pairs" cross (matching genotypes: Southern all-female population female × Southern mixed-sex population male), "Northern pairs" cross (matching genotypes: Northern all-female population female × Northern mixed-sex population male), and "Northern female x Southern male" cross (non-matching genotypes: Northern all-female population female × Southern mixed-sex population male). If female resistance caused the low fertilization success we observed in crosses between Southern all-female population females and Northern mixed-sex population males in Experiment 1, then the Southern pairs cross in Experiment 2 should also produce more female-biased offspring sex ratios (i.e., we should see a female genotype effect). But if the low fertilization success was due to genetic incompatibility, then the Northern female × Southern male cross should produce more female-biased offspring sex ratios (i.e., we should see a genotype-matching effect).

We used lab-reared insects that had been collected from natural populations as eggs or hatchlings in August 2022 (except for one lab-colony male). All females were collected from all-female populations (and were therefore impaternate), and all males were descended purely from Northern or Southern mixed-sex populations. Southern all-female population females were collected as first-instar hatchlings from population CB (Figure 1B). The Northern all-female population females (from population NS) and Southern mixedsex population males (from population VS) were collected as eggs. Three of the Northern mixed-sex population males were collected as hatchlings from population CO, and the fourth was lab-bred (from Northern mixed-sex population stock). Females were housed and paired as described above. However, the average age at pairing was 17.4 days (SD = 10.1d) for females and 61.5 days (SD = 26.1 d) for males. Four of the Southern mixed-sex population males were used twice (paired once with a Southern all-female population female and once with a Northern all-female population female, in

Table 2. Experiment 2 sample sizes. Females were either from aNorthern or Southern all-female population, and males descendedfrom either Northern or Southern mixed-sex populations. Each femalewas paired either with a male of the same genotype (Southern Pairs,Northern Pairs) or a differing one (Northern female × Southern male).*One Southern Pair produced no offspring, leaving only six pairs of thattreatment for offspring sex ratio analyses.

Cross type	Female genotype	Genotype matching	Number of pairs	Number of hatchlings
Southern pairs	Southern	Yes	7*	112
Northern female × South- ern male	Northern	No	6	85
Northern pairs	Northern	Yes	4	60

alternating order, with at least 4 days of rest between pairings to minimize sperm depletion effects). We collected eggs (n = 437 total; mean = 27.3; SE = 1.3) from each pair after 20 days of laying (but 21 days for one female). Four females started laying before pairing (two from the Southern pairs cross treatment and two from the Northern female × Southern male cross treatment); for these four females, any pre-pairing eggs were removed, and eggs were collected 20 days after pairing; the offspring sex ratios produced from these four females were similar to those produced by other females in their respective treatments. We again quantified the number of eggs, hatching success, and number of hatchlings (Supplementary Material, Fecundity and Viability), and the hatchlings (n = 257 total; mean = 16.1 per female; SE = 1.5) were sexed as described above.

Based on the data from these three crosses, we used AICc to compare two models of offspring sex ratio, each containing a single fixed effect, as well as a null (intercept-only) model. In the first model, the fixed-effect was female genotype (Southern vs. Northern); whereas in the second model, it was genotype matching (matching or non-matching). All three were generalized linear mixed models with a binomial family error structure with logit link, and they included an observation-level random effect to account for overdispersion.

Experiment 3: Is resistance driven by genotypic or maternal effects? And are male traits decaying in all-female populations?

In Experiment 1, as in nature, impaternity was confounded with female population type and genotype, since females from all-female populations are always impaternate, and females from mixed-sex populations are usually paternate. To disentangle these effects, we performed another set of crosses using our second generation of lab-reared insects (Experiment 3, Figure 2). These crosses included impaternate females (i.e., females produced by unmated mothers in Experiment 1) descended from both Northern mixed-sex and Southern all-female populations. If resistance to fertilization is due to a trait in Southern all-female populations (i.e., a genotypic effect), both paternate and impaternate females descended from Northern mixed-sex populations should lay more fertilized eggs and produce more sons than Southern all-female population females when paired with males. If impaternity results in female resistance, then impaternate females descended from Northern mixed-sex populations should produce more female-biased offspring sex ratios than paternate Northern females.

To test the male trait decay hypothesis, we also included two types of males in these crosses (Experiment 3, Figure 2): fully Northern males (descended from Northern mixed-sex populations only, from Experiment 1) and North-South intraspecific hybrid males (produced by Southern all-female population mothers and Northern mixed-sex population fathers in Experiment 1). If male traits have decayed in the Southern all-female populations, intraspecific hybrid males should have poor fertilization success. If there is no such decay, intraspecific hybrid males should have similar success to fully Northern males (or greater success than them with Southern females, in case of incompatibilities between Northern and Southern genotypes). For comparison, we also included North-South intraspecific hybrid females in our crosses.

We thus paired four types of females (11 impaternate Southern all-female population females, 11 impaternate Northern mixed-sex population females, 19 paternate Northern mixed-sex population females, and 12 North-South intraspecific hybrid females) with the two types of males (26 fully Northern males and 27 North-South intraspecific hybrid males), in a full-factorial design (Table 3). Paternate females from Northern mixed-sex populations were descended from primarily sexual lineages; impaternate Southern all-female population females were descended from entirely parthenogenetic lineages; and impaternate Northern mixed-sex population females were descended from primarily sexual lineages via a single generation of parthenogenesis.

Subsequent heterozygosity analysis showed that three of the putatively paternate North-South intraspecific hybrid females used in this experiment were actually impaternate (see Supplementary Material, Heterozygosity). We did not remove these from our analyses because we were not able to sequence DNA from all focal females. Our estimates of the differences between paternate and impaternate females are therefore conservative.

We reared and paired the insects and collected their eggs as in Experiment 1, except that the average pairing age (days since final molt) was 10 days (SD = 4.6) for females and 34.7 days (SD = 11.6) for males, and we collected eggs laid over 15 days instead of 20 days. We also kept 63 additional females laying parthenogenetically (Table 3) for comparison. The eggs (n =1811; mean = 15.6; SE = 0.4) were checked daily for hatching until 21 weeks after the last egg was collected, and hatchlings (n = 1281; mean = 11; SE = 0.5) were sexed as described above. In addition, we propagated 20 intraspecific hybrid females parthenogenetically for an additional generation to check for infertility (Supplementary Material, Performance of second-generation intraspecific hybrid females). We again collected fecundity and viability data (Supplementary Material, Fecundity and Viability), and sequenced DNA from a subset of daughters to quantify their heterozygosity (Supplementary Table S1; Supplementary Material, Heterozygosity). We used AICc model selection to investigate the effects on offspring sex ratio. We tested three main effects: focal female impaternity (paternate vs impaternate), focal females' maternal genotype (Southern vs. Northern), and male (mate) genotype (fully Northern vs. North-South intraspecific hybrid). We used only the female's mother's genotype (ignoring that of her father) because all paternate females necessarily had Northern mixed-sex population fathers and all impaternate females had none. We also tested interactions among these factors. We therefore compared 18 models (see Supplementary Table S14 for the full list of models and the predictions they test). All were generalized linear mixed models, and all used a binomial distribution and included an observation-level random effect to account for overdispersion.

Results

Experiment 1: Females from long-established allfemale populations are resistant to fertilization

When paired with males, females from Northern mixed-sex populations tended to produce near-even proportions of sons and daughters (41% males, on average), as expected when most or all eggs are fertilized. In contrast, females from Southern all-female populations tended to produce highly female-biased offspring sex ratios (17% males, on average), suggesting that fewer of their eggs were fertilized (Figure 3A). All but one of the females from Southern all-female **Table 3.** Experiment 3 sample sizes. Females were either putatively paternate (had a father) or impaternate (had no father), and they were either descended from Southern all-female populations, Northern mixed-sex populations, or a cross between the two (North-South intraspecific hybrids had a mother from a Southern all-female population and a father from a Northern mixed-sex population). Males were either fully Northern (descended from Northern mixed-sex populations) or North-South intraspecific hybrids (sons of a mother from a Southern all-female population and a father from a Northern mixed-sex population). Each female was either left alone to reproduce parthenogenetically (unpaired) or paired with one of the two types of males. Offspring sex ratio analyses only included male-paired females, and *egg viability analyses only included females that laid eggs (one of the unpaired hybrid females laid none).

Female type	Unpaired focal females	Focal females paired with fully Northern male	Focal females paired with North- South intraspecific hybrid male
Impaternate from Southern all-female populations	24	5	6
Impaternate from Northern mixed-sex populations	5	4	7
Paternate from Northern mixed-sex populations	14	10	9
Paternate North-South intraspecific hybrid	20*	7	5

populations produced female-biased offspring sex ratios, but 80% of them produced at least one son, confirming that they received viable sperm. The effect of female population type on offspring sex ratio was strongly supported (best model; Δ AICc = 10.43 for the null model; Supplementary Table S4). All control (i.e., unpaired, parthenogenetically reproducing) females produced only daughters.

We found no evidence that male embryo deaths could explain the differences we found in offspring sex ratios. Instead, mating tended to increase both hatching success and total hatching numbers (Figure 4A–C; Supplementary Tables S5–S8; Supplementary Material, Fecundity and Viability). Offspring heterozygosity analysis confirmed that observed variation in offspring sex ratio reflected variation in fertilization rate (Figures 5A; Supplementary Figure S2A and B; Supplementary Material, Heterozygosity). When paired with males, both types of females produced mixed clutches of paternate (high-heterozygosity) and impaternate (low-heterozygosity) daughters, but females descended from Southern all-female populations produced many more low-heterozygosity daughters (69.6%) compared to females descended from Northern mixed-sex populations (10.5%).

Experiment 2: Low fertilization rate is not explained by genetic incompatibility

We did not find evidence of incompatibility between the Northern and Southern genotypes: Northern females from all-female populations produced approximately even offspring sex ratios (47% males, on average), whether paired with Northern or Southern males, whereas Southern females from all-female populations produced female-biased offspring sex ratios (2% males, on average) when paired with Southern males (Figure 3B and C). The effect of female genotype (whether the female came from a Northern or Southern all-female population) on offspring sex ratio was very strongly supported (best model, Supplementary Table S10), just as it had been in Experiment 1 (see Supplementary Figure S7 for a comparison). There was no evidence of an effect of genotype matching (genotype matching model: \triangle AICc = 31.13; Supplementary Table S10). The Southern pair crosses tended to result in somewhat higher fecundity, but this effect was not supported by AIC (the null model was the best model; Supplementary Table S11), and all three cross types resulted in similar egg hatching success and total numbers of

hatchlings (Figure 4D–F; Supplementary Tables S12 and S13; Supplementary Material, Fecundity and Viability).

Together, the results of Experiments 1 and 2 suggest that evolved resistance, not genetic distance, explains low fertilization rates of male-paired females descended from Southern all-female populations (Figure 3C). In all-female Southern populations (which appear to be long-established), females are resistant to fertilization by males of both the Northern genotype (Experiment 1) and the Southern genotype (Experiment 2), indicating that females in these populations possess an evolved trait that confers resistance to fertilization regardless of genetic distance to the male. By contrast, in the Northern all-female population (which is both geographically and genetically very close to nearby mixed-sex populations, and appears to be recently established), females are not resistant to fertilization by males of either Northern or Southern genotype (Experiment 2), suggesting an absence of both genetic incompatibility and evolved resistance.

Experiment 3: Genotypic and maternal effects on female resistance, and decay of male traits in all-female populations

We found very strong support for both genotypic and maternal effects on offspring sex ratio (both factors included in the three best models; null model \triangle AICc = 18.63, Supplementary Table S15). Paternate females from Northern mixed-sex populations produced approximately even offspring sex ratios (47% males, on average), as expected when all or most eggs are fertilized (Figure 6A). In contrast, females descended from Southern all-female populations produced severely female-biased offspring sex ratios (10% males, on average), as expected when many offspring are produced by parthenogenesis. Impaternate Northern mixed-sex population females and North-South intraspecific hybrid females produced intermediate (i.e., moderately female-biased) offspring sex ratios (respectively, 36% and 38% males, on average; Figure 6A). Heterozygosity analysis of a subset of female offspring again revealed mixed clutches containing both paternate and impaternate daughters and confirmed that variation in offspring sex ratio reflected variation in fertilization rate (Figure 5B; Supplementary Table S16).

We also found evidence that male (mate) genotype affected the proportion of fertilized eggs, but its effect depended on female impaternity (best model, Supplementary Table S15).



Figure 3. Females from Southern (S) genotype all-female populations produced proportionally fewer sons than females from the Northern (N) genotype, regardless of their mate's genotype. (A) Experiment 1. Offspring sex ratios (i.e., proportion of offspring that were sons) of females from either Southern all-female populations or Northern mixed-sex populations, each of which had been paired with a male from a Northern mixed-sex population. There was very strong evidence of an effect of female genotype on offspring sex ratio (Supplementary Table S4). (B) Experiment 2. Offspring sex ratios of females from either Southern- or Northern-genotype all-female populations that had been paired with either a Southern- or Northern-genotype male. Southern pairs (with matching genotypes: Southern females × Southern males) are orange (left); Northern female × Southern male pairs (with differing genotypes) are pink (middle); Northern pairs (with matching genotypes: Northern females × Northern males) are sky blue (right). We again found very strong support for the effect of female genotype on offspring sex ratio, and no support for an effect of genotype matching (Supplementary Table S10). In both boxplots (A and B), lower hinges represent the 25th percentiles, middle lines represent medians, and upper hinges represent the 75th percentiles. Each dot represents the offspring sex ratio of one experimental female. The horizontal dotted line marks an even proportion of sons and daughters, expected when all eggs are fertilized. A sex ratio of zero would mean that all offspring are daughters, as expected when no eggs are fertilized. (C) Summary of cross outcomes. Arrows represent types of crosses performed and their outcome, with the start of the arrow indicating the male's population of origin and the point of the arrow representing the female's population of origin. Red (lighter) arrows indicate low fertilization rate (i.e., resistance to fertilization); black arrows indicate high fertilization rate (no resistance to fertilization). Diamonds are mixed-sex populations; circles are all-female populations (each labeled with its code). Fill color represents genotype: Northern (N) is blue (darker); Southern (S) is gold (lighter). Asterisks highlight the three cross types performed in Experiment 2 (as depicted in panel B).

Intraspecific hybrid males tended to sire approximately half as many sons as fully Northern males when paired with impaternate females (17% vs. 32% sons, on average), but both types of males sired approximately equal proportions of sons when paired with paternate Northern females (Figure 6B).

cific hybrid females tended to produce the most offspring

(Figure 4I), and their sexually and parthenogenetically

when paired with paternate Northern females (Figure 6B). We again found no evidence of negative effects of pairing on reproductive output that could explain the female-biased offspring sex ratios we observed (Figure 4G–I; Supplementary Figure S8; Supplementary Tables S17–S22; Supplementary Material, Fecundity and Viability). Interestingly, intraspe-

produced offspring were viable. Their parthenogenetic offspring were propagated for a further generation and found to be fully fertile, confirming an absence of hybrid unviability (Supplementary Material, Second-generation intraspecific hybrid females).

We found evidence of three processes that could facilitate the persistence of all-female populations in *Megacrania batesii*: a maternal effect causing resistance to fertilization after the first generation of asexual reproduction, a genotypic



Figure 4. Reproductive output from Experiments 1 (panels A–C), 2 (panels D–F), and 3 (panels G–I). Experiment 1: Females reared from eggs collected from each population type (Southern all-female populations on the left, and Northern mixed-sex populations on the right) were paired with a male and allowed to mate (filled boxes) or were kept alone and allowed to reproduce parthenogenetically (white boxes). Pairing had little effect on the number of eggs produced over 20 days of oviposition (A), but tended to enhance egg viability (B) and the total number of hatchlings produced (C). Experiment 2: Southern pairs tended to lay more eggs than Northern pairs or Northern female × Southern male pairs, but statistical analysis did not support this effect, and there was no difference between pair types in egg viability or number of hatchlings. Experiment 3: We manipulated whether females were paternate or impaternate, and either descended from Southern all-female populations, Northern mixed-sex populations, or a cross between the two. They were either paired with a male and allowed to reduce reproductive performance of females descended from Southern all-female populations; by contrast, mating tended to enhance the reproductive performance of females descended from Northern mixed-sex populations (see Supplementary Material). In each boxplot: lower hinges are the 25th percentiles; middle lines are medians; and upper hinges are 75th percentiles. Each dot represents the eggs or hatchlings produced by one female.

effect indicating evolved resistance to fertilization in longestablished all-female populations, and decay of male traits that could reduce the fertilization success of sons produced in such populations by invading males. Unfertilized eggs develop into daughters in this species, so reduced fertilization, either from female resistance or male trait decay, results in fewer males in the next generation. Our findings thus suggest that long-established (Southern-genotype) all-female populations in this species remain reproductively isolated and asexual, despite the potential for introgression from neighboring mixed-sex populations, because they have evolved resistance to sex. Similar processes could contribute to the formation of distinct all-female (parthenogenetic) and mixed-sex (sexual) populations in other facultatively parthenogenetic species. Our findings also provide insight on the role of sexual conflict in the evolution of reproductive modes.

We found that *M. batesii* females produce some daughters parthenogenetically even after mating, but the rate of parthenogenesis varies markedly among populations. Mated females descended from Northern mixed-sex populations produced approximately 10% of their daughters parthenogenetically-a similar rate of parthenogenetic origin to that observed in field-collected females from Northern mixed-sex populations (Miller et al., 2024a). By contrast, mated impaternate females descended from Southern all-female populations produced 70% of their daughters parthenogenetically. These Southern females thus produced very female-biased offspring sex ratios after mating, regardless of whether the males were genetically similar (Southern genotype) or distant (Northern genotype). Together, these results suggest that Southern all-female populations of Megacrania batesii (which appear to be relatively long-established) are undergoing the evolution of reproductive isolation via resistance to fertilization. However, isolation in our experiments was not complete, so the evolution of resistance might be in its incipient stages. Indeed, females from a Northern all-female



Figure 5. Offspring sex ratio reflects fertilization rate. Weighted regression (solid black line) of the proportion of daughters that were produced sexually by each female, as determined by offspring heterozygosity, vs. that female's offspring sex ratio, in Experiments 1 (A) and 3 (B). Each point represents the offspring sex ratio and fertilization rate of one female, colored by female type (genotype in Experiment 1; genotype and impaternity in Experiment 3). Females were either descended from Southern all-female populations (Southern-AFP), Northern mixed-sex populations (Northern-MSP), or a cross between the two (North-South hybrids). Point size represents the number of daughters that were sequenced. The dashed line represents the expected relationship between the proportion of daughters produced sexually (inferred from high heterozygosity) and the offspring sex ratio, if female-biased sex ratios result from reduced fertilization rate and an increased proportion of parthenogenetically produced daughters (slope = 2.0). The confidence intervals for the observed relationship overlap this value in both Experiment 1 (slope = 1.55, CL = 1.07, 2.25, $R^2 = 0.53$) and Experiment 3 (slope = 1.8, CL = 1.33, 2.43, $R^2 = 0.491$).

population (which appears to be much more recently established) were not resistant to fertilization, perhaps illustrating an earlier stage in this process. Work on other facultatively parthenogenic stick insects has also revealed variation in fertilization success. There is a similar difference in fertilization success between females from all-female and mixed-sex populations in *Clitarchus hookeri* (Morgan-Richards et al., 2010; Morgan-Richards, 2023), and there is variation among different lineages in *Timema douglasi* (Larose et al., 2023). Female resistance could, therefore, be a common mechanism contributing to the formation of these animals' reproductive mosaics.

Reproductive isolation could be maintained by genetic or gametic incompatibility between diverged populations (Schluter, 2001), but we did not find evidence of such incompatibility in this system. In M. batesii, most mixedsex populations are of the Northern genotype while most all-female populations are of the Southern genotype (Miller et al., 2024a). In Experiment 1, we only used males from Northern mixed-sex populations and crossed them with females from either Southern all-female or Northern mixedsex populations. However, in Experiment 2, we also used males from a Southern mixed-sex population, crossing them with females from a Northern all-female population to test for incompatibility between the Northern and Southern genotypes. When crossed with Southern males, females from a Southern all-female population (which appears to be longestablished) still produced female-biased offspring sex ratios. By contrast, females from the Northern all-female population (which appears to be relatively recently established) produced near-even offspring sex ratios whether crossed with Northern or Southern males. Moreover, the intraspecific hybrid females produced by crossing Southern females and Northern males in Experiment 1 were viable and fertile, as were their parthenogenetically produced descendants. Together, these results confirm that the reduction in fertilization success we observed was due to a trait in females from Southern all-female populations, and not due to overall genetic divergence between Southern and Northern genotypes. This evolved trait in long-established all-female populations is consistent with theoretical predictions that elevated sexual conflict in facultative parthenogens can lead to the evolution of female resistance and the extinction of males (Burke & Bonduriansky, 2019; Kawatsu, 2013).

We found that both female genotype and impaternity affected offspring sex ratio and the rate of fertilization. We teased these factors apart in Experiment 3, which included not just the types of females typically found in the wild (impaternate females from all-female populations and paternate females from mixed-sex populations), but also experimentally generated impaternate females from mixed-sex population stock. We found that, when allowed to mate, paternate females descended from Northern mixed-sex populations produced approximately even offspring sex ratios, but impaternate females from the same populations produced more female-biased offspring sex ratios. We also found a clear difference in offspring sex ratio between Southern and Northern impaternate females. And once again, offspring heterozygosity analysis showed that differences in offspring sex ratio corresponded to differences in fertilization rate. These results show that female resistance in *M. batesii* can result both from genotypic effects that evolve in long-established all-female populations, and from a maternal effect that can occur when females from mixed-sex populations undergo a single generation of parthenogenetic reproduction. This maternal effect confers a degree of resistance in impaternate daughters, potentially enabling them to take advantage of lower male densities by engaging in parthenogenetic reproduction. This maternal effect might therefore have evolved in response to a history of fluctuating male densities and alternating sexual and parthenogenetic generations. A similar maternal effect has been reported for another facultatively parthenogenetic stick insect, Extatosoma tiaratum (Burke & Bonduriansky, 2022).

Our findings also suggest that male traits are decaying in Southern all-female populations of *M. batesii*. When females from Southern all-female populations mated with Northern males and produced sons, we found that these intraspecific



Female Type

Figure 6. Experiment 3 offspring sex ratio results. The four vertical bands across both panels indicate female type (impaternity and genotype): Females were either from Southern all-female population stock, Northern mixed-sex population stock, or a cross (North-South intraspecific hybrid, from a Southern mother and Northern father). Impaternate females had no father (i.e., were parthenogenetically produced); putatively paternate females were produced by mothers that were paired with a male. (A) Offspring sex ratios (i.e., proportion of offspring that were sons) by female type. Females produced a smaller proportion of sons if they were descended from Southern all-female populations or if they were impaternate. These effects were very strongly supported (Supplementary Table S15). (B) Offspring sex ratios by female type (as labeled above panel A) and male type (as labeled below the x-axis). Females were paired either with a fully Northern (N) male or a North-South intraspecific hybrid (H) male (produced by a Southern mother and Northern father). Intraspecific hybrid males had reduced fertilization success (produced relatively fewer sons) than fully Northern males, when paired with impaternate females. The interaction between female impaternity and male genotype was strongly supported (Supplementary Table S15). In both boxplots (A and B), each dot represents the offspring sex ratio from one female. The dotted line across the middle of each panel marks an even proportion of sons and daughters, expected when all eggs are fertilized. A sex ratio of zero (all daughters) is expected when no eggs are fertilized. Boxplot lower hinges are the 25th percentiles; middle lines are medians; and upper hinges are the 75th percentiles. The best model included an interaction between impaternity and genotype (Supplementary Table S15); but the plot suggests only an additive effect (panel A). This apparent discrepancy occurs because we only included maternal (not paternal) genotype in our models, such that North-South intraspecific hybrid females were modeled as belonging to the Southern genotype. While paternate and impaternate Northern mixed-sex population females produced only mildly different offspring sex ratios from each other, impaternate females from Southern all-female populations produced much lower offspring sex ratios than paternate North-South intraspecific hybrid females (panel A). If the only difference between impaternate Southern all-female population females and North-South hybrid females had been their impaternity, then this greater difference between their offspring sex ratios would suggest an interaction (i.e., a greater effect of impaternity on females from the Southern genotype than on those from the Northern genotype). However, since intraspecific hybrid females also carry Northern genes, this pattern suggests only an additive effect of impaternity and genotype (i.e., intraspecific hybrid females produce more sons because they are both paternate and half-Northern).

hybrid males went on to have lower fertilization success than their fully Northern counterparts. Male traits are predicted to decay over many generations of asexual reproduction through genetic drift as well as selection for phenotypes that optimize female performance, and such decay has been found in other asexual animals (van der Kooi & Schwander, 2014). This could also contribute to reproductive isolation and the maintenance of all-female populations in a positive feedback loop. If a male were to invade a long-established all-female population and mate with a female, any sons resulting from such a mating would likely have very low fitness. These intraspecific hybrid sons would mostly encounter impaternate females and fertilize very few eggs. Male trait decay could thus limit the spread of sex in these populations. Interestingly, the effect of male hybrid origin in our experiments all but disappeared if the female was paternate and from a Northern mixed-sex population. This suggests that, rather than the ability to fertilize eggs in general, it is the ability to overcome female resistance that is impaired in intraspecific hybrid males. Alternatively, it is possible that this effect reflects cryptic female mate choice (Eberhard, 1994; Thornhill, 1983), where impaternate females discriminate against intraspecific hybrid males.

The poor fertilization success of intraspecific hybrid males in our experiment stood in sharp contrast to the reproductive success of intraspecific hybrid females. Intraspecific hybrid females tended to have higher egg viability and produce more hatchlings than either fully Southern or fully Northern females, providing some evidence against outbreeding depression (but see Wu et al., 1996). The success of intraspecific hybrid females suggests that there may in fact be hybrid vigor in this system, and that the poor performance of intraspecific hybrid males is due specifically to the decay of male reproductive traits (or reduced post-copulatory attractiveness). Alternatively, the poor success of intraspecific hybrid males could be a case of Haldane's Rule, whereby interspecies hybrids of the heterogametic sex tend to be inviable or sterile more often than those of the homogametic sex (Haldane, 1922). However, the high success of intraspecific hybrid males that mated with paternate Northern females shows that these males are not sterile. If both hybrid vigor and male trait decay occur in this system, their interplay could have complex effects on the outcome of male invasion into asexual populations.

The reductions in fertilization that we observed appear to be driven by post-mating isolation via resistance to fertilization. Impaternate females from Southern all-female populations produced very few sons, demonstrating that very few of their eggs were fertilized, even when sperm transfer was confirmed by the presence of at least one son (i.e., at least one paternate offspring). Post-copulatory mechanisms, such as small or non-functional spermathecae, could prevent or reduce fertilization after mating has taken place, as suggested by work on asexual stick insect species (Schwander et al., 2013). However, behavioral resistance to mating may also be at play. Impaternate females from Southern all-female populations were more likely than their Northern mixed-sex population counterparts to produce female-only clutches after being paired with a male, suggesting that the Southern females may be more likely to avoid mating or sperm transfer altogether. Work in another facultative stick insect (Extatosoma tiara*tum*) shows that females can employ resistance behaviors and chemical signaling to prevent mating (Burke et al., 2015). Chemical signals are also modified in M. batesii females

originating from Southern all-female populations (Ying et al., 2024), and these changes could potentially mediate mating avoidance as well.

We found that sexual reproduction tends to enhance female reproductive success, at least in the protected conditions of the lab environment. Why, then, has resistance to fertilization evolved in some *M. batesii* populations?

The reduction in fertilization we observed could be due to the neutral decay of female sexual traits. Passive decay or vestigialization of female sexual traits is expected after many generations of asexuality (Carson et al., 1982). Indeed, stunted or decayed female sexual traits and impaired fertilization have been reported in many asexually reproducing animals (Carson et al., 1982; Schwander et al., 2013; van der Kooi & Schwander, 2014). However, many of these changes appear to be the result of selection rather than neutral decay (Schwander et al., 2013; van der Kooi & Schwander, 2014). In fact, sexual traits can be costly (e.g., sexual signals can attract predators) and are expected to be under strong negative selection in asexually reproducing populations where they are no longer needed (van der Kooi & Schwander, 2014). Moreover, vestigialization would not explain the maternal effect we found on fertilization, whereby impaternate females descended from mixed-sex populations also produced fewer of their offspring sexually. Instead, sexual conflict and the costs of sex itself (Lehtonen et al., 2012) could play a role in the evolution of female resistance.

We found no evidence from life history measures that parthenogenetic reproduction is more advantageous for females than sex. For females descended from Northern mixed-sex populations, fertilized eggs had higher hatching success than unfertilized eggs; and for females descended from Southern all-female populations, mating did not have a consistent effect on reproductive performance. However, there could be other costs of sexual reproduction that are not manifested in the lab environment, such as the costs of constant mate-guarding by males. Adult M. batesii females in mixed-sex populations are subject to near-continuous mate guarding (Boldbaatar et al., 2024), and males even engage in combat on top of females (DW, pers. obs.). These interactions could impose energetic costs on females, injure them, or attract predators. Fertilization itself could also lead to poor offspring quality, for example, due to inbreeding depression in small populations. In fact, mixed-sex M. batesii populations tend to have high levels of inbreeding (Miller et al., 2024a). Furthermore, if the allfemale populations of *M. batesii* are very well adapted to their local environment, there may be selection against reproducing sexually because mating with incoming males can result in offspring with less locally adapted alleles (Tilquin & Kokko, 2016) or because recombination tends to break-up beneficial allele combinations (Lynch, 1984; Maynard Smith, 1978).

Moreover, the social environment in all-female populations could greatly reduce the benefit to females of producing sons. If females gain more descendants from daughters than from sons, they could obtain a greater payoff from unfertilized eggs (100% of which develop into daughters) than from fertilized eggs (~50% of which develop into sons). As noted above, any sons produced through mating with invading males in long-established all-female populations are likely to encounter mostly resistant females and have poor fertilization success. And since *M. batesii* are flightless and have very poor dispersal ability (Boldbaatar et al., 2024), any sons produced in an all-female population would have little opportunity to

disperse to other populations and find more receptive mates. It is also possible that kin selection could select against females producing harmful males in all-female populations. These populations typically consist of very closely related (nearly clonal) individuals (Miller et al., 2024a), and if neighboring females are more closely related to each other than to their sexually produced sons, then the inclusive fitness costs of producing harmful sons could outweigh the benefits. Thus, mean offspring production from pairings in the lab might underestimate the costs relative to the benefits of mating in the wild. Overall, our findings thus suggest a role for sexual conflict, but more research is needed on the costs and benefits of mating in natural populations.

Our findings support theoretical predictions that the evolution of female resistance can play a critical role in geographical parthenogenesis by facilitating the establishment and maintenance of all-female populations (Burke & Bonduriansky, 2018, 2019). When an all-female population is first established, our findings suggest that the daughters of founding females would be immediately resistant to fertilization due to a maternal effect associated with impaternity. This initial resistance could hinder subsequent invasion by males and allow further resistance to evolve. In addition, over many generations of asexuality, both male and female sexual traits could decay in the population. Thus, if a male did manage to invade, his intraspecific hybrid sons would have poor reproductive success. These processes could thereby jointly promote local transitions from sex to obligate asexuality. Alternatively, theory suggests that the evolution of effective male coercion could maintain sexual reproduction, or enable males to invade all-female populations (Burke & Bonduriansky, 2019). In order to understand the evolution of resistance in all-female populations and the evolution of the maternal effect on female resistance, further research is needed on the costs, benefits, and outcomes of sex and resistance in the wild. In particular, further work is needed to establish whether sexual reproduction confers net benefits on females, or whether it imposes net costs, causing sexual conflict over mating in natural populations. In addition, the physiological and behavioral mechanisms behind female resistance and the poor performance of intraspecific hybrid males in these facultative parthenogens remain unknown. Understanding the processes that promote and maintain distinct reproductive modes in facultatively parthenogenic populations could provide broad insights into the evolution of sex and asexuality.

Supplementary material

Supplementary material is available online at Evolution.

Data availability

The data and code underlying this article are available in the Dryad Digital Repository, at https://doi.org/10.5061/dryad. k98sf7mhf

Author contributions

Experiment 1: designed by D.W., R.B., J.B., and N.B.; carried out by J.B., R.B., and D.W. Experiments 2 and 3: designed and carried out by D.W. and R.B. S.M. and D.W. extracted DNA and analyzed heterozygosity data. D.W. performed all other analyses, constructed the figures, and wrote the original draft, with input from R.B. All authors contributed to editing. R.B. provided advice, feedback, and support throughout.

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