Genetic and phenotypic consequences of local transitions between sexual and parthenogenetic reproduction in the wild

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Abstract:

Transitions from sexual to asexual reproduction have occurred in numerous lineages across the tree of life but many questions remain about how such transitions occur and why asexual populations rarely persist. In facultatively parthenogenetic animals, all-female populations can arise when males are absent or become extinct and, if such populations can persist asexually for many generations, they could ultimately give rise to obligately asexual species. However, the initial stages of this process remain poorly understood. The facultatively parthenogenetic Australian phasmid *Megacrania batesii* exhibits a spatial mosaic of mixed-sex populations that reproduce predominantly sexually and all-female sites that reproduce exclusively via parthenogenesis. We used this system to compare genetic and phenotypic parameters among multiple natural populations that vary in reproductive mode and geographic location. Analysis of single nucleotide polymorphisms (SNPs) collected from reduced representation whole genome data showed populations grouping by geographic location rather than reproductive mode, with little gene-flow between them. Mixed-sex populations had drastically higher heterozygosities than all-female populations. Phenotypic analysis revealed considerable inter-population variation in rates of deformities in (non-functional) wings, with higher mean frequency and severity of deformities in all-female sites. All-female sites also harbored more parasites, but only in swamp habitats. However, reproductive mode did not explain variation in mean female fecundity, egg hatching success, or missing appendages. These results indicate that local transitions to parthenogenetic reproduction can lead to increased rates of developmental abnormalities and increased vulnerability to disease but can also occur without substantial fitness consequences despite dramatic reductions in genetic diversity and heterozygosity.

Key words: Asexual reproduction, heterozygosity, fitness consequences, facultative parthenogenesis

Introduction

The pervasiveness of sexual reproduction in animals has remained a mystery for over a century. Parthenogenetic strategies avoid physically costly sexual encounters, enjoy increased colonialization ability, and do not spend resources creating males that contribute minimally to offspring (Maynard Smith, 1978). Yet sex is nearly ubiquitous across the animal kingdom.

Efforts to explain the prevalence of sexual strategies have sought to identify the advantages that sexual reproduction offers, particularly in the long term (reviewed in Barton & Charlesworth, 1998; Otto & Gerstein, 2006; Hadany & Comeron, 2008; Neiman & Shwander, 2011). Sex can be considered beneficial because it generates genotypic diversity, thereby potentially facilitating adaptation (Fisher-Muller effect: Fisher, 1930; Muller, 1932; Crow & Kimura, 1965; Park and Krug, 2013; Cooper, 2007; Becks & Agrawal, 2012; Colegrave, 2002). Alternative hypotheses focus instead on the long-term consequences of asexuality (Bell, 1982; Agrawal, 2006; Howard & Lively, 1994; Park, Jokela & Michalakis, 2010). In particular, the accumulation of deleterious alleles in non-recombining animals could result in a reduction of mean fitness due to increased mutation load (i.e., Mullers ratchet, Muller 1964) which can lead to population extinction (Lynch & Gabriel, 1990; Lynch et. al., 1993; Chao, 1990; Loewe & Cutter, 2008). These theories focus on the consequences of reproducing asexually in the long-term but, to explain the prevalence of sexual reproduction in nature, it is also important to understand what happens in the short-term, during the initial stages of transitions to asexuality. In particular, what are the factors that often enable sexual populations to outcompete or prevent invasion by asexual strategies?

In animals, asexual lineages always arise from sexual ancestors (Butlin, 2002) but the process of transition from sexual to asexual reproduction and the short-term consequences of such transitions are not well understood, especially across natural populations. A common approach is to compare costs and benefits of sex and parthenogenesis between closely related sexual and asexual species (Bast et. al., 2018; Kimmerer 1994; Tarkhnishvili et. al., 2010). Consequently, most knowledge of shifts to asexuality comes from studying ancient transitions (e.g., Loewe & Lamatsch, 2008; Schlupp, 2010; Fontaneto, 2012; Larose, Parker & Schwander, 2018). While such an approach has yielded important insights into how asexual lineages may have persisted following ancient transitions to parthenogenesis, it

cannot explain how such transitions are likely to proceed. Comparing related species also introduces potential confounding factors since genuine consequences of reproducing asexually cannot be differentiated from genetic changes associated with speciation. There is therefore little certainty that differences between sexual and asexual species are due to differences in reproductive mode.

Facultatively parthenogenetic species, in which every female can engage in sexual or asexual reproduction depending on whether they mate (Normark, 2003), provide an opportunity to disentangle species-specific factors from direct consequences of asexuality. Facultatively parthenogenic animals can reap the benefits of both modes of reproduction while avoiding many of the costs (D'Souza & Michiels, 2010). Facultative parthenogenesis can also be an intermediate stage between obligate sexuality and obligate asexuality (Schwander et. al., 2010), and so studying the genetic and phenotypic differences between sexually and asexually reproducing populations within the same species of a facultative parthenogen could help reveal factors that promote or impede transitions to obligate asexuality and the long-term persistence or extinction of asexual populations. Many facultatively parthenogenetic animals exhibit a geographic pattern where some populations reproduce sexually while others reproduce asexually. This phenomenon, known as geographic parthenogenesis (GP), has been reported in several taxa, including Opiliones (Burns, Hedin & Tsurusaki, 2018), mayflies (Tojo, Sekiné & Matsumoto, 2006), phasmids (Law & Crespi, 2002; Morgan-Richards, Trewick & Stringer, 2010), and others (reviewed in Glesener & Tilman, 1978). The geographic patterns found in GP animals provide an opportunity to investigate the immediate effects that follow the establishment of asexual populations, a process that can involve unmated females colonizing new areas, or males becoming locally extinct. The short-term genetic and phenotypic effects of such transitions could determine whether asexual populations persist or suffer rapid extinction.

The Australian Peppermint Stick Insect, *Megacrania batesii*, is a facultatively parthenogenic species endemic to the wet tropics of northern Queensland, Australia (Cermak & Hasenpusch, 2000), as well as the Solomon Islands and other Pacific islands (Van Herwaarden, 1998). When *M. batesii* females mate, they create a mixed-sex brood of sexually produced offspring. However, females can also reproduce via thelytoky, a process by which males are not required and females that avoid mating produce all-female broods

from unfertilized eggs. While some phasmids can produce male offspring from unfertilized eggs (Pijnacker & Ferwerda, 1980; Scali, 2009; Brock et. al., 2018), it is currently unknown how common parthenogenetically produced males are in this species. Cermak & Hasenpusch (2000) reported the existence of two isolated all-female populations at the southern end of the range of *M. batesii* in Australia, but pilot surveys (conducted by RB and NWB) identified a geographic mosaic of populations with contrasting sex ratios throughout the species range. Females from all-female locations do not show obvious morphological differentiation from females found in mixed-sex locations and experimental population crosses in our laboratory (to be reported elsewhere) showed that these populations are inter-fertile. Since sexual reproduction is believed to be the ancestral reproductive mode in phasmids (Schwander & Crespi, 2009), we assume that all-female populations of this species are descended from mixed-sex, sexual populations. We therefore took advantage of this natural system to investigate the consequences of recent and ongoing local transitions from sexual reproduction to parthenogenesis.

Our first aim was to describe the geographic variation in sex ratio and reproductive mode in wild populations of *M. batesii*. Our second aim was to use these populations to investigate the consequences of transitions from sexual to asexual reproduction. In particular, we wanted to determine whether incipient transitions to asexuality (i.e., parthenogenetic reproduction) in the wild are associated with reduced genetic diversity, heterozygosity, and phenotypic performance, as predicted by theory. *Megacrania batesii* occurs in two types of habitat: rainforest along beach margins (where it feeds on *Pandanus* sp.), and swamp within closed-canopy rainforest (where it feeds on *Benstonea* sp.). We therefore also asked whether phenotypic and genotypic parameters are affected by habitat type. Because we are interested in how reproductive mode transitions affect functionality in natural environments, we report phenotypic data collected from individuals observed in natural populations, rather than from their lab-reared descendants.

Methods

Study locations

We investigated a spatial mosaic of populations with either mixed sex or all-female sex ratios along ~ 33km of coastline between Forest Creek and Emmagen Creek. We also surveyed two isolated all-female locations (reported previously by Cermak & Hasenpusch, 2000) located ~ 160-180 km south of this area at Etty Bay and Bingil Bay (see Fig. 4a). *M. batesii* were observed and sampled from habitat along Cape Tribulation Road and Kimberley Road, in Forest Creek Village, Cow Bay Village, Cape Tribulation Village and adjacent beaches, at Emmagen Creek, and at Etty Bay and Bingil Bay, in Queensland, Australia.

Population sex ratio and phenotyping

A total of 1,324 wild *M. batesii* individuals were photographed on their host plants at 25 locations in 2019 (N = 258), 2020 (N = 283), 2021 (N = 259), and 2022 (N = 524) to quantify sex ratio and collect phenotypic data. At each location, we scanned host plants (*Pandanus* sp. or *Benstonea* sp.) and recorded the presence, developmental status (nymph or adult), sex of adults and some nymphs that were developed enough to distinguish sex, and pairing status (single female, single male or male-female pair) of each *M. batesii* individual seen. Given the very effective crypsis of *M. batesii*, it is likely that we failed to spot some individuals at each location, but our surveys should nonetheless represent differences between locations in the relative abundance, sex ratio and phenotype of *M. batesii* individuals. The *M. batesii* sites surveyed were classified as “all-female” if only females were seen, and “mixed-sex” if both sexes were seen (see Fig. 3a). Localities with numerous individuals could be differentiated as “all-female” or “mixed-sex” because males, when present, are easy to distinguish from females by their body shape and wing length (see Fig. 1). In mixed-sex sites, many adult females are guarded by males (Boldbaatar et al. 2022). One site where a few females (but no males) were found was designated a “putative all-female” location (B1). One site (NN) was consistently all-female for 2019-2021 but, in 2022, males were found at that site. This site is hereafter referred to as a “transitional” site and was excluded from phenotypic analyses comparing sexual and asexual locations.

Each adult and nymph seen in the field was imaged in situ on its host plant using a Sony RX10-IV camera. When possible, a transparent plastic scale attached to a thin metal rod was held next to the adult insects so that body size measurements could be obtained from the image (Fig. S1). In most cases, this procedure did not appear to disturb the insects since no movement or defensive reaction (retreating, jumping off the leaf, or spraying of defensive fluid) was observed. From the images, ImageJ (Schneider, Rasband & Eliceiri, 2012) was used to measure the length of the pro-thoractic notum (protonum) and scale. Pronotum length was then calculated in cm as a measure of body size. We measured pronotum length because this part of the body was consistently visible in the photos, including photos of females being guarded by males. The photos were also used to record the number of missing legs and antennae. Leg and antennae loss in arthropods is an indication of poor condition that could result from predator attack or problems with moulting (Maruzzo et. al., 2005). For each individual, we also recorded the presence of black spots indicative of fungal infection, the presence of ectoparasites (mites or biting midges), and the presence and severity of wing deformities. Normal wings are held flat over the dorsal thorax of the insect, with one wing overlapping the other. Mild wing deformities were defined as normally shaped wings held apart (not overlapping), whereas severe wing deformities were defined as wings that were widely separated, partly or entirely missing, or highly abnormal in shape.

In 2019 and 2020, we collected 1,418 eggs from several wild-collected *M. batesii* females and sexed 719 hatchlings from those eggs to verify that females at the all-female locations were reproducing asexually while females at the mixed-sex locations were reproducing sexually. Wild-collected females were kept in mesh cages in for 7-12 days and fed on *Benstonea* sp. or *Pandanus* sp. leaves, and then released at the site of capture. Females that were mating or guarded by males when collected were housed together with the male. Eggs laid by wild-collected females were counted and then kept in containers with moist cocopeat at ~27°C until hatching in controlled-temperature rooms at The University of New South Wales, Sydney. Fecundity was quantified for each wild-collected female as the number of eggs laid per day, and egg viability was quantified as the hatching rate of these eggs. Hatchlings were sexed based on the morphology of the 8th and 9th abdominal sternites (Fig. S2), and hatchling sex ratio was used to assess reproductive mode in the natural source

210 locations. Fertilized eggs hatch into both males and females whereas unfertilized eggs
211 typically hatch into females only. Thus, when only female hatchlings were obtained from
212 eggs laid by wild-collected females, we inferred that that the females at that location
213 reproduced only via parthenogenesis; whereas when male hatchlings were obtained, we
214 inferred that sexual reproduction had taken place. Based on data from laboratory crosses
215 (not shown), spontaneous production of males from unfertilized eggs is impossible or
216 extremely rare (< 1/1000 eggs) in *M. batesii* (RB, unpublished data).

217

218 **DNA sampling, extraction and purification**

219 In 2020 and 2021, mid-legs were collected from wild nymphs or adults for DNA
220 sequencing by pinching the leg with sterile tweezers, causing the insect to autotomize the
221 pinched leg. A few additional samples were obtained from adults that died in cages during
222 egg collection, or from whole first-instar nymphs collected in the field. Samples were stored
223 in pure ethanol at 4°C or without ethanol at -80°C until DNA extraction.

224 Isolation of DNA was conducted using the Gentra Puregene Tissue Kit (4g) from
225 Qiagen according to the manufacturer's protocol, "DNA Purification from Mouse Tail Tissue
226 Using the Gentra Puregene Mouse Tail Kit," with slight modifications to suit the study
227 species (www.qiagen.com). Specifically, prior to adding 1.5 µl Puregene Proteinase K to the
228 lysate, the samples were subsampled and placed in 2ml free standing screw-capped tubes.
229 Then, 3 x 5mm glass beads and ~5-10 x 1mm silicon beads were placed in the tubes with the
230 samples. The tubes were placed in a FastPrep-24 (MP Biomedical) homogenizer and run on
231 a 2 x 25-second cycle to break down the sample exoskeleton. Following homogenization, 1.5
232 µl Puregene Proteinase K was added to the lysate and the rest of the manufacturer's
233 protocol was followed.

234 Isolates from a total of 188 *M. batesii* individuals were sent to Diversity Arrays
235 Technology Inc. (Canberra, Australia) for whole-genome reduced representation genotyping
236 using the DArT-seq protocol. Genome complexity reduction was performed using a
237 restriction enzyme double digest of PstI and HpaII. Next-generation sequencing of
238 amplification fragments was conducted on the Illumina Hiseq 2500 (www.illumina.com),

239 producing 1,384,779 single-end reads of raw data across all 188 samples. SNPs were then
240 called using the DArTsoft analytical pipeline (Kilian et. al., 2012).

241

242 **Quality control filtering**

243 The DArTsoft pipeline was used to calculate quality parameters, including call rate,
244 reproducibility, and polymorphic information content (PIC). The initial data set provided by
245 DArTseq consisted of 12,977 SNPs across the 188 samples. Due to a high percentage of
246 missing data (>30%) and possible mislabeling, 33 samples were omitted from this analysis
247 (see supplementary file "omitted_samples.xls"). A total of 12,518 polymorphic DArT
248 markers were generated for the retained 155 samples. Before quality control filtering, the
249 mean repeatability was 0.987 and over 50% of SNPs had reproducibility over 99% (see Fig.
250 S4). The mean minor allele frequency by locus was 0.18 but varied across locations with
251 asexual sites having higher levels of minor alleles compared to sexual sites. The mean call
252 rate was 0.95 and 95% of SNPs had a call rate above 75%. The PIC value of SNP markers
253 ranged from 0.0 to 0.5 with a mean of 0.239 and median of 0.228.

254 DArTSeq™ sequences were filtered using dartR (Gruber et. al., 2018). Various filter
255 parameters were tested to determine suitable filters and SNP sequences matching the
256 following parameters were removed from the dataset: average reproducibility < 95%, call
257 rate < 75%, minor allele frequency (MAF) < 0.05, read depth < 3, and Hamming distance >
258 0.80. Remaining SNPs were assessed for linkage disequilibrium and all SNPs with a hamming
259 distance less than 0.2 were excluded from further analysis. Quality control filtering removed
260 5,603 SNPs or 43.17% of SNPs (see Table S5). After quality control, 7,374 high quality SNPs
261 were identified for further analysis.

262

263 **Phenotypic analysis**

264 All phenotypic analyses were conducted in R, v. 4.0.2 (R Core Team, 2020). The
265 package "MCMCglmm" was used to model variation in phenotypic traits using a mixed
266 model approach (Hadfield, 2010). For each response variable, reproductive mode (assumed
267 to be sexual for mixed-sex sites and asexual for all-female sites), and habitat type

(*Benstonea* swamp or *Pandanus* beach) were modeled as fixed effects, and none were transformed. All models also included year, and a matrix representing genetic structure, as random effects.

Each phenotypic response variable was modeled separately with different specifications depending on deviance information criterion (DIC) (see Table S7). Sample sizes differed for different phenotypic variables (for all summary stats see Table S2). Pronotum length was analysed with a Gaussian model. Presence of fungal infections and ectoparasites were combined into one response variable ("parasites") showing the presence/absence of fungal infections or ectoparasites and analysed with a categorical (binary) model. The numbers of missing legs and antennae were added together and this sum was analysed as a single response variable. The variable was analysed with a Poisson model because there were no instances of a stick insect with all appendages missing, thus there was no upper bound on the variable. Presence/absence of mild and severe wing deformities were analysed with a categorical (binary) model. Hatching success of eggs collected from wild females was also analysed as a binary matrix of successes and failures. Fecundity (number of eggs laid) was analysed using a Poisson model.

Host plant type is strongly correlated with habitat type, with *Pandanus* sp. host plants typically growing along beach margins, and *Benstonea* sp. host plants growing along margins of streams and swamps in closed-canopy rainforest. We therefore characterized habitat type as "*Pandanus*-beach" or "*Benstonea*-swamp". All models initially included the interaction between reproductive mode and habitat, but this interaction was dropped if its inclusion increased DIC. However, the main effects of reproductive mode and habitat type were retained in all models (see Table S1). For each phenotypic variable, the matrix representing population-level phylogenetic information (i.e., genetic structure) was used to calculate the broad sense phylogenetic heritability (H^2) and assess the amount of phylogenetic signal. Phylogenetic information used in the models was collected using the *gl.dist.pop()* function in the R package "dartR" and inverse phylogenetic covariance matrices were created using the function *inverseA().* Phylogenetic information was retained in all models.

Figure 1: *Megacrania batesii* individuals and pairs: (A) adult female from an all-female site; (B) adult female from a mixed-sex site; (C) adult male; (D) female guarded by male.

Population structure and genetic diversity

We investigated the genetic relationships between locations by calculating a dissimilarity matrix using the *bitwise.dist()* function in the R package "poppr" (Kamvar, Tabima, & Gřunwald, 2014) based on Hamming distances (see Wang et. al., 2015). This matrix was then used with *pvclust()* to create a dendrogram based on correlation distance between individuals and 10,000 bootstrap iterations were done to determine the level of support for each node (Suzuki & Shimodaira, 2006). Several clustering methods were compared (see supp materials "testing_clustering_methods.html"), all methods yielding similar genetic structure. Pairwise F_{st} values were calculated using the function *stamppFst()* with 10,000 bootstrap iterations used to obtain confidence limits for each pairwise difference (Pembleton, Cogan, & Forster, 2013). Non-Metric Multidimensional Scaling

325 (NMDS) using *metaMDS()* was then used to visualize the structure among samples as a
326 complement to the cluster analysis (Dixon, 2003). Observed and expected heterozygosities
327 as well as F_{is} of each locality was obtained by *gl.get.diversity()* (see Table S6). Sites with less
328 than two sampled individuals were excluded from these analyses.

329 Gene flow between sites was estimated using discriminant analysis of principal
330 components (DAPC). DAPC uses K-means and model selection to determine genetic clusters
331 in lieu of known populations or genetic groups (Jombart, Devillard & Balloux, 2010).
332 Seventeen optimal genetic clusters were identified using the Bayesian Information Criterion
333 (see Fig. S3) and DAPC cross validation using the function *xvalDapc()*. Six discriminant
334 analysis axes were retained based on the α score. Membership probabilities were estimated
335 for each individual for each identified group and *ggplot()* was then used to visualize the
336 admixture proportions of each individual's genotype.

337 Allelic diversity, Shannon's diversity, and heterozygosity were quantified using the
338 function *gl.report.diversity()* as described by Sherwin et. al. (2017), and these values were
339 compared between sites (see Fig. S5). Observed heterozygosity (Ho) values were calculated
340 using the function *gl.report.heterozygosity()* and used to visualize heterozygosity of each
341 sample (see Fig. 5c). Isolation by distance was determined using the function *gl.ibd().* All
342 functions used are from the package "dartR" (Gruber et al., 2018). The data that support
343 the findings of this study are openly available in dryad at http://doi.org/[...], reference
344 number […].

345 **Results**

346 **Sex ratio and reproductive mode**

347 Sex ratio was bimodally distributed, with either only females or an approximately
348 equal number of females and males observed at nearly all locations (see Table 2). With one
349 exception (see below), we did not find any locations where adult males were present but
350 rare (and the absence of such locations is supported by population genomic analysis,
351 described below). In several cases, all-female and mixed-sex sites were found to occur in
352 close proximity, and with no obvious barriers to dispersal. For example, mixed-sex locations
353 B4 and VR are separated by < 2km of contiguous rainforest from all-female locations B1 and
354 CB, and the all-female location NS is separated by < 1km of rainforest from adjacent mixed-

355 sex locations (See Fig. 4). Despite the close proximity of males to some all-female sites, sex
356 ratios were consistent over four years of surveys at nearly all locations (see Supplementary
357 file "sex_ratio_by_year.csv").

358

359 Table 2: Sample sizes and sex of *M. batesii* adults and nymphs that could be sexed found in
360 the field at each of the surveyed locations (summed over the four years of the study). At
361 sites where we only found females, a binomial two-tailed test was used to determine the
362 probability that it is a mixed-sex location (even sex ratio) based on the number of individuals
363 seen. At sites where males and females have been seen, the location is mixed-sex by
364 definition so we used the binomial test to calculate the probability that the sex ratio of
365 individuals photographed at each location deviated significantly from 50%. At the
366 "transitional" location (NN), only females were seen in 2019-2021 but males were found in
367 2022.

Location	Site type	No. males	No. females	Total	Sex Ratio	p-value
EC	All-female	0	27	27	1	< 0.001
MS	Mixed-sex	85	94	179	0.53	0.10
MK	Mixed-sex	69	58	127	0.46	0.09
MB	Mixed-sex	24	34	58	0.59	0.09
CO	Mixed-sex	105	116	221	0.52	0.08
RP	Mixed-sex	20	37	57	0.65	0.02
BK	Mixed-sex	21	36	57	0.63	0.03
NW	Mixed-sex	21	33	54	0.61	0.06
NN	Transitional	4	30	34	0.88	< 0.001
NS	All-female	0	65	65	1.00	< 0.001
TB	All-female	1	46	47	0.98	< 0.001
TS	All-female	0	12	12	1.00	< 0.001
B1	Putative all-female	0	2	2	1.00	0.50
B4	Mixed-sex	2	2	4	0.50	0.75
CB	All-female	0	155	155	1.00	< 0.001
CS	All-female	0	68	68	1.00	< 0.001
VR	Mixed-sex	13	15	28	0.54	0.28
VS	Mixed-sex	6	10	16	0.63	0.24
MA	All-female	0	16	16	1.00	< 0.001
KB	All-female	0	31	31	1.00	< 0.001
KR	All-female	0	24	24	1.00	< 0.001
FC	All-female	0	7	7	1.00	< 0.001
EB	All-female	0	27	27	1.00	< 0.001
BL	All-female	0	18	18	1.00	< 0.001

368

369 Eggs collected from most all-female locations produced 100% female hatchlings
370 whereas eggs collected from mixed-sex locations produced approximately equal numbers of
371 female and male hatchlings (see Table S4). We observed only two exceptions to this pattern.
372 A sample of eggs from the all-female site TB produced one hatchling that was
373 phenotypically male-like, and genotyping showed that this hatchling was not a hybrid
374 between locations. This hatchling was therefore either an extremely rare spontaneous male
375 (see Discussion) or a phenotypically atypical female. In addition, at one location (NN) where
376 only females had been observed before 2022, eggs collected from one female in 2021
377 produced several male hatchlings, and genotyping indicated that this female was either a
378 migrant from the adjacent, genetically similar mixed-sex location BK or that this female had
379 mated with a male migrant from location BK. The following year, adult males were observed
380 at NN and this location therefore appears to be undergoing a transition from all females to
381 mixed-sex.

382

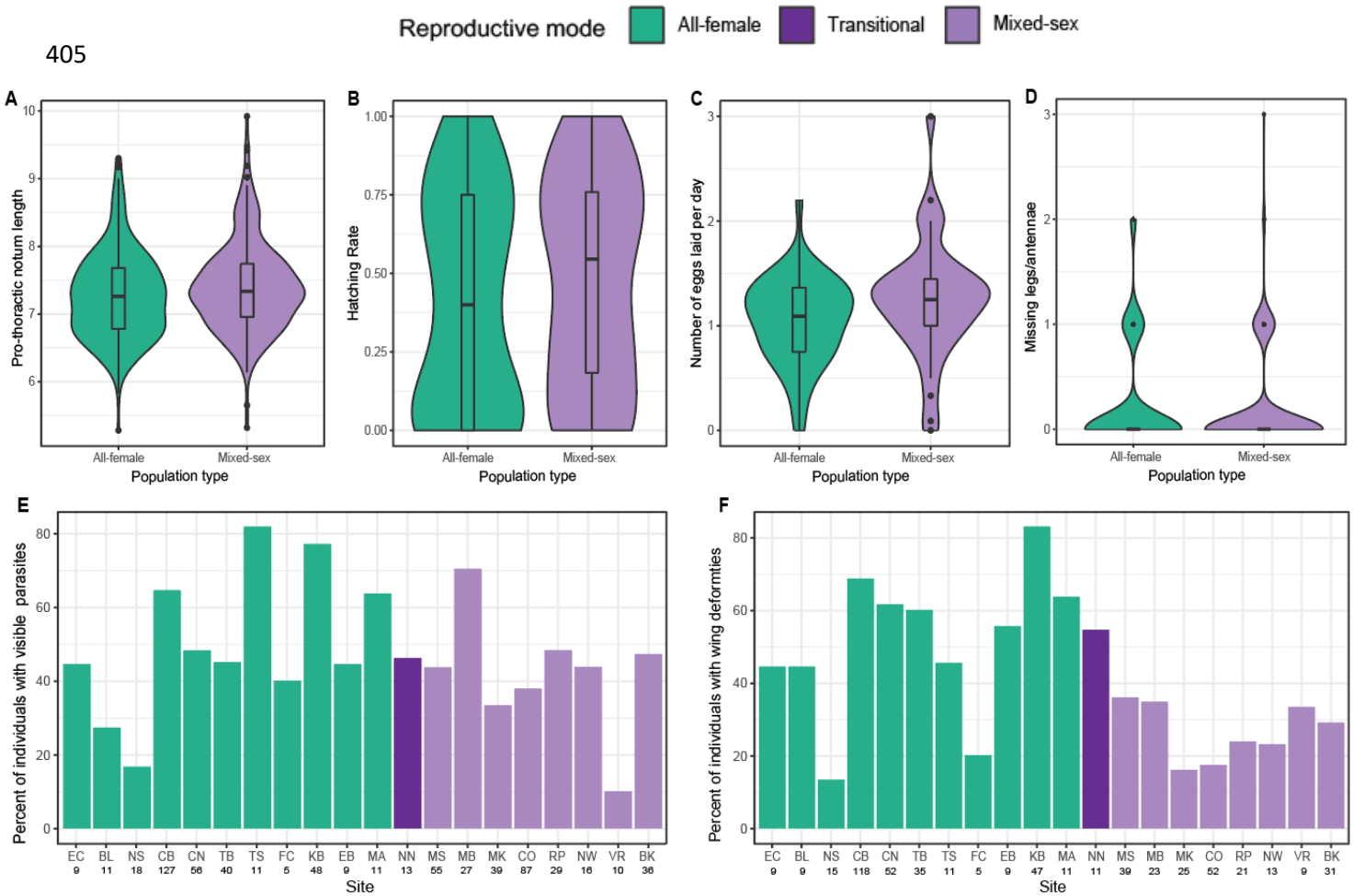
383 **Morphology, fecundity, and egg hatching success**

384 We did not find clear and consistent evidence of an effect of reproductive mode
385 across the fitness-related phenotypic traits measured (see Fig. 2b and 2c; for all summary
386 statistics see Table S2). Mean fecundity and egg hatching success were ~12 and 19% lower,
387 respectively, at all-female than at mixed-sex locations, but there was a great deal of
388 variation in these variables among both mixed-sex and all-female locations (Fig. S7). Adult
389 females at all-female locations were more likely to exhibit mild (pMCMC < 0.001) and severe
390 (pMCMC < 0.005) deformities in their non-functional wings than were females at mixed-sex
391 locations (see Fig. S6). Relative to females at mixed-sex locations, adult females at all-female
392 locations had more parasites in *Benstonea*-swamp habitats, but not in *Pandanus*-beach
393 habitats (pMCMC < 0.005; see Fig. 3). Overall, mean rates of parasitism and wing
394 deformities were ~19% and 91% higher, respectively, in females from all-female locations.
395 Additionally, mean numbers of missing legs and antennae were ~40% higher in adult
396 females at all-female locations, but there was no support for an overall difference in this
397 variable between mixed-sex and all-female locations (pMCMC = 0.34). Estimated

398 phylogenetic heritability (H^2) values suggested effects of genotype for fecundity ($H^2 = 0.74$),
399 the presence of deformities ($H^2 = 0.64$), the presence of parasites ($H^2 = 0.64$), and the
400 hatching success of eggs collected from females at several locations ($H^2 = 0.87$). There was
401 moderate support for an effect of genotype on missing antennae and legs ($H^2 = 0.41$) but
402 little support for effects of genotype for mild ($H^2 = 0$) or severe ($H^2 = 0$) wing deformities, or
403 for pronotum length ($H^2 = 0.12$).

404

405



417 Figure 2: Phenotypic values for *M. batesii* females at mixed-sex and all-female locations: A)
418 Pronotum length as a measure of body size; B) Hatching rate of eggs; C) Number of eggs laid
419 per day as a measure of fecundity; D) Number of missing legs and antennae per individual;
420 E) Percent of adult females observed at each location with visible parasites (fungus, biting
421 midges, or mites); F) Percent of adult females from each location with wing deformities
422 (mild or severe). In panels A – D, means (bars), inter-quartile ranges (boxes) and non-
423 outliner ranges (whiskers) are shown. Violins indicate the distribution of individual values.

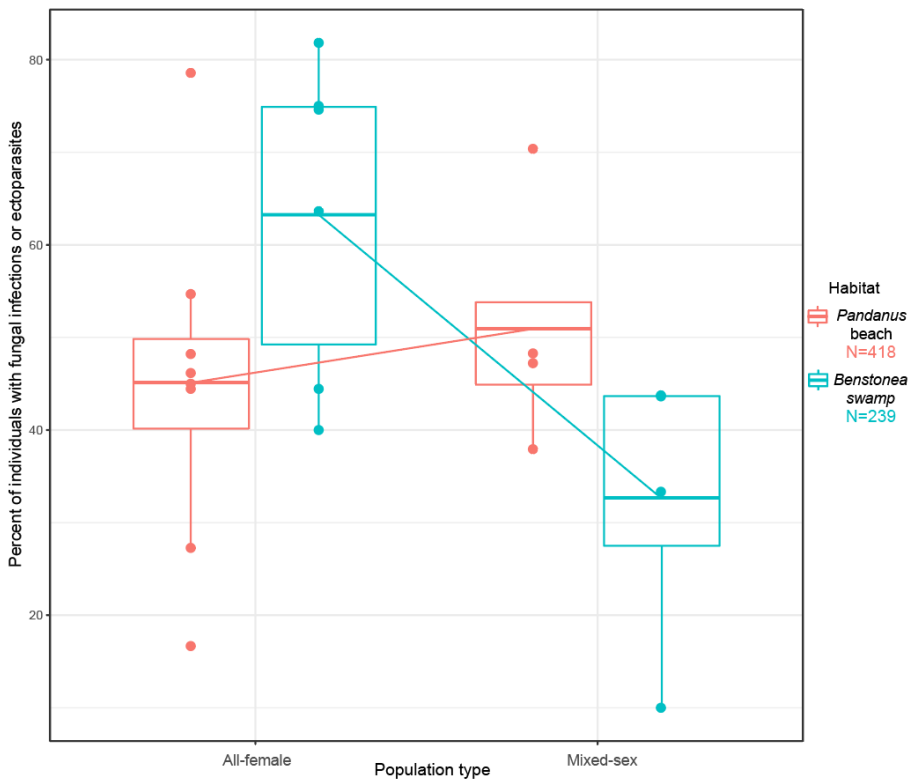


Figure 3: Effects of habitat type and reproductive mode on the rate of visible parasites (fungus, mites or biting midges) in *M. batesii* females at all-female and mixed-sex locations. Each point represents a location mean. Bars indicate means across all populations in each group, boxes represent inter-quartile ranges and whiskers show non-outliner ranges.

Population structure

Sampling sites of *M. batesii* clustered by geographical location rather than by sex ratio. Two major genetic clusters were observed, a "northern" cluster north of the Noah Creek mouth and a "southern" cluster south of the Noah Creek mouth. The Noah Creeks mouth (~ 150 m wide at its widest point) therefore appears to serve as a major barrier to *M. batesii* dispersal and gene-flow. The northern cluster consists mostly of mixed-sex sites while the southern cluster consists mostly of all-female sites, but both genetic clusters contain both all-female and mixed-sex sites (see Fig. 5). Pairwise F_{st} values reflect the same genetic structure as the cluster analysis, with sites separated by greater geographic distances showing less relatedness (see Table S3). Isolation by distance analysis indicated that genetic differentiation is affected by geographic proximity (Mantel statistic based on Pearson's product-moment correlation, mantel statistic $r = 0.364$, $p < 0.005$). F_{st} values

between the sites varied between 0.16 and 0.96 with lower values between mixed-sex sites in the northern cluster and higher values between all-female sites in the southern cluster.

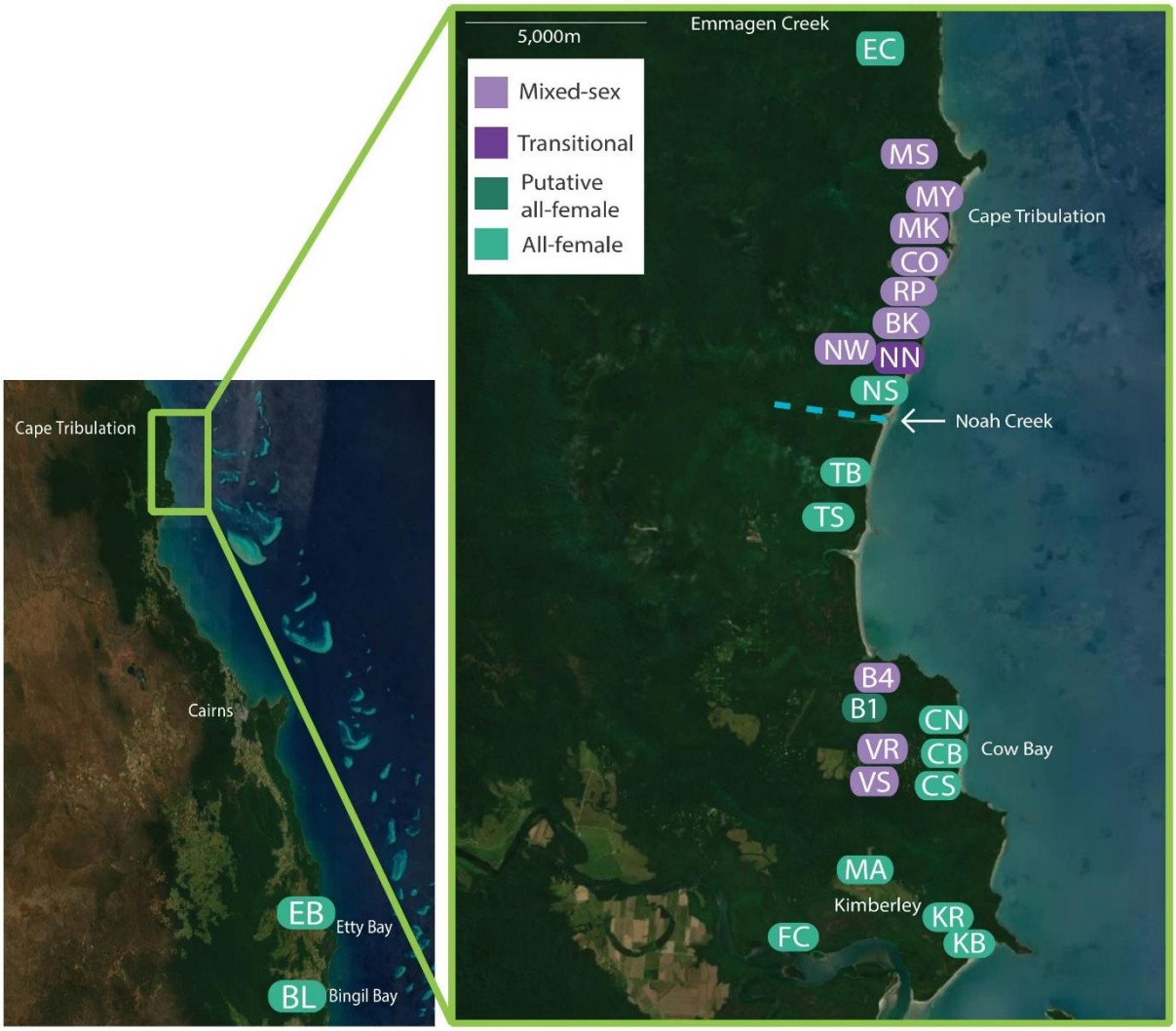


Figure 4: Geographical variation in sex ratio and reproductive mode in *M. batesii*. Two isolated all-female sites occur at the southern limit of the known range of *M. batesii* in Australia, and a spatial mosaic of all-female and mixed-sex sites occurs ~160-180km north of this area between Forest Creek and Emmagen Creek. The blue dashed line represents the mouth of the Noah Creek, which is the main gene flow barrier separating the northern and southern genetic clusters. Locations are characterised as “mixed-sex”, “putative all-female” or “all-female” based on the sex of adults and nymphs photographed at each location (See Table 2). The “transitional” location was all-female in 2019-2021, but males were found at this location in 2022.

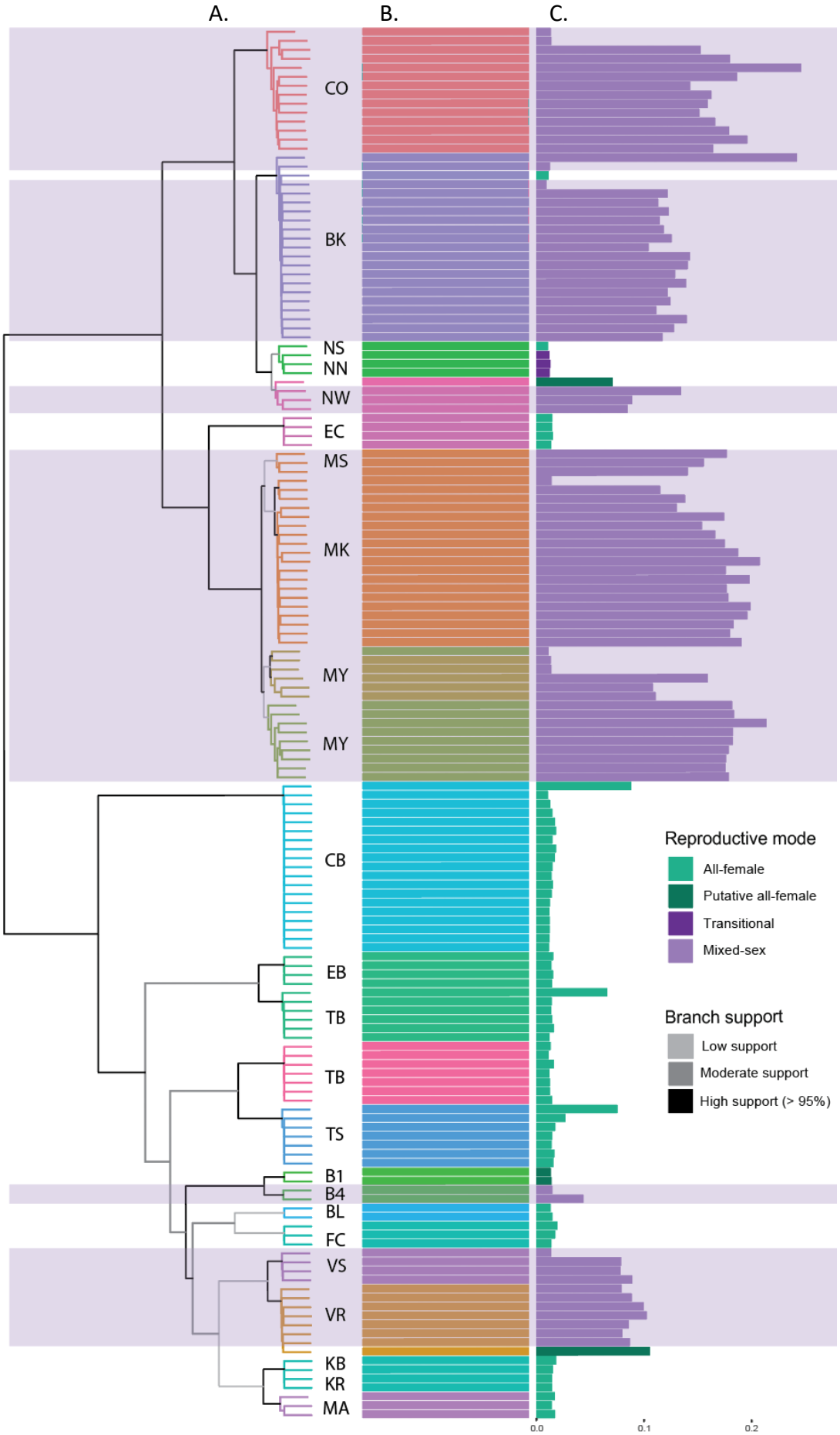

A.
B.
C.
CO
BK
NS
NN
NW
EC
MS
MK
MY
MY
CB
EB
TB
TB
TS
B1
B4
BL
FC
VS
VR
KB
KR
MA
0.0
0.1
0.2
Reproductive mode
All-female
Putative all-female
Transitional
Mixed-sex
Branch support
Low support
Moderate support
High support (> 95%)

Figure 5: Population structure and heterozygosity of *M. batesii* sites based on 7374 high-quality SNPs from 154 individuals sampled from the field. A) Dendrogram showing relatedness of sampled individuals, with branches colored according to the level of bootstrapped support (based on 10,000 bootstrap iterations) and tips colored to match their highest membership probability. B) Admixture plot showing percent membership in the 17 genetic populations supported by the analysis (Fig. S3); C) Observed heterozygosity values. In plots A and B, different colors represent the primary assigned cluster as indicated by admixture analysis. In plot C, colors represent the observed sex ratio and inferred reproductive mode for each location. Areas shaded in purple show mixed-sex sites.

Admixture analysis revealed that the 155 individuals sampled across the study area belong to 17 genetic populations, with little gene flow detected between sites. Most individuals had 100% membership probability in a single population (Fig. 5b). However, one all-female location (TB) contained multiple genotypes within the one location. Two sets of locations found near one another (< 1000 m) had the same genotype but differing reproductive modes and heterozygosity levels (B1-B4 and NN-NS-NW).

NMDS analysis (Fig. 6) produced a pattern consistent with the population structure obtained from genetic cluster analysis, with the biggest separation found between sites north vs. south of the Noah Creek mouth.

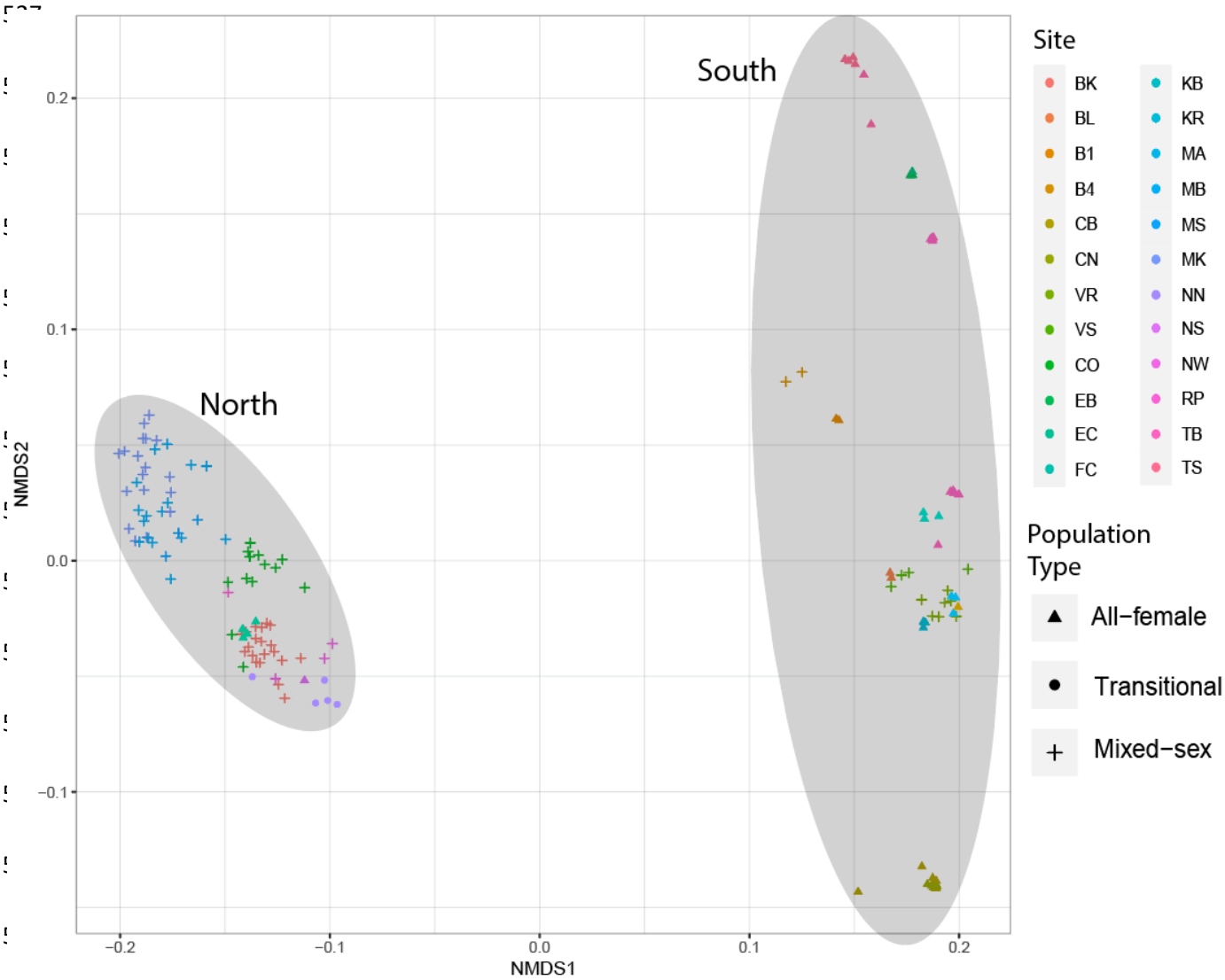


Figure 6: Nonmetric Multidimensional scaling (NMDS) plot of 155 individual *M. batesii* samples based on 7374 high-quality SNPs. Shaded ovals represent individuals found in locations relative to the Noah Creek gene flow barrier.

Genetic diversity and heterozygosity

Relative to mixed-sex locations, all-female sites had less allelic diversity and Shannon's diversity, and individuals from all-female sites typically had much lower heterozygosity. All three diversity metrics showed similar patterns across locations (See Fig. S7). There was also substantial variation in genetic diversity between mixed-sex locations: for example, allelic richness was more than 50% higher at CO than at B4. A few individuals sampled from mixed-sex sites had heterozygosity levels comparable to individuals from all-

552 female sites, suggesting that these individuals were produced asexually (see Fig. 5c). On
553 average, heterozygosity of individuals from mixed-sex (sexual) sites was approximately
554 seven times greater than heterozygosity of individuals from all-female (asexual) sites ($p <$
555 0.0001: Welsh's two sample t-test).

556

557 **Discussion**

558 The facultative nature of asexual reproduction in *M. batesii* created a spatial pattern
559 of variation in reproductive modes in their natural range, and this pattern has remained
560 relatively stable over four years of sampling. We took advantage of this system to
561 investigate apparently recent shifts from sexual to asexual reproduction and their
562 subsequent genetic and phenotypic consequences. At one location, we also observed a
563 recent re-invasion of males into a previously all-female site. We found genetic relatedness
564 and admixture indicative of multiple independent transitions from sexual reproduction to
565 parthenogenesis. The establishment of female-only locations led to the loss of allelic
566 diversity and a 7-fold reduction in observed heterozygosity, but the phenotypic and fitness
567 consequences of these genetic changes appear to be highly variable among sites. All-female
568 sites had higher rates of mild and severe deformities in their non-functional wings. These
569 sites also had higher rates of fungal and arthropod parasites, but only in *Benstonea*-swamp
570 habitats where parasites may be generally more abundant. However, we found little
571 statistical support for an overall difference in mean body size, fecundity or egg hatching
572 success between all-female and mixed-sex sites. All of the phenotypic traits that we
573 examined varied substantially among both all-female and mixed-sex sites, suggesting that
574 genotype might be more important than mode of reproduction in determining these traits
575 in *M. batesii*.

576 In this study, we describe a geographic pattern of mixed-sex (sexual) and all-female
577 (asexual) sites in the natural range of *M. batesii* in northern Queensland. This spatial mosaic
578 of all-female and mixed-sex sites has not been previously described in *M. batesii*, although
579 Cermak and Hasenpusch (2000) reported isolated all-female populations at the southern
580 end of the known range of this species in Australia. Cluster analyses showed that sites
581 within this range grouped together based on geographic location, regardless of reproductive

582 mode, and that asexual sites did not form a single monophyletic clade. We can infer at least
583 three independent transitions from sexual to parthenogenetic reproduction, resulting in all-
584 female sites (EC, NBS, B1) that are very close spatially and genetically to adjacent mixed-sex
585 sites. Additionally, admixture analysis showed little to no gene flow between the locations
586 despite their geographical proximity (see Fig. 5b). Although we cannot conclusively reject
587 the possibility that spontaneous male generation from unfertilized eggs resulted in the
588 sexual locations south of Noah Creek, these phylogenetic patterns are most consistent with
589 multiple independent origins of asexual populations of *M. batesii* from ancestral sexual
590 populations. It is not known how long ago the asexual sites were established, because no
591 historic samples or previous literature is available that could be used to quantify time of
592 establishment. However, the close geographical proximity and genetic relatedness of some
593 sexual and asexual sites suggest recent establishment. For example, sites B1 and B4 as well
594 as NW and NS exhibit differing reproductive modes, but they are still considered to be from
595 the same population based on admixture analysis. Additionally, results of inter-population
596 crosses (to be presented in a separate paper) show that individuals from sexual and asexual
597 sites are interfertile and can therefore be regarded as a single species.

598 We identified two main genetic groups, separated by a large estuary (Noah Creek)
599 that serves as a gene flow barrier. The northern group consists of mainly sexual sites and the
600 southern group consists of mainly asexual sites. This appears to reflect the same geographic
601 pattern found in other geographically parthenogenetic phasmids in the southern
602 hemisphere (Buckley, Marske & Attanayake, 2009; Morgan-Richards, Trewick & Stringer,
603 2010) and the opposite pattern as geographically parthenogenic phasmids in the northern
604 hemisphere (Law & Crespi, 2002). However, the northernmost site that we sampled in this
605 study (EC) is all-female. It therefore remains unclear whether *M. batesii* exhibits a latitudinal
606 gradient like other geographically parthenogenic phasmids, a single sexual area surrounded
607 by asexual populations, or a mosaic of sexual and asexual populations across the range.
608 Asexually reproducing populations of species that display geographical parthenogenesis are
609 often found in marginal habitats, typically at a higher altitude or latitude than sexually
610 reproducing populations (Lynch, 1984; Kearney, 2005; Tilquin & Kokko, 2016). However, the
611 locations examined in our study are found in similar tropical coastal-lowland rainforest
612 habitats spanning a small latitudinal range (0.24°; 1.8° including the isolated southern

populations), with little variation in temperature, humidity, or precipitation between locations. Additionally, sexual and asexual sites were both found in each of the two major habitat types (*Pandanus*-beach and *Benstonea*-swamp), so climate or habitat differences do not appear to explain the variation in reproductive mode.

The lack of obvious environmental barriers or differences in habitat between the sexual and asexual sites suggests that this spatial pattern of variation in reproductive mode results from factors intrinsic to the biology of *M. batesii*. Four intrinsic processes might have contributed to the observed spatial pattern: dispersal and colonization of new habitats by unmated females, male invasion of established all-female populations, spontaneous male genesis from unfertilized eggs, and local losses of sex through male extinction.

First, if females disperse more than males, they could travel further from the mixed-sex sites and establish all-female sites that could avoid male invasion for many generations. All-female sites could persist once established if parthenogenetically produced females exhibit reduced propensity of females to mate, and reduced benefits of mating, as reported for *Extatosoma tiaratum* (Burke & Bonduriansky, 2022). However, this explanation seems implausible because *M. batesii* adults of both sexes of this species disperse similar distances (Boldbaatar, 2022).

Second, if sexual sites south of the gene-flow barrier (VR, VS, B4) arose as a result of males from the north invading southern all-female sites, then these sites would show a genetic admixture pattern consistent with hybrid origin. We found no such pattern. Rather, all the southern sites clustered together, and none showed evidence of genetic admixture from northern sites. However, we observed an apparent on-going transition from all-female to mixed-sex at a northern site (NN), showing that male invasion of all-female sites is possible and might be relatively frequent at locations where all-female sites arise in close proximity to mixed-sex sites.

Third, sex may re-invade after it is lost through the spontaneous production of males from unfertilized eggs. One possibility from our results is that the sexual sites in the south arose via such spontaneous male genesis. Spontaneous male genesis is possible in diploid species with XX XO sexual karyotypes; due to a meiotic/developmental error, the second X chromosome can be lost in situ in parthenogenically produced offspring, resulting in a

genetic male (Pijnacker & Ferwerda, 1980; Scali, 2009). The viability and reproductive success of spontaneous males has not been studied in any phasmid species, so the ability for spontaneous males to successfully turn an established asexual population to sexual is currently unknown (see Morgan-Richards, Langton-Myers & Trewick, 2019). However, in an obligate asexual marine snail *Potamopyrgus antipodarum*, low rates of male production and reduced functionality in asexually-produced males were found to make male invasion in asexual populations unlikely (Neiman et. al., 2012; Jalinsky, Logsdon Jr., & Neiman, 2020) The sexual karyotype of *M. batesii* is currently unknown, but diploid phasmids are known to have either XX XY or XX XO sexual karyotypes (Scali, 2009; Schwander & Crespi, 2009), making spontaneous male genesis a possibility. If spontaneous males are possible in *M. batesii*, they are extremely rare (< 1/1000 parthenogenetic eggs). Out of 533 eggs from all-female sites that were collected in the field, we found one morphologically male-like hatchlings, although morphologically ambiguous hatchlings are occasionally found. This hatchling was not reared to adulthood, so it was not conclusively determined to be male. Also, out of 1083 of eggs produced parthenogenetically in the lab, no males were found. Thus, the spontaneous re-emergence of sex in female-only locations appears unlikely in this species.

Fourth, female-only sites could be established if males go extinct in some parts of the range. This seems the most likely explanation for the geographic mosaic of sexual and asexual localities that we report. Male extinction could occur stochastically in *M. batesii* populations, and such a pattern of extinction may be especially likely in small, isolated populations (Pianka, 2000). Male extinction might also occur as a result of the evolution of female resistance to mating. There are many costs of sexual reproduction (Lehtonen, Jennions & Kokko, 2012; but see Otto & Lenormand, 2002). In a facultatively parthenogenetic species, such costs could generate sexual conflict and favour female resistance to mating. Sexual conflict can play a large role in sexual/asexual population dynamics, involving evolution of male traits that harm females and female counter-adaptations that minimize the harm (reviewed in Parker, 2006 and Hosken, Archer & Mank, 2019). *Megacrania batesii* males guard females in mixed-sex sites for two weeks or more, and most adult females at these sites carry guarding males (Boldbaatar, 2022). This guarding behavior could interfere with female foraging, reducing fitness of *M. batesii* females. Gerber

674 and Kokko (2016) found that female resistance tactics are most beneficial at low population
675 densities, which occurs in many of the sites described in this paper. Thus, opportunity for
676 the evolution of female resistance tactics appears to exist in this species. However,
677 modeling by Kawatsu (2013) found that antagonistic evolution driven by sexual conflict
678 ultimately leads to the prevalence of sex or the co-occurrence of both reproductive modes
679 unless strong reproductive isolation evolves. *Megacrania batesii* investigated in this study
680 do not show complete reproductive isolation across sites: females from asexual locations
681 can mate with males from mixed-sex locations and produce sons when paired in the lab (to
682 be presented in a separate paper). However, we found that some individuals from sexual
683 sites have heterozygosity values comparable to those of asexual sites. This suggests that
684 females from mixed-sex sites occasionally reproduce asexually despite an abundance of
685 males. It is possible that some females fail to mate or experience fertilization failure after
686 mating, and selection favouring asexual reproduction could potentially lead to the evolution
687 of female mating avoidance or resistance to fertilisation (Burke & Bonduriansky, 2022).

688 The apparent local transitions from sexual to parthenogenetic reproduction
689 identified in this study allowed us to investigate the genetic and phenotypic consequences
690 of reproducing asexually by comparing heterozygosity, genetic diversity, and multiple
691 phenotypic traits from individuals sampled in sexual and asexual sites. We found a striking
692 contrast in genetic diversity between the sexual and asexual sites. Asexual sites had on
693 average 85% less allelic richness, 85% less Shannon's diversity, and 83.5% less
694 heterozygosity than their sexual counterparts. This dramatic difference in genetic diversity
695 between the sexual and asexual lineages could be explained by the mechanism of
696 parthenogenesis in *M. batesii*. The cytological mechanisms underlying asexual reproduction
697 can vary between species and could have a significant effect on the amount of
698 heterozygosity maintained when restoring the parental chromosome number (Stenberg &
699 Saura, 2009; Moritz et al. 1990). Preliminary results suggest that parthenogenesis in *M.*
700 *batesii* results in substantial (but not complete) loss of heterozygosity over a single
701 generation of parthenogenetic reproduction, consistent with automixis via either terminal
702 or central fusion (Stenberg & Saura, 2009). Interestingly, the dramatic heterozygosity
703 differences we observed did not seem to severely disadvantage asexually reproducing *M.*
704 *batesii*. We found no consistent overall effect of reproductive mode on body size, number of

missing appendages, or offspring production. Average fecundity and egg viability were 12% and 19% lower, respectively, in all-female sites compared to mixed-sex sites, but there was no statistical support for an effect of reproductive mode on these traits. This reflects the considerable variation between sites within each reproductive mode. Asexual reproduction could be having a modest negative effect on some aspects of performance, but other factors such as inbreeding in smaller sexual populations or the prevalence of highly locally adapted genotypes in some asexual populations could be having a large effect as well.

While there was little support for a negative effect of asexual reproduction on some important phenotypic and fitness components, *M. batesii* from asexual sites had higher rates of mild and severe wing deformities, in addition to large inter-site variation in these traits (see Fig. 2d and 2e). The apparent differences in heterozygosity levels between the reproductive modes could explain the higher rates of wing deformities in all-female sites. Parthenogenetically produced *Extatosoma tiaratum* females exhibit higher rates of wing deformities than do sexually produced females (Burke & Bonduriansky 2022). Likewise, multiple homozygous loss of function mutations cause wing defects in *Drosophila* (Terriente-Felix, López-Varea & de Celis, 2010; George et. al., 2019). If *M. batesii* shares any of these genes, asexually produced females may be more at risk of displaying a homozygous genotype resulting in the loss of function of certain genes relating to wing formation during development. Additionally, the high rates of wing deformities in asexual sites may reflect developmental instability as seen with wing asymmetry in pea aphids (Hammelman et. al., 2020). While wing deformities may be more prevalent in asexual sites, both sexes of *M. batesii* are flightless and wing deformities might have little or no effect on the fitness of these individuals.

We also found elevated rates of fungal and arthropod parasitism of females in all-female sites inhabiting *Benstonea*-swamp habitats. Many *M. batesii* individuals had brown or black spots on their head, thorax, abdomen, or legs that appeared to be a fungal infection, and some individuals carried small mites or biting midges. Elevated rates of parasites in asexual lineages are consistent with Red Queen dynamics, such that sexual populations are better equipped than asexual populations in evolutionary arms races against parasites due to their higher levels of recombination and genotypic diversity (Hamilton, Axelrod & Tanese, 1990; Hamilton & Zuk, 1982). Indeed, Hite (2017) and Lively

736 (1987) found higher frequencies of males and sexually produced offspring in the sea snail,
737 *Dentistyla dentifera*, and the New Zealand mud snail, *Potomopyrgus antipodarum*
738 respectively, during fungal epidemics. Some asexual *M. batesii* sites (KB, CBB, and TS) may
739 be more susceptible to parasites due to their low heterozygosity levels but these patterns
740 were not consistent across all asexual sites. However, we found an interaction between
741 reproductive mode and habitat type, suggesting that asexual reproduction leads to elevated
742 rates of infections either by fungus or arthropod ectoparasites specifically for *M. batesii*
743 found in swamps on *Benstonea* host plants, but not in *Pandanus*-dominated beach habitat.
744 This might be because high humidity in swamp habitats allows for more fungal abundance
745 and growth. Our results therefore suggest that asexual *M. batesii* populations are
746 disadvantaged in their ability to cope with parasites only in certain parts of their range.

747 This study is among the first to investigate the immediate consequences of
748 transitions to asexual reproduction in natural populations. While other facultative
749 parthenogens are known to exhibit geographic parthenogenesis (Morgan-Richards, Trewick ,
750 & Stringer 2010; Kellner, Seal, & Heinze, 2013; Burns, Hedin, & Tsurusaki, 2018), and low
751 heterozygosity and genetic diversity have been reported in many asexual animals (Johnson
752 & Leefe, 1999; Rebecchi et. al., 2005; Goudie et. al., 2012; Nougué et. al., 2015; Sekiné, Tojo,
753 & Bae, 2015; Calvete et. al., 2018; Jaron et. al., 2022), we combined population genetic data
754 with detailed phenotypic data on natural populations to gain a better understanding of
755 what happens to animals directly following transitions to asexual reproduction. We
756 uncovered a dramatic reduction in genetic diversity, but this did not translate into
757 consistent differences in fitness-related traits between sexual and asexual sites. Indeed, our
758 results imply that some asexual *M. batesii* sites have genotypes that are well-adapted to
759 their stable tropical rainforest environment and thus perform well despite extremely low
760 heterozygosity and allelic diversity. These subtle phenotypic effects suggest that the loss of
761 sex may not drive asexual lineages to extinction by itself. Rather, asexual sites might persist
762 and even thrive until outside factors like changes in environment impose strong selection,
763 and extinction might occur as a result of failure to adapt to such changes.

764 Understanding the consequences of sexual vs. asexual reproduction in *M. batesii* is
765 also relevant to conservation because this species has a restricted natural range and could
766 be quite vulnerable to global heating and habitat loss. It remains unclear how well the

populations described in this study would be able to tolerate or adapt to changing conditions, such as increased temperatures, reduced precipitation, or novel pathogens. Cermak and Hasenpusch (2000) designated this species as vulnerable due to its range being less than 100km^2, but also noted that if the populations were found to be genetically distinct they may represent declining remnants of larger ancestral populations, and therefore conservation management should be considered. Indeed, we did find that the genetic groups were spatially isolated from one another. This puts them more at risk of extinction events as small, isolated populations are more likely to undergo Muller's ratchet and accumulate deleterious mutations (Muller, 1964). Asexual populations might be especially at risk of extinction in changing environments. The importance of field-based insect studies increases as concerns grow about a substantial, ongoing decline in terrestrial insect abundances around the world (van Klink et. al., 2020). Long-term monitoring of natural insect populations could help to better estimate the level of threat to their survival and to inform future management.

Acknowledgements:

We would like to thank Jigmidmaa Boldbaatar, Ana Vasconcelos, and Braxton Jones with their assistance in the field. We would also like to thank Holly Trim for assistance with DNA extractions. This research was funded by the Australian Research Council through Discovery Grant DP200101971 to RB.

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