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

Abstract:

Transitions from sexual to asexual reproduction have occurred in numerous lineages across the tree of life, but it remains unclear why asexual populations rarely persist. In facultatively parthenogenetic animals, all-female populations can arise when males are absent or become extinct, and such populations can give rise to obligately asexual species. Facultative parthenogens could therefore shed light on the initial stages of transitions to asexuality, and the factors that determine the success or failure of asexual populations. Here, we describe a novel spatial mosaic of mixed-sex and all-female populations of the facultatively parthenogenetic Australian phasmid *Megacrania batesii*, and use this system to investigate the consequences of reproductive mode variation in the wild. Analysis of single nucleotide polymorphisms (SNPs) indicated multiple, independent transitions between reproductive modes. As expected, all-female populations had drastically reduced heterozygosity and genetic diversity relative to mixed-sex populations. However, we found few consistent differences in fitness-related traits between population types. All-female populations exhibited more frequent and severe (non-functional) wing deformities, but did not show higher rates of appendage loss. All-female populations also harbored more parasites, but only in certain habitats. Reproductive mode explained little variation in female body size, fecundity, or egg hatch-rate. Our results confirm that transitions to parthenogenetic reproduction can lead to dramatic reductions in genetic diversity and heterozygosity. However, our findings also suggest that asexual *M. batesii* populations consist of high-fitness genotypes that might be able to thrive for many generations, perhaps until they encounter a drastic environmental change to which they are unable to adapt.

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 reveal the factors and processes involved in the early stages of transitions to asexual reproduction. Comparing related species also introduces potential confounding factors since genuine consequences of reproducing asexually cannot be differentiated from genetic changes associated with speciation.

Facultatively parthenogenetic animals, in which every female can engage in sexual or asexual reproduction depending on whether mating and fertilization occurs (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 the long-term persistence of asexual populations, and the potential for transitions to obligate asexuality. 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 immediate genetic and phenotypic effects of such transitions could determine whether asexual populations persist or suffer rapid extinction. Yet, very little is known about the relative performance of females in all-female (asexual) versus mixed-sex (sexual) populations of such species.

The Australian Peppermint Stick Insect, *Megacrania batesii*, is a facultatively parthenogenic species endemic to the wet tropics of far-north 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, whereby

116 females that avoid mating produce all-female broods from unfertilized eggs. While some
117 phasmids can produce male offspring from unfertilized eggs (Pijnacker & Ferwerda, 1980;
118 Scali, 2009; Brock et. al., 2018), it is currently unknown how common parthenogenetically
119 produced males are in *M. batesii*. Cermak & Hasenpusch (2000) reported the existence of
120 two isolated all-female populations at the southern end of the Australian range of *M.*
121 *batesii*, but pilot surveys identified a complex geographic mosaic of populations with
122 contrasting sex ratios throughout the species range. Females from all-female locations do
123 not show obvious morphological differentiation from females found in mixed-sex locations
124 and experimental population crosses in our laboratory (to be reported elsewhere) showed
125 that these populations are inter-fertile. Since sexual reproduction is believed to be the
126 ancestral reproductive mode in phasmids (Schwander & Crespi, 2009), we assume that all-
127 female populations of this species are descended from mixed-sex, sexual populations.

128 Our first aim was to describe geographic variation in sex ratio and reproductive
129 mode in wild populations of *M. batesii*. Our second aim was to use these populations to
130 investigate the consequences of transitions to asexual reproduction in wild populations
131 within their natural habitats. In particular, we aimed to determine whether incipient
132 transitions to asexuality (i.e., parthenogenetic reproduction) are associated with reduced
133 genetic diversity and heterozygosity, as predicted by theory and shown by empirical studies
134 on other species (Stenberg & Saura, 2009). We also wanted to determine whether such
135 genetic changes are associated with reduced phenotypic performance in fitness-related
136 traits, including female body size, fecundity, egg hatching success, appendage deformity and
137 loss, and parasite load. A number of studies have compared the performance of sexual and
138 asexual animals in the laboratory (Browne et. al., 1988; Kenny 1996; Cullum 1997; Mee et.
139 al., 2011; Sukumaran & Grant, 2013). However, given the strong environment-dependence
140 of life history and fitness (Saether & Engen 2015), there is a need for research on wild
141 populations in fully natural environments. *M. batesii* occurs in two types of habitat:
142 rainforest along beach margins (where it feeds on *Pandanus* sp. host-plants), and swamp
143 within closed-canopy rainforest (where it feeds on *Benstonea* sp. host-plants). We therefore
144 also asked whether phenotypic and genotypic parameters are affected by habitat type.
145 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

175 all-female" location (B1). One site (NN) was consistently all-female for 2019-2021 but, in
176 2022, males were found at that site. This site is hereafter referred to as a "transitional" site
177 and was excluded from phenotypic analyses comparing sexual and asexual locations.

178 Each adult and nymph seen in the field was imaged in situ on its host plant using a
179 Sony RX10-IV camera. When possible, a transparent plastic scale attached to a thin metal
180 rod was held next to the adult insects so that body size measurements could be obtained
181 from the image (Fig. S1). In most cases, this procedure did not appear to disturb the insects
182 since no movement or defensive reaction (retreating, jumping off the leaf, or spraying of
183 defensive fluid) was observed. From the images, ImageJ (Schneider, Rasband & Eliceiri,
184 2012) was used to measure the length of the pro-thoractic notum (protonum) and scale.
185 Pronotum length was then calculated in cm as a measure of body size. We measured
186 pronotum length because this part of the body was consistently visible in the photos,
187 including photos of females being guarded by males. The photos were also used to record
188 the number of missing legs and antennae. Leg and antennae loss in arthropods is an
189 indication of poor condition that could result from predator attack or problems with
190 moulting (Maruzzo et. al., 2005). For each individual, we also recorded the presence of black
191 spots indicative of fungal infection, the presence of ectoparasites (mites or biting midges),
192 and the presence and severity of wing deformities. Normal wings are held flat over the
193 dorsal thorax of the insect, with one wing overlapping the other. Mild wing deformities
194 were defined as normally shaped wings held apart (not overlapping), whereas severe wing
195 deformities were defined as wings that were widely separated, partly or entirely missing, or
196 highly abnormal in shape.

197 In 2019 and 2020, we collected 1,418 eggs from several wild-collected *M. batesii*
198 females and sexed 719 hatchlings from those eggs to verify that females at the all-female
199 locations were reproducing asexually while females at the mixed-sex locations were
200 reproducing sexually. Wild-collected females were kept in mesh cages in for 7-12 days and
201 fed on *Benstonea* sp. or *Pandanus* sp. leaves, and then released at the site of capture.
202 Females that were mating or guarded by males when collected were housed together with
203 the male. Eggs laid by wild-collected females were counted and then kept in containers with
204 moist cocopeat at ~27°C until hatching in controlled-temperature rooms at The University of
205 New South Wales, Sydney. Fecundity was quantified for each wild-collected female as the

206 number of eggs laid per day, and egg viability was quantified as the hatching rate of these
207 eggs. Hatchlings were sexed based on the morphology of the 8th and 9th abdominal sternites
208 (Fig. S2), and hatchling sex ratio was used to assess reproductive mode in the natural source
209 locations. Fertilized eggs hatch into both males and females whereas unfertilized eggs
210 typically hatch into females only. Thus, when only female hatchlings were obtained from
211 eggs laid by wild-collected females, we inferred that that the females at that location
212 reproduced only via parthenogenesis; whereas when male hatchlings were obtained, we
213 inferred that sexual reproduction had taken place. Based on data from laboratory crosses
214 (not shown), spontaneous production of males from unfertilized eggs is impossible or
215 extremely rare (< 1/1000 eggs) in *M. batesii* (unpublished data).

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217 **DNA sampling, extraction and purification**

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

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

233 Isolates from a total of 188 *M. batesii* individuals were sent to Diversity Arrays
234 Technology Inc. (Canberra, Australia) for whole-genome reduced representation genotyping
235 using the DArT-seq protocol. Genome complexity reduction was performed using a

236 restriction enzyme double digest of PstI and HpaII. Next-generation sequencing of
237 amplification fragments was conducted on the Illumina Hiseq 2500 (www.illumina.com),
238 producing 1,384,779 single-end reads of raw data across all 188 samples. SNPs were then
239 called using the DArTsoft analytical pipeline (Kilian et. al., 2012).

240

241 **Quality control filtering**

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

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

261

262 **Phenotypic analysis**

263 All phenotypic analyses were conducted in R, v. 4.0.2 (R Core Team, 2020). The
264 package "MCMCglmm" was used to model variation in phenotypic traits using a mixed

model approach (Hadfield, 2010). For each response variable, reproductive mode (assumed 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, & Gr̈unwald, 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 (NMDS) using *metaMDS()* was then used to visualize the structure among samples as a complement to the cluster analysis (Dixon, 2003). Observed and expected heterozygosities

323 as well as F_{is} of each locality was obtained by *gl.get.diversity()* (see Table S6). Sites with less
324 than two sampled individuals were excluded from these analyses.

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

333 Allelic diversity, Shannon's diversity, and heterozygosity were quantified using the
334 function *gl.report.diversity()* as described by Sherwin et. al. (2017), and these values were
335 compared between sites (see Fig. S5). Observed heterozygosity (Ho) values were calculated
336 using the function *gl.report.heterozygosity()* and used to visualize heterozygosity of each
337 sample (see Fig. 5c). Isolation by distance was determined using the function *gl.ibd().* All
338 functions used are from the package "dartR" (Gruber et al., 2018). The data that support
339 the findings of this study are openly available in dryad at
340 https://doi.org/10.5061/dryad.n02v6wx1g.

341

342 **Results**

343 **Sex ratio and reproductive mode**

344 Sex ratio was bimodally distributed, with either only females or an approximately
345 equal number of females and males observed at nearly all locations (see Table 2). With one
346 exception (see below), we did not find any locations where adult males were present but
347 rare (and the absence of such locations is supported by population genomic analysis,
348 described below). In several cases, all-female and mixed-sex sites were found to occur in
349 close proximity, and with no obvious barriers to dispersal. For example, mixed-sex locations
350 B4 and VR are separated by < 2km of contiguous rainforest from all-female locations B1 and
351 CB, and the all-female location NS is separated by < 1km of rainforest from adjacent mixed-
352 sex locations (See Fig. 4). Despite the close proximity of males to some all-female sites, sex

353 ratios were consistent over four years of surveys at nearly all locations (see Supplementary
354 file “sex_ratio_by_year.csv”).

355

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

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

378

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

380 We did not find clear and consistent evidence of an effect of reproductive mode
381 across the fitness-related phenotypic traits measured (see Fig. 2b and 2c; for all summary
382 statistics see Table S2). Mean fecundity and egg hatching success were ~12 and 19% lower,
383 respectively, at all-female than at mixed-sex locations, but there was a great deal of
384 variation in these variables among both mixed-sex and all-female locations (Fig. S7). Adult
385 females at all-female locations were more likely to exhibit mild (pMCMC < 0.001) and severe
386 (pMCMC < 0.005) deformities in their non-functional wings than were females at mixed-sex
387 locations (see Fig. S6). Relative to females at mixed-sex locations, adult females at all-female
388 locations had more parasites in *Benstonea*-swamp habitats, but not in *Pandanus*-beach
389 habitats (poptype:habitat interaction: pMCMC < 0.005; see Fig. 3). Post-hoc Tukey tests
390 show this interaction is driven by a near-significant difference between parasitism rates
391 between all-female and mixed-sex sites within swamp habitats (p = 0.054) whereas no other
392 pairwise comparisons approached statistical significance (all p > 0.2; see Table S8).

393 Overall, mean rates of parasitism and wing deformities were ~19% and 91% higher,
394 respectively, in females from all-female locations. Additionally, mean numbers of missing

395 legs and antennae were ~40% higher in adult females at all-female locations, but there was
396 no support for an overall difference in this variable between mixed-sex and all-female
397 locations (pMCMC = 0.34). Estimated phylogenetic heritability (H^2) values suggested effects
398 of genotype for fecundity ($H^2 = 0.74$), the presence of deformities ($H^2 = 0.64$), the presence
399 of parasites ($H^2 = 0.64$), and the hatching success of eggs collected from females at several
400 locations ($H^2 = 0.87$). There was moderate support for an effect of genotype on missing
401 antennae and legs ($H^2 = 0.41$) but little support for effects of genotype for mild ($H^2 = 0$) or
402 severe ($H^2 = 0$) wing deformities, or for pronotum length ($H^2 = 0.12$).

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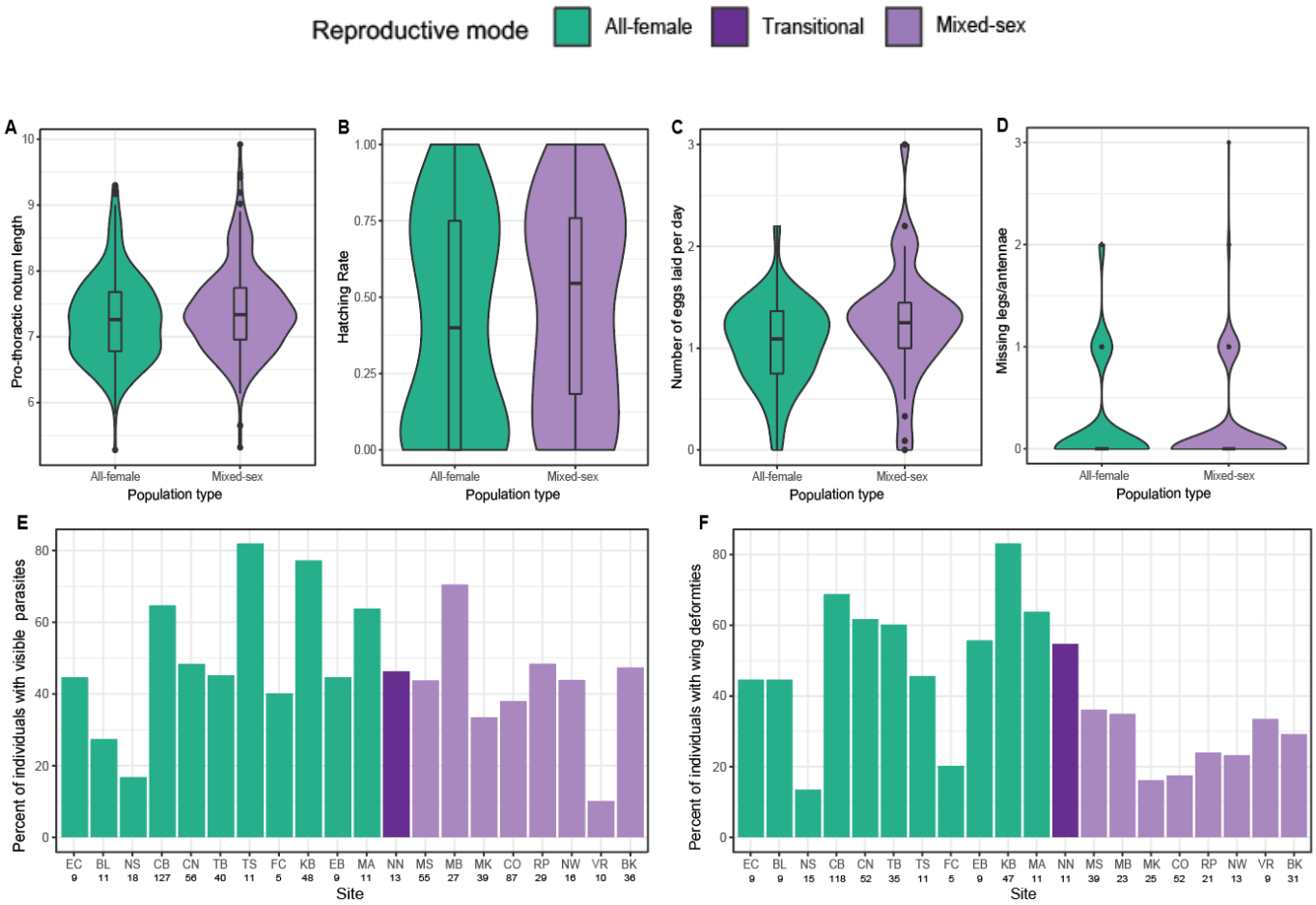

416 Figure 2: Phenotypic values for *M. batesii* females at mixed-sex and all-female locations: A)
417 Pronotum length as a measure of body size; B) Hatching rate of eggs; C) Number of eggs laid
418 per day as a measure of fecundity; D) Number of missing legs and antennae per individual;
419 E) Percent of adult females observed at each location with visible parasites (fungus, biting
420 midges, or mites); F) Percent of adult females from each location with wing deformities

421 (mild or severe). In panels A – D, means (bars), inter-quartile ranges (boxes) and non-
422 outliner ranges (whiskers) are shown. Violins indicate the distribution of individual values.

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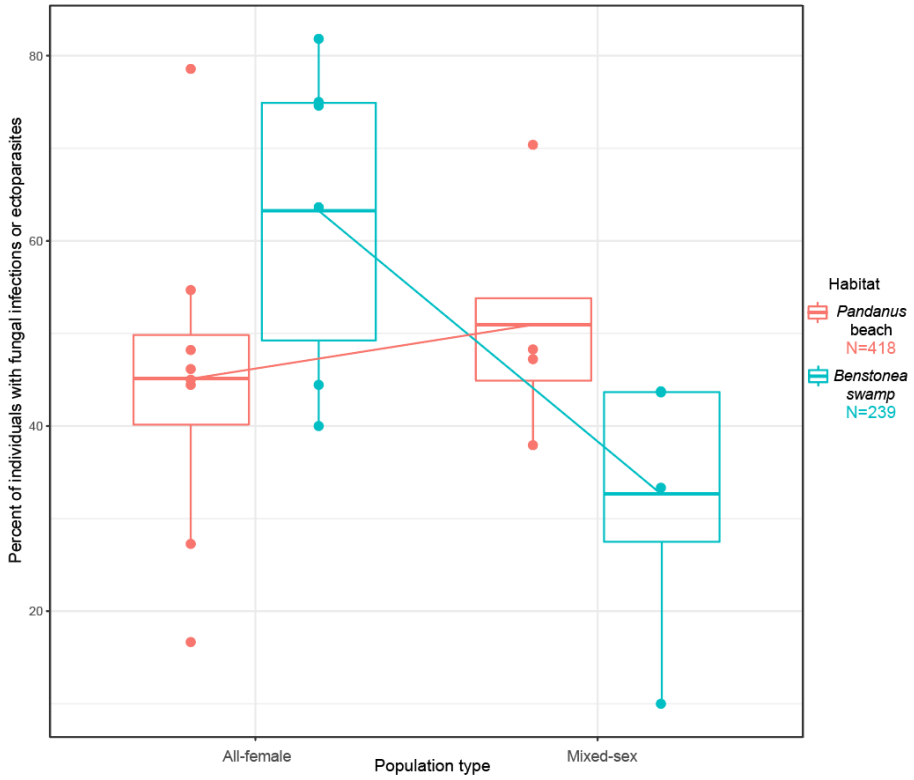


433 Figure 3: Effects of habitat type and reproductive mode on the rate of visible parasites
434 (fungus, mites or biting midges) in *M. batesii* females at all-female and mixed-sex locations.
435 Each point represents a location mean. Bars indicate means across all populations in each
436 group, boxes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

437

438 **Population structure**

439 Sampling sites of *M. batesii* clustered by geographical location rather than by sex
440 ratio. Two major genetic clusters were observed, a "northern" cluster north of the Noah
441 Creek mouth and a "southern" cluster south of the Noah Creek mouth. The Noah Creeks
442 mouth (~ 150 m wide at its widest point) therefore appears to serve as a major barrier to *M.*
443 *batesii* dispersal and gene-flow. The northern cluster consists mostly of mixed-sex sites
444 while the southern cluster consists mostly of all-female sites, but both genetic clusters
445 contain both all-female and mixed-sex sites (see Fig. 5). Pairwise F_{st} values reflect the same
446 genetic structure as the cluster analysis, with sites separated by greater geographic
447 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.

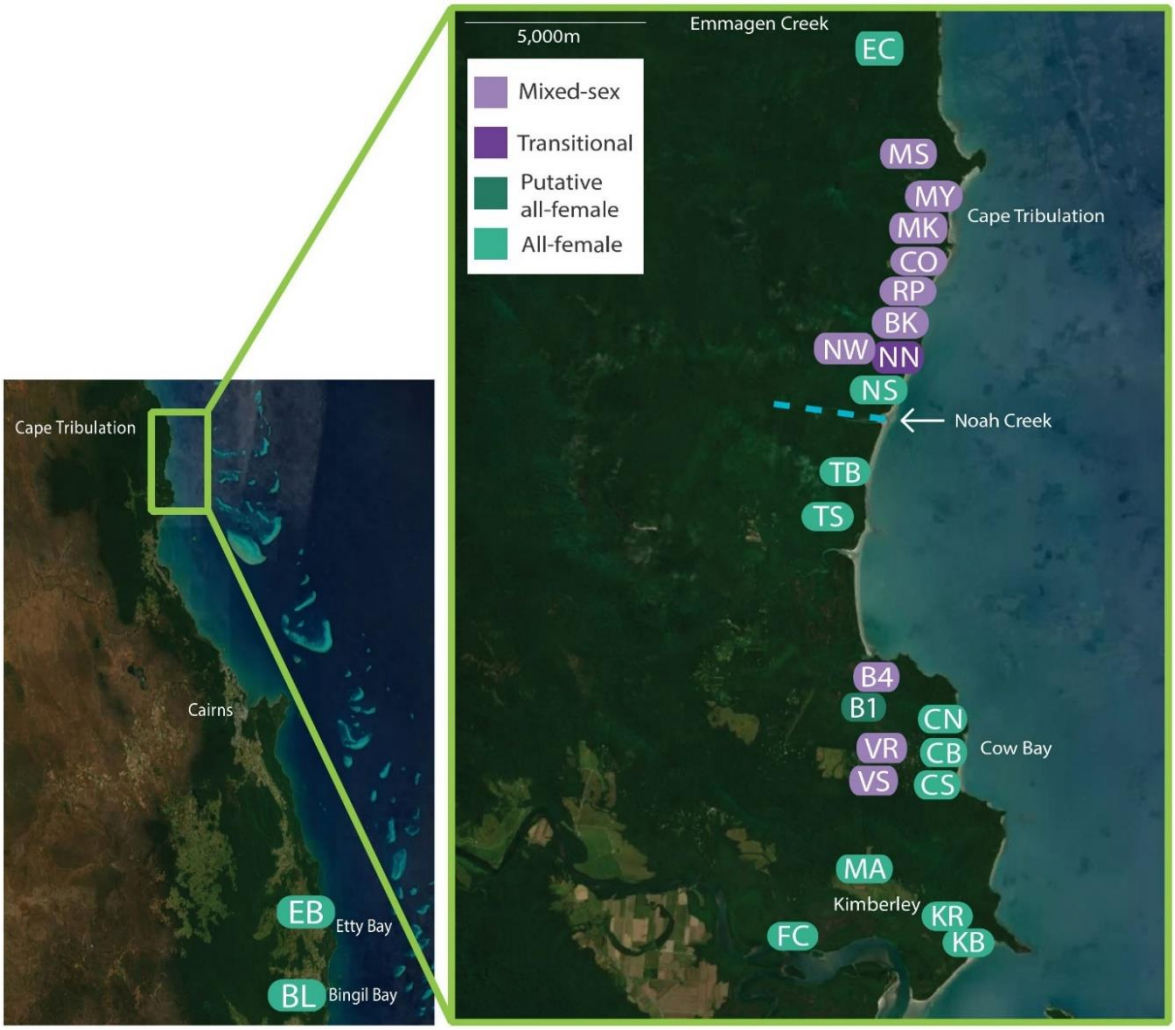
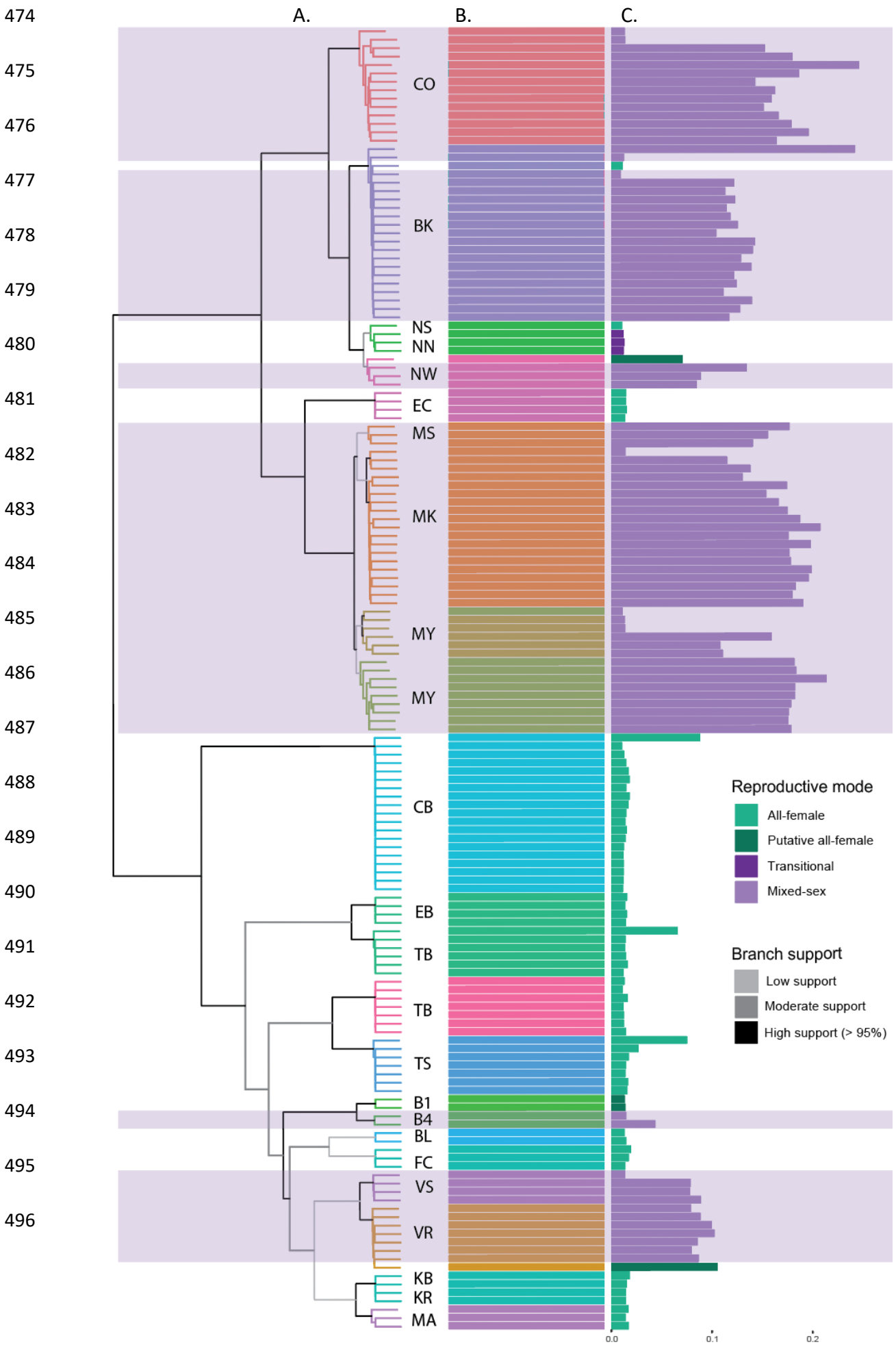


Figure 4: Geographical variation in sex ratio and reproductive mode in *M. batesii*. Two isolated all-female sites occur at the southern limit of the known range of *M. batesii* in Australia, and a spatial mosaic of all-female and mixed-sex sites occurs ~160-180km north of this area between Forest Creek 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.
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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.

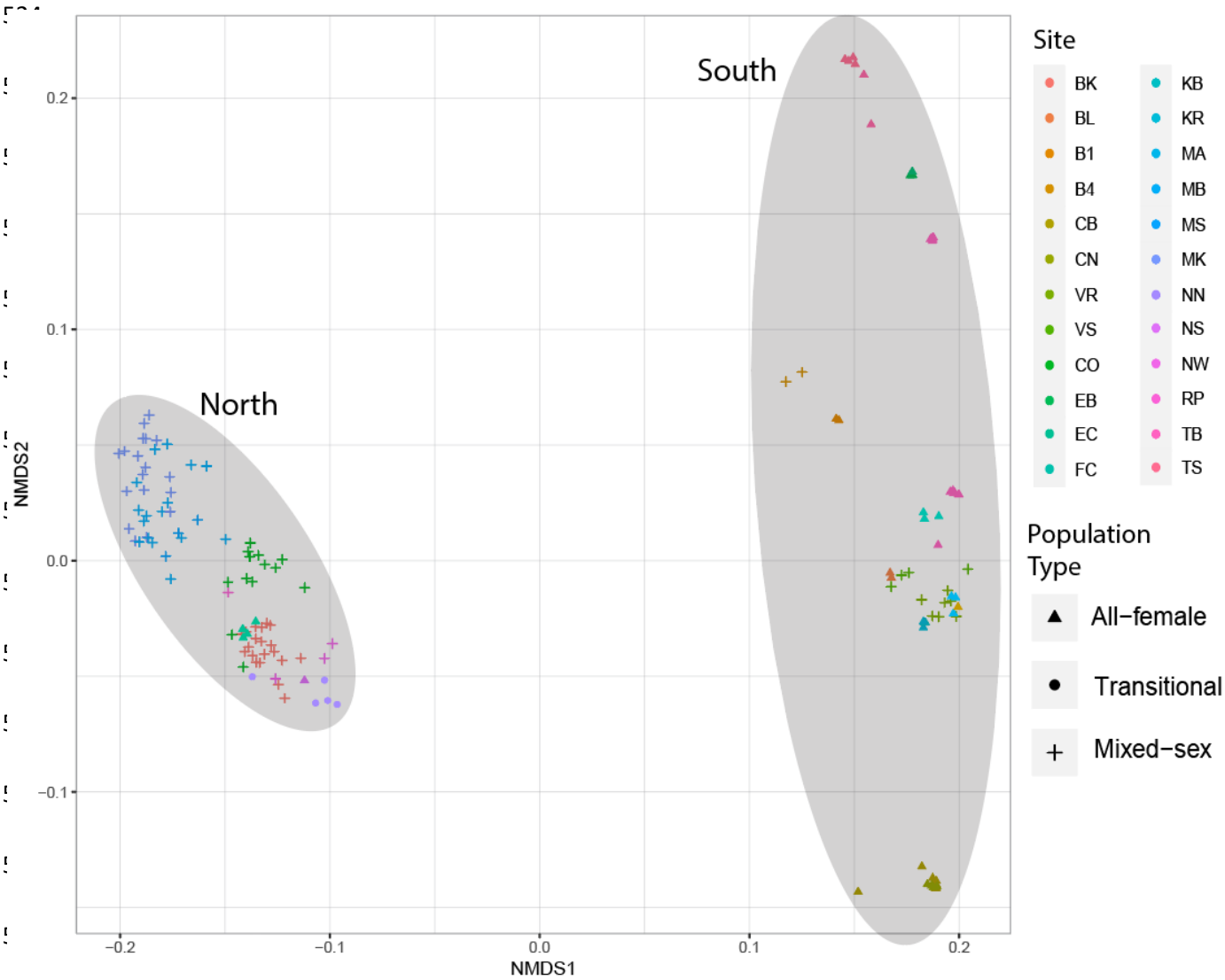

Figure 6: Nonmetric Multidimensional scaling (NMDS) plot of 155 individual *M. batesii* samples based on 7374 high-quality SNPs. Shaded ovals represent individuals found in locations relative to the Noah Creek gene flow barrier.

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-female sites, suggesting that these individuals were produced asexually (see Fig. 5c). On

550 average, heterozygosity of individuals from mixed-sex (sexual) sites was approximately
551 seven times greater than heterozygosity of individuals from all-female (asexual) sites ($p <$
552 0.0001: Welsh's two sample t-test).

553

554 **Discussion**

555 We discovered a novel geographical mosaic of all-female and mixed-sex populations
556 of the facultative parthenogen *M. batesii* across its known natural range in Australia. Using
557 this natural system, we carried out the first detailed comparison of both genetic and
558 phenotypic parameters between wild all-female and mixed-sex populations in a facultative
559 parthenogen. Genetic relatedness and admixture indicate multiple independent transitions
560 from sexual reproduction to parthenogenesis. At one location, we also observed a recent re-
561 invasion of males into a previously all-female site. The establishment of female-only
562 locations led to the loss of allelic diversity and a 7-fold reduction in observed heterozygosity,
563 but the phenotypic and fitness consequences of these genetic changes appear to be highly
564 variable among sites. All-female sites had higher rates of mild and severe deformities in
565 their non-functional wings, but did not exhibit increased rates of appendage loss. These
566 sites also had higher rates of fungal and arthropod parasites, but only in *Benstonea*-swamp
567 habitats where parasites may be generally more abundant. However, we found little
568 statistical support for an overall difference in mean body size, fecundity or egg hatching
569 success between all-female and mixed-sex sites. All of the phenotypic traits that we
570 examined varied substantially among both all-female and mixed-sex sites, suggesting that
571 genotype might be more important than mode of reproduction in determining these traits
572 in *M. batesii*, and that high-fitness genotypes are able to thrive without sexual reproduction.

573 Cluster analyses showed that *M. batesii* populations grouped together based on
574 geographic location, regardless of reproductive mode, and that asexual sites did not form a
575 single monophyletic clade. With one exception (the transition site NN), this pattern
576 remained stable over four years of sampling. We can infer at least four independent
577 transitions from sexual to parthenogenetic reproduction, resulting in all-female sites: the
578 assumed transition from a sexually reproducing ancestral population to an all-female
579 population that gave rise to the genetic cluster of asexual populations south of the gene-

flow barrier at Noah Creek, plus three more recent transitions resulting in all-female populations EC, NS, and B1, all of which are very close spatially and genetically to adjacent mixed-sex sites. Admixture analysis showed little gene flow between the locations despite their geographical proximity (see Fig. 5b). It is not known how long ago these reproductive mode transitions occurred, because no historic samples or previous literature is available that could be used to quantify time of establishment. However, the close geographical proximity and genetic relatedness of some sexual and asexual sites suggest recent establishment. For example, sites B1 and B4 as well as NW and NS exhibit differing reproductive modes, but they are still considered to be from the same population based on admixture analysis. Additionally, results of inter-population crosses (to be presented in a separate paper) show that individuals from sexual and asexual sites are interfertile and can therefore be regarded as a single species.

We identified two main genetic groups, separated by a large estuary (Noah Creek) that serves as a gene flow barrier. The northern group consists of mainly sexual sites and the southern group consists of mainly asexual sites. This partially resembles the geographic pattern found in other geographically parthenogenetic phasmids in the southern hemisphere (Buckley, Marske & Attanayake, 2009; Morgan-Richards, Trewick & Stringer, 2010), and contrasts with the pattern in the northern hemisphere (Law & Crespi, 2002). However, the northernmost site that we sampled in this study (EC) is all-female, while some southern populations (VS, VR, B4) are mixed-sex. Thus, *M. batesii* appears to exhibit a mosaic of sexual and asexual populations across its known range in Australia, rather than a clear latitudinal gradient. Asexually reproducing populations of species that display geographical parthenogenesis are often found in marginal habitats, typically at a higher altitude or latitude than sexually reproducing populations (Lynch, 1984; Kearney, 2005; Tilquin & Kokko, 2016). However, the locations examined in our study are found in similar tropical coastal-lowland rainforest 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.

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

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

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

631 Third, sex may re-invade after it is lost through the spontaneous production of males
632 from unfertilized eggs. The sexual sites in the south (VS, VR, B4) could have arisen via such
633 spontaneous male genesis. Spontaneous male genesis is possible in diploid species with XX
634 XO sexual karyotypes; due to a meiotic/developmental error, the second X chromosome can
635 be lost in situ in parthenogenically produced offspring, resulting in a genetic male (Pijnacker
636 & Ferwerda, 1980; Scali, 2009). The viability and reproductive success of spontaneous males
637 has not been studied in any phasmid species, so the ability for spontaneous males to
638 successfully turn an established asexual population to sexual is currently unknown (see
639 Morgan-Richards, Langton-Myers & Trewick, 2019). However, in the obligate asexual marine
640 snail *Potamopyrgus antipodarum*, low rates of male production and reduced functionality in

641 asexually-produced males were found to make male invasion in asexual populations unlikely
642 (Neiman et. al., 2012; Jalinsky, Logsdon Jr., & Neiman, 2020) The sexual karyotype of *M.*
643 *batesii* is currently unknown, but diploid phasmids are known to have either XX XY or XX XO
644 sexual karyotypes (Scali, 2009; Schwander & Crespi, 2009), making spontaneous male
645 genesis a possibility. If spontaneous males are possible in *M. batesii*, they are extremely rare
646 (< 1/1000 parthenogenetic eggs). Out of 533 eggs from all-female sites that were collected
647 in the field, we found one morphologically male-like hatchling, although morphologically
648 ambiguous hatchlings are occasionally found. This hatchling was not reared to adulthood, so
649 it was not conclusively determined to be male. Out of 1083 of eggs produced
650 parthenogenetically in the lab, no males were found. Thus, the spontaneous re-emergence
651 of sex in female-only locations appears unlikely in this species.

652 Fourth, female-only sites could result from local extinction of males. This seems the
653 most likely explanation for the geographic mosaic of sexual and asexual localities that we
654 report: northern all-female sites (EC, NS) could have resulted from male extinction, while
655 southern mixed-sex sites (VS, VR, B4) could represent remnants of an ancestral mixed-sex
656 population south of Noah Creek. Male extinction could occur stochastically in *M. batesii*
657 populations, and such a pattern of extinction may be especially likely in small, isolated
658 populations (Pianka, 2000). Male extinction might also occur as a result of the evolution of
659 female resistance to mating. There are many costs of sexual reproduction (Lehtonen,
660 Jennions & Kokko, 2012; but see Otto & Lenormand, 2002). In a facultatively
661 parthenogenetic species, such costs could generate sexual conflict and favour female
662 resistance to mating. Sexual conflict can play a large role in sexual/asexual population
663 dynamics, involving evolution of male traits that harm females and female counter-
664 adaptations that minimize the harm (Kawatsu, 2013; Gerber & Koko, 2016; Burke &
665 Bonduriansky, 2019). *Megacrania batesii* males guard females in mixed-sex sites for two
666 weeks or more, and most adult females at these sites carry guarding males (Boldbaatar,
667 2022). This guarding behavior could interfere with female foraging, reducing fitness of *M.*
668 *batesii* females. Gerber and Kokko (2016) found that female resistance tactics are most
669 beneficial at low population densities, which occurs in many of the sites described in this
670 paper. Thus, opportunity for the evolution of female resistance tactics appears to exist in
671 this species. However, modeling by Kawatsu (2013) found that antagonistic evolution driven

by sexual conflict ultimately leads to the prevalence of sex or the co-occurrence of both reproductive modes unless strong reproductive isolation evolves. *Megacrania batesii* investigated in this study do not show complete reproductive isolation across sites: females from asexual locations can mate with males from mixed-sex locations and produce sons when paired in the lab (to be presented in a separate paper). However, we found that some individuals from sexual sites have heterozygosity values comparable to those of asexual sites. This suggests that females from mixed-sex sites occasionally reproduce asexually despite an abundance of males. It is possible that some females fail to mate or experience fertilization failure after mating, and selection favouring asexual reproduction could potentially lead to the evolution of female mating avoidance or resistance to fertilisation (Burke & Bonduriansky, 2022).

We found a striking contrast in genetic diversity between the sexual and asexual populations. Asexual sites had on average 85% less allelic richness, 85% less Shannon's diversity, and 83.5% less heterozygosity than their sexual counterparts. This dramatic difference in genetic diversity could be explained by the mechanism of parthenogenesis in *M. batesii*. The cytological mechanisms underlying asexual reproduction determines how much heterozygosity is maintained when restoring the parental chromosome number (Stenberg & Saura, 2009; Moritz et al. 1990). Preliminary results suggest that parthenogenesis in *M. batesii* results in substantial (but not complete) loss of heterozygosity over a single generation of parthenogenetic reproduction, consistent with automixis via either terminal or central fusion (Stenberg & Saura, 2009).

Yet, we found no consistent overall effect of reproductive mode on body size, number of missing appendages, or female reproductive output. 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 given the considerable variation between sites within each reproductive mode. These findings suggest that, while asexual reproduction could negatively affect some aspects of performance, other factors such as inbreeding in smaller sexual populations or the prevalence of highly locally adapted genotypes in some asexual populations could affect performance to a comparable degree.

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). Low heterozygosity 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 fitness.

We also found elevated rates of fungal and arthropod parasitism of females in all-female sites when compared to mixed-sex sites within *Benstonea*-swamp habitats but not within *Pandanus*-beach 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 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 (1987) found higher frequencies of males and sexually produced offspring in the sea snail, *Dentistyla dentifera*, and the New Zealand mud snail, *Potomopyrgus antipodarum* respectively, during fungal epidemics. Some asexual *M. batesii* sites (KB, CB, and TS) may be more susceptible to parasites due to their low heterozygosity levels but these patterns were not consistent across all asexual sites. Instead, we found an interaction between reproductive mode and habitat type, suggesting that asexual reproduction leads to elevated rates of infections for *M. batesii* found in closed-canopy rainforest swamps on *Benstonea* host plants, but not in *Pandanus*-dominated beach habitat. This might be because high humidity in swamp habitats allows for more fungal abundance

732 and growth. Our results therefore suggest that asexual *M. batesii* populations are
733 disadvantaged in their ability to cope with parasites only in certain parts of their range.

734 Our finding of considerable among-site variation in performance suggests that some
735 asexual *M. batesii* lineages have genotypes that are well-adapted to their environment and
736 thus perform well despite extremely low heterozygosity and allelic diversity. Thus, the loss
737 of sex and consequent genetic changes may not be sufficient to drive asexual lineages to
738 extinction. Rather, locally adapted asexual lineages might persist and even thrive, perhaps
739 for many generations. Ultimately, however, it is possible that outside factors, like changes in
740 climate or the invasion of new competitors or parasites, might impose strong selection, and
741 asexual populations might suffer extinction as a result of failure to adapt to such changes.

742 Understanding the consequences of sexual vs. asexual reproduction in *M. batesii* is
743 also relevant to conservation because this species has a restricted natural range and could
744 be quite vulnerable to global heating, habitat loss, or novel pathogens. Cermak and
745 Hasenpusch (2000) designated this species as vulnerable due to its range being less than
746 100km^2, but also noted that if the populations were found to be genetically distinct they
747 may represent declining remnants of larger ancestral populations, and therefore
748 conservation management should be considered. Indeed, we did find that the genetic
749 groups were spatially isolated from one another. This puts them more at risk of extinction
750 events as small, isolated populations are more likely to undergo Muller's ratchet and
751 accumulate deleterious mutations (Muller, 1964). Asexual populations might be especially
752 at risk of extinction in changing environments. The importance of field-based insect studies
753 increases as concerns grow about a substantial, ongoing decline in terrestrial insect
754 abundances around the world (van Klink et. al., 2020). Long-term monitoring of natural
755 insect populations could help to better estimate the level of threat to their survival and to
756 inform future management.

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