Varroa mites escape the evolutionary trap of haplodiploidy

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Keywords: haplodiploidy, inbreeding, parasite evolution, Varroa

Abstract

Genetic diversity is essential for populations adapting to environmental-changes. Following genetic bottlenecks, invasive species often have reduced diversity, yet must rapidly adapt to novel environments. This paradox is especially pronounced in parasites, which face repeated transmission bottlenecks and anthropogenic countermeasures. The challenge of maintaining diversity in the short-term intensifies in haplodiploid species, where males inherit and transmit only half of their mother's genome, limiting effective population size. To examine how such species may maintain adaptability, we studied inheritance in Varroa mites (*Varroa destructor*), globally invasive honey bee parasite. Using three-generation pedigrees, we found that Varroa is not haplodiploid, as previously thought. Rather, females produce diploid sons who transmit either maternal allele to daughters. This system slows reduced heterozygosity loss under sib-mating, compared to ancestral haplodiploidy, increasing effective population size and adaptability. Our findings reveal a rare reversion from haplodiploidy, long considered an evolutionary end state, and suggest reproductive plasticity underlies the resilience of invasive parasites like Varroa.

Introduction

Genetic diversity is the basis for biodiversity, being required for species to evolve in response to environmental changes[1]. For a given selection coefficient, the effectiveness of natural selection *vs.* genetic drift is determined by the effective population size[2]. While selection dominates at larger population sizes, beneficial alleles can be lost due to drift in small populations. Biological invasions are often accompanied by genetic bottlenecks that reduce effective population size. Parasites are particularly at risk, as they often experience bottlenecks as part of their life cycle: an introduction to a new host often involves one or a few individuals, and the host-parasite population dynamics also involve frequent and drastic changes in the population's size[3]. In addition, because of difficulties involved in finding unrelated mates within a host, many parasites frequently inbreed, which further decreases genetic variability[4–6]. Although all the above scenarios are expected to decrease the effective population size, by definition, invasive parasites are successful species that adapt to different environments, hosts, and in many cases, the chemical pesticides used to control them[7,8]. This 'genetic paradox of invasion'[9,10] raises the question of how invasive

species with expected low genetic diversity are so successful. A few solutions have been suggested for this paradox. These include physiological explanations[11–13], and multiple invasions that compensate for the initial low genetic diversity[13–15]. Occasionally, organisms evolve unusual reproductive strategies that help them avoid inbreeding. For example, in some ants, males and females constitute genetically distinct clonal lineages that combine to produce sterile workers but otherwise do not intermix[16,17]. Detecting unusual reproduction mechanisms typically requires multi-generation-controlled crosses, which are often difficult if not impossible to recreate (especially in non-domesticated organisms), suggesting that many unusual reproductive modes remain to be discovered.

Here we examined the reproductive biology of the Varroa mite (*Varroa destructor*), a global invasive parasite of honey bees. These mites have experienced a range of genetic bottlenecks. They went through a genetic bottleneck when switching from their original host, the Eastern honey bee (*Apis cerana*) to the Western honey bee (*Apis mellifera*)[18]. They also lose genetic diversity when colonizing new hives. Lastly, they routinely sib-mate in small families (typically one male mates with three to four female sisters)[19,20]. This mating behavior further increases the strength of genetic drift, and makes natural selection less effective. Yet, Varroa shows high adaptability: it efficiently switches between host species[21–23], and can rapidly evolve resistance to pesticides via various mechanisms[24]. While a high level of adaptability explains the mite's successful global spread, the mechanisms underlying this adaptability remain puzzling in the light of regular genetic bottlenecks and routine inbreeding.

Varroa is believed to be haplodiploid, whereby fertilized eggs develop into diploid females, while unfertilized eggs develop into haploid males[20,25–27]. Haplodiploid species have a smaller effective population size relative to diplodiploid systems[28,29], though haplodiploidy may confer long-term advantages in inbreeding systems such as more effective purging of deleterious alleles[30]. In Varroa, haplodiploidy was inferred from two karyotyping studies[31,32]. Yet, as those studies examined Varroa embryos only, sex could not be directly determined. Häußermann et al.[20] found that unmated females can produce males—consistent with arrhenotokous parthenogenesis *vs.* paternal genome elimination (PGE) that

is found in many other mites. Yet, while consistent with haplodiploidy, these lines of evidence are indirect.

Haplodiploidy is believed to be evolutionarily stable, sometimes being referred to as an 'evolutionary trap', suggesting limited potential for an evolutionary reversion to diploidy[33,34]. Cruickshank and Thomas[35] used phylogenetic reconstruction to infer that Varroa evolved from diploid ancestors, via intermediate pseudo-arrhenotokous species. While Varroa was included in that study, its haplodiploidy was assumed based on the fact that all members of its family were haplodiploid. However, the taxon sampling was sparse and until recently, the phylogenetic placement of Varroa was uncertain, opening conclusions to criticism[36]. Indeed, more complete investigations were hampered by the uncertain taxonomic placement of Varroa, until a recent study placed it as a subfamily within Laelapidae, allowing a more targeted comparison[37]. Of the 19 sexually reproducing Laelapid mites in the Tree of Sex database[38], only a single one is not haplodiploid: *Dicrocheles phalaenodectes*, a parasite inhabiting the ear canal of some Noctuid moths. However, the placement of *Dicrocheles* in Laelapidae is based solely on morphological data, and has been debated[39]; it is certainly only distantly related to Varroa. Thus, based on the sum total of the evidence, haplodiploidy was ancestral to Varroa.

Theory suggests that reversal to diploidy from arrhenotoky is unlikely[34], and, indeed, no reversals of arrhenotokous sex determination are known to date[40]. Yet, despite strong circumstantial evidence suggesting the existence of arrhenotokous parthenogenesis in Varroa, no study has examined Varroa's mode of genetic inheritance, making its success in spite of genetic handicaps somewhat of a mystery. Here, we explicitly examine this species' mode of genetic inheritance using multi-generational pedigrees.

Results

Two lines of evidence suggest that Varroa males are functionally diploid: [1] their somatic cells inherited and expressed both maternal alleles (Fig 1 and 2), and [2] they randomly transmited these alleles to their daughters (Fig 1). Modeling shows that this increases the mite's effective population size relative to ancestral haplodiploidy (Fig 3).

116

117 *Males are produced by diploid arrhenotoky preserving their mothers' genotype.*

118 Adult males inherited both of their mother's alleles, as evident from the three-generational
119 pedigree study. Looking at the cross between a heterozygous F1 female (AB) and a
120 homozygous F1 male (AA), we found that all variants of the F2 grandson in a family
121 maintained their mother's heterozygosity (Fig 1A, example of family 478). This was
122 consistent across families, as pooled data showed 97% of the genotypes in F2 males
123 maintained their mother's heterozygosity (Fig 1C). Similarly, when crossing a homozygous
124 F1 female (AA) and a heterozygous F1 male (AB), variants of F2 grandson were
125 homozygous like the F1 mother (Fig 1B), and the pooled-family's data were in consistent
126 (Fig 1D). Both alleles were also expressed, as evidenced from heterozygosity in RNA-seq
127 data of adult female mites and their sons (Fig 2, Table S1). The two sexes showed similar
128 heterozygosity proportions in their RNA across five families (mean difference = 0.022 ±
129 0.10, paired t-test: t = 0.46, df = 4, P-value = 0.672).

130

131 *Males transmit their two alleles to their daughters with equal probability*

132 Males not only inherited two alleles, but they also randomly transmitted each allele to their
133 daughters, in a typical Mendelian manner. Looking at the cross between a homozygous F1
134 female (AA) and a heterozygous F1 male (AB), we observed that about ~50% of the
135 variants in F2 females were homozygous (like mother) and the rest were heterozygous
136 (like the father) (Fig 1B). F2 male offspring of the same cross were homozygous (AA), just
137 like their mother. This was consistent across families, as shown by the pooled data (Fig
138 1D).

139

140 *All else being equal, Varroa diploid male arrhenotoky has greater population size relative to*
141 *ancestral haplodiploidy*

142 We conducted genetic transmission path modelling, which traced gene flow from ancestors
143 to descendants and quantified genetic relatedness through path coefficients, following
144 Wright [41]. This showed that Varroa lost slightly but consistently less heterozygosity under
145 inbreeding conditions, compared to haplodiploids, and therefore had a correspondingly
146 higher effective population size (Fig 3). The difference arose because the two alleles in

147 male genomes lowered the correlation between uniting gametes and attenuated inbreeding
148 compared with haplodiploid mating, whereby the male inherited a single allele.
149 Consequently, for the same census size, the limit of Ne was higher under diploid
150 arrhenotoky across the explored ranges of mothers per generation, daughters per mother,
151 and brood-cell occupancy probability.
152
153 **Discussion**
154 In this study, we found that *V. destructor* has evolved from ancestral haplodiploidy [35] to a
155 novel reproductive strategy with diploid arrhenotokous males that are clones of their
156 mothers (see Fig 4 for a scheme of the inferred mode of genetic inheritance). This strategy
157 potentially has both short-term, and long-term benefits for Varroa, each overcoming a
158 different evolutionary 'paradox'. In the short-term, the production of clonal males increases
159 the females' fitness by assuring transmission of both of her genetic variants. This strategy
160 can overcome the "paradox of sex" by blending the benefits of sexual and asexual
161 reproduction. Namely, we found that females can produce sons containing both copies of
162 their genes, but female eggs are produced meiotically. In the long term, functional
163 diplodiploidy can potentially increase the effective population size (vs. haplodiploidy) (Fig
164 3). A higher effective population size imparts greater evolutionary potential, and indeed
165 Varroa has been remarkably adaptable despite repeated bottlenecks exemplifying the
166 "genetic paradox of invasion".
167
168 Another evolutionary surprise from this study is the effective loss of haploid arrhenotoky.
169 Once evolved, asymmetric inheritance appears to reach an evolutionarily stable state,
170 sometimes referred to as an 'evolutionary trap'[34]. Obstacles to re-evolving Mendelian
171 inheritance are diverse. One obstacle is mechanistic, in that males would need to re-evolve
172 meiosis, which was always thought to be difficult[42]. However, recent work from asexual
173 stick insects indicates that male meiotic regulatory mechanisms are preserved in females[43],
174 so re-evolving male meiosis may be easier than expected. The other obstacle is
175 evolutionary, as examined in detail by Bull[44]. In that scenario, females reach a fitness
176 optimum in haplodiploid systems, being able to control male production and thus the sex
177 ratio. Thus, re-emergence of diploid males would have to be a male trait, and biparental

178 diploid males must carry mutations that allow them to produce diploid sons. However,
179 given the level of arrhenotokous female control over fertilization and sex ratios, those traits
180 are theoretically unlikely to spread[44]. Indeed, the only evolutionary feasible transition from
181 haplodiploidy appears to be towards parthenogenesis, though these are short lived[45]. Yet,
182 Varroa overcame the constraint on the production of diploid sons by having females solely
183 control both, the sex ratio, and diploid male production. The production of meiotic sperm
184 and the evolution of diploid arrhenotokous male production illustrate how the unforeseen
185 complexity of biological mechanisms may challenge long-standing elegant mathematical
186 models.
187
188 Although the mechanism through which sex is determined in Varroa still needs further
189 investigation, other facultatively parthenogenetic systems provide insights into how it may
190 hypothetically occur. For instance, Cape honey bees (*Apis mellifera capensis*) store new eggs
191 in their oviducts arrested in metaphase I, while meiosis II takes place only after
192 oviposition[46]. If the egg is not fertilized, it develops into a female by central fusion. If it is
193 fertilized, a diploid female is sexually produced. A similar or parallel mechanism may be at
194 play in Varroa, except that unfertilized eggs undergo central fusion to develop into males
195 while preserving their mother's genotype (Fig 1). Unlike honey bees, where sex is
196 controlled genetically, an epigenetic factor would be required for sex determination in
197 Varroa. Such a factor may be provided by the female, for instance by controlling
198 fertilization.
199
200 *Conclusions*
201 Our work underscores the importance of comprehensive experimental inquiries into
202 diverse reproductive mechanisms, even in organisms that appeared to be well studied.
203 Recent investigations have unveiled a broader spectrum of reproductive strategies than
204 were previously recognized[42], exemplifying the dynamic nature of biological systems. Yet,
205 the discovery of a novel reproductive system, such as that in Varroa, can have wide-ranging
206 fundamental and applied consequences. For instance, Varroa's haplodiploidy is assumed
207 when considering how it evolves in response to countermeasures, such as pesticides,
208 breeding honey bees for parasite tolerance, or meiotic drive[47–49]. Furthermore, Varroa has

209 emerged as a compelling model for the study of the evolutionary dynamics in reproductive
210 systems, offering insights into mechanisms for overcoming evolutionary constraints and
211 the intricacies of sex determination. Our findings open promising avenues for future
212 research, contributing to a deeper understanding of evolutionary innovation and
213 reproductive plasticity in Varroa and beyond.
214
215 **Materials and methods**
216
217 *Honey bees and mites*
218 For the pedigree study: Varroa mites and honey bees (*Apis mellifera* L.) were
219 obtained from colonies at the Onna village apiary and the experimental apiary of the
220 Okinawa Institute of Science and Technology (OIST) in Okinawa, Japan. All colonies used in
221 the experiment were maintained without any treatment against Varroa and received a 60%
222 w:v sugar solution and 70% pollen patties as supplemental feed when necessary.
223 Experiments were conducted between July and November 2020.
224 For comparative transcriptomic analysis of Varroa gene expression heterozygosity
225 in mothers and sons: Varroa mites were collected from *A. mellifera* colonies at the Zhejiang
226 University in CITI, China, in November 2024. A total of ten mites were collected from five
227 families, each consisting of a mother and her son.
228
229 *Artificial infestation for Varroa pedigree construction*
230 To obtain a three-generational mite pedigree (F0, F1, and F2), we used a semi-natural
231 infestation system. This started with the collection of a naturally infesting mated Varroa
232 female (F0). Her adult daughter (F1) was then artificially introduced into a honey bee pre-
233 pupal cell and allowed to commence the reproductive phase of its life cycle. The F1 mite
234 and her offspring (F2) were then collected at the end of the reproductive cycle. For a
235 detailed description of the experimental design, see Fig 5 and the sections below. Using this
236 approach, we could genotype all members of the pedigree, except for the male that mated
237 with the F0 female.
238
239 Collection of naturally infesting families (F0 and F1 generations)

240 To obtain mites from the first two generations (F0 and F1), naturally infested families were
241 collected from within the cells of 18- to 20-day-old worker bee pupae. At this
242 developmental stage of the bee host, there is typically one dark brown female mite (the
243 foundress, F0), one adult male (the foundress' son, F1), at least one light-colored female
244 (the first foundress' daughter, F1), a few nymphs females, and an egg[25]. We determined
245 each mite's sex and developmental stage according to external morphology and cuticle
246 coloring based on[50]. For the color index, see Fig S1. To ensure that all offspring belonged to
247 a single family, we thoroughly examined the cell content to confirm that it was infested by a
248 single foundress mite and that no other adults were present. Except for the adult female
249 daughters (F1), the rest of the family members were preserved in a separate
250 microcentrifuge tube kept at -20 °C for later DNA extraction. The adult F1 daughters were
251 easily distinguished from the F0 foundress mite by their lighter cuticle color (color index 0-
252 2, see Fig S1), and were assumed to have already copulated with their brother, the F1 male,
253 by the time of the collection[20]. Next, the F1 young adult females were prepared to be
254 artificially introduced into a freshly capped 5th instar larva cell to start their first
255 reproductive cycle.
256
257 Incubation and dispersal phases of F1 female mites
258 Based on our and others' observations[51], the survival and fecundity of young adult mites
259 increase if they stay for a few days on an adult nurse bee before starting their first
260 reproductive cycle. This period is presumably imitating the "dispersal phase" (former
261 called the "phoretic phase"), in which the adult female mite infests and feeds on an adult
262 honey bee[51,52]. Therefore, the collected young adult females (F1 generation) spent three
263 days on a nurse bee before infestation. However, during the experiment, we noticed that
264 the very young adult daughter's cuticle was light and fragile, and most of the mites did not
265 survive when they were transferred directly from the pupae onto a nurse bee. Therefore,
266 we let the young mites first feed on a white-eyed pupa in a gelatin capsule for one to four
267 days ("incubation phase", Fig 5B1) until their cuticle was sclerotized and darker (color
268 index 3-4, see Fig S1). After this intermediate period, the mites were marked on their
269 dorsal idiosoma (PC-1MR POSCA, Japan), and transferred onto an adult nurse bee in a cage
270 for another three days, for the above-mentioned "dispersal phase" (Fig 5B2). In all

271 incubation periods, the mites were kept in a controlled dark environment, imitating hive
272 conditions (34-34.5 °C, 60-75% RH).
273
274 Artificial infestation of F1 female mites
275 After the artificial "dispersal phase", the mites were brushed off the bees using a fine paint-
276 brush following anesthesia by a 20-s exposure to CO_2, 0.2mPa at 10 L/hour, and placed on a
277 moist filter paper to avoid desiccation until the infestation procedure. The mites were then
278 introduced to a worker bee cell, one mite per cell, as described by[53] (Fig 5C) . In detail, six
279 hours before infestation, cells containing a 5th instar larva (about to be sealed) were
280 marked on an acetate sheet attached to a frame from a mite-free hive. The frame was then
281 placed back into the hive so that the bees could seal the cells. Five hours later the frame
282 was taken into a warm room (25-28 °C) for the F1 females to be introduced into the freshly
283 sealed cells. For the artificial infestation, a precise incision (3-5 mm) was made at the
284 margin of the cell-capping using a surgical blade (N 11). After the incision was made, the
285 cell capping was slightly lifted, just enough for the mite to crawl under the capping and into
286 the cell. The mite was then placed at the entrance of the cell using a moist fine paintbrush,
287 with its head facing the cell opening. After the mite entered the cell, it moved under the
288 larva to the cell bottom. The cap was then resealed by gently pushing it down using the
289 back end of the paintbrush. The infested frame was then incubated in a dark controlled
290 environment (34-34.5 °C, 60-75% RH) for ten days, during which the mites were
291 reproducing (Fig 5D). During this period emerging bees were removed daily.
292
293 Collection of an artificially infested family (F2 generation)
294 Ten days after the F1 mites' infestation, the cells were opened and their contents were
295 examined (Fig 5E). The family members' sex, developmental stage (adult, nymph, or egg),
296 and viability, were recorded. To assure the parenthood of the offspring (F2), double-
297 infested cells and unmarked mites were not included in the final analysis. In total, out of 78
298 successful infestations, 14 were double-infested by a non-marked mite. Each individual was
299 snap-frozen in a separate microcentrifuge tube at -20 °C for later DNA extraction. In total,
300 we collected 223 mites belonging to 30 families. Each family was composed of a foundress

301 adult female (F0), her male and female adult offspring (F1), and their F2 offspring (at least
302 one male/female mite in various developmental stages).
303
304 *Genomic analysis of the Varroa pedigree*

305 DNA extraction, library preparation, and sequencing

306 Genomic DNA was extracted from each mite following the protocol described previously[23].
307 In brief, after surface sterilization using absolute ethanol, each mite was crushed in a 1.5
308 mL microcentrifuge tube dipped in liquid nitrogen. The crushed mite was then processed
309 using a QIAamp DNA Micro Kit (© Qiagen) following the manufacturer's instructions and
310 eluted in 20 µL of water. Total dsDNA quantity was measured using a Qubit™ 4
311 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and DNA quality was verified using a
312 Bioanalyzer 2100 with high-sensitivity DNA kits (Agilent, Japan). Short inserts of 150-bp
313 paired-end libraries were prepared for each individual using a Nextera XT DNA Library
314 Preparation Kit (Illumina, Japan) and 16 PCR cycles. cDNA was then cleaned and size-
315 selected using CA-magnetic beads (Dynabeads® MyOne Carboxylic Acid, Invitrogen;
316 Thermo Fisher Scientific, USA), and 11-11.5% PEG 6000 (Sigma-Aldrich, Japan). The
317 library's quality was measured using a Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS
318 Assay Kit, and its size was assessed using a 4200 Tapestation with a high-sensitivity D5000
319 kit (Agilent, Japan). Libraries were run on a NovaSeq6000 in 150 bp x 2 paired-end mode
320 (Illumina, Japan) at the OIST Sequencing Center. All biosamples are available in the
321 Sequence Read Archive (SRA) under the accession number PRJNA794941. Biosample
322 details including sex, developmental stage, pedigree information, and sequencing coverage,
323 are provided in Table S2.

324 Read mapping, variant calling, and filtering, of genomic data

325 We mapped all reads to the *V. destructor* reference genome GCF_002443255.1[54] using the
326 very sensitive mode of NextGenMap v0.5.0[55]. Reads were sorted and duplicates were
327 removed using SAMtools[56]. We called variants against the reference genome using
328 FreeBayes v1.1.0[57] with the following parameters: minimum alternative allele counting = 5,
329 minimum alternative allele fraction = 0.2, minimum mapping quality = 8, minimum base

330 quality = 5, and using the four best SNP alleles. The variant calling resulted in a total of
331 73,903,468 variated sites. As our population consisted of closely related individuals (mite
332 families from the same apiary, in a high-inbreeding system), we did not expect to detect
333 many variants. Therefore, we set the minor allele frequency threshold to 0.2, which
334 includes sites with a minor allele frequency of 20% or more, to exclude any rare variants
335 due to mutations or possible mapping errors. We included variants only for the seven
336 chromosomes (excluding the mitochondrial DNA and unscaffolded regions). In addition, we
337 filtered for the following site quality parameters: we included only bi-allelic sites, a depth
338 range between 16-40, and maximum missing data per site = 0.5. The variants' quality
339 distribution showed a clear bimodal distribution with a cutoff at ~10,000 (Fig S2). To
340 ensure the quality of the variants for the genetic inheritance analysis, we chose a strict
341 minimum quality filter of 15,000. Filters were applied using VCFtools v0.1.12b[58], resulting
342 in 33,925 final variants from 223 individuals. The pipeline from fastq files down to the VCF
343 file was constructed using Snakemake[59]. Codes are available and reproducible on GitHub
344 (https://github.com/nurit-eliash/Varroa-pedigree-study). For further security of site
345 quality, we applied two additional site-quality parameters to account for biases in alternate
346 allele representation and to ensure high-confidence genotype calls based on allele
347 frequency and quality. Variant allele frequency (VAF) was calculated as $VAF = AO/DP$,
348 where AO (Alternate Observations) represents the number of reads supporting the
349 alternate allele, and DP (Depth) is the total read depth at the site. Similarly, Quality Allele
350 Frequency (QAF) was computed as $QAF = QA/QA+QO$, where QA (Quality of Alternate
351 Allele) is the sum of Phred-scaled base quality scores for reads supporting the alternate
352 allele, and QO (Quality of Reference Allele) is the sum of Phred-scaled base quality scores
353 for reads supporting the reference allele. To minimize errors in genotyping due to
354 sequencing artifacts or depth-related biases, we applied genotype-specific thresholds for
355 VAF and QAF based on observation of values from F2 females of which parents and
356 grandmother genotypes are fixed, and so their own genotype is assumed with high
357 confidence (Fig S3, S4 and S5 for QAF and VAF distribution, in genotypes AA, BB and AB, in
358 accordance). The thresholds (Table 1), ensure that homozygous reference (AA) sites
359 exhibit low alternate allele support (VAF and QAF < 0.01), heterozygous (AB) sites

360 maintain a balanced allele distribution (0.2< VAF < 0.8, 0.2 < QAF < 0.8), and homozygous
361 alternate (BB) sites demonstrate high alternate allele support (VAF and QAF > 0.9). This
362 filtering approach enhances the accuracy of genotype classification, while reducing the
363 inclusion of low-confidence variant calls.
364
365 **Table 1.** Quality Allele Frequency (QAF) and Variant allele frequency (VAF) threshold and
366 their ideal values used for sites' filtration in the genotype flow of mite pedigrees.

Genotype	VAF threshold	Ideal value, interpretation
AA (Homozygous Reference)	< 0.01	~0, Strong reference support
AB (Heterozygous)	0.2 - 0.8	~0.5, Balanced allele representation
BB (Homozygous Alternate)	> 0.9	~1, Strong alternate support

367
368 *Genotype validation using Sanger sequencing*
369 Several genotype calls obtained from whole genome sequencing were validated by Sanger
370 sequencing using the same genomic DNA extracted. For the validation, we randomly
371 selected three genes, located on different chromosomes, each containing at least one
372 variant site. In total, we re-sequenced 14 individual mites from four families on six variant
373 sites. The sequenced region was located on the exons of three genes: alpha-mannosidase
374 2x-like (LOC111243621 on chromosome NW_019211455.1), 5-oxoprolinase-like
375 (LOC111252938, on chromosome NW_019211459.1), and probable E3 ubiquitin-protein
376 ligase RNF144A-A (LOC111254119, on chromosome NW_019211460.1). Primers were
377 designed with the NCBI primer design tool (utilizing Primer3 and BLAST), with default
378 parameters and product size set to 300-1,000 bp. Primer sequence, product length, and
379 gene accession numbers are provided in Table S3. The size of the PCR product was checked
380 by running it in 1% agarose gel (135V, 20 min), purified, and size selected using the solid
381 phase reverse immobilization (SPRI) method. The purified PCR products were Sanger
382 sequenced on a ThermoFisher SeqStudio Genetic Analyzer using the original reverse
383 primer and BigDye® Direct Cycle Sequencing kit (Thermo Fisher Scientific, USA), following
384 the manufacturer's instructions. We then aligned the reverse complementary sequences to
385 the Varroa reference genome (GCF_002443255.2_Vdes_3.0) to detect and validate the

386 variant sites using Geneious Prime 2022.1.1. (https://www.geneious.com). For sequence
387 alignments and the validated sites, see Fig S6.
388
389 *Transcriptomic analysis of Varroa mother and son*

390 RNA extraction, library preparation, and sequencing

391 Total RNA was extracted from each single mite and sequenced by Metware Biotechnology
392 Co., Ltd. (Wuhan, China) following an in-house protocol. RNA integrity was assessed using a
393 Qubit™ 4 Fluorometer (Invitrogen; Thermo Fisher Scientific, USA) and a Qsep400
394 Bioanalyzer. For mRNA enrichment, polyadenylated RNA was isolated using Oligo(dT)
395 beads, followed by fragmentation with fragmentation buffer. First-strand cDNA was
396 synthesized using random hexamers and reverse transcriptase, while second-strand
397 synthesis was carried out with dNTPs and DNA polymerase I. The resulting double-
398 stranded cDNA was purified, subjected to end-repair, A-tailing, and adapter ligation,
399 followed by PCR amplification. Library quality was assessed using a Qubit™ 4 Fluorometer
400 with an Invitrogen dsDNA HS Assay Kit, and insert size distribution was confirmed using a
401 Bioanalyzer (Agilent, Japan). The final libraries were pooled and sequenced on an Illumina
402 platform in paired-end mode. All biosamples are available in the Sequence Read Archive
403 (SRA) under the accession number PRJNA1216898. Biosample details including sex, family,
404 and mapped reads, are provided in Table S1.

405 Read mapping, variant calling, and filtering, of RNAseq data

406 RNA-seq reads were mapped to the *V. destructor* reference genome
407 (GCF_002443255.2_Vdes_3.0) using HISAT2 v2.2.1 in default settings[60]. The mapping rate
408 varied between samples, with uniquely mapped reads ranging from ~5.88% to 15.71% of
409 the total reads. Clean reads were obtained after quality filtering using fastq v0.23.2[60], with
410 adapters removed, reads containing more than 10% N bases discarded, and bases with
411 quality scores below 20 trimmed. Variant calling was performed using GATK [62], and SNPs
412 and InDels were annotated with ANNOVAR[41]. To ensure high-confidence variant sites, we
413 filtered out low-quality SNPs and retained only sites with adequate coverage and quality

414 metrics, although the exact thresholds were not specified in the company report. We
415 observed thousands of exonic variants per sample, including both synonymous and
416 nonsynonymous SNVs, as well as stop-gain mutations. The resulting variant dataset
417 included annotations for exonic, 5' and 3' UTR regions.

418 *Mathematical modeling*

419 We followed the classical approach used by Wright[63] to mathematically model declines in
420 heterozygosity due to inbreeding within haplodiploid systems. This approach involves
421 constructing path diagrams representing genetic transmission paths from ancestors to
422 descendants and quantifying genetic relatedness through path coefficients. By enumerating
423 paths and calculating their coefficients, we derived two mathematical relationships
424 describing the expected declines in heterozygosity per generation under the reproductive
425 life cycle of Varroa, one for the reproductive system with arrhenotokous diploid males and
426 the other for haploid males. These two equations allowed a direct comparison between the
427 two reproductive systems. Figure 3 plots the two equations in terms of how the rate of
428 decline in heterozygosity, translated into effective population size, changes with the degree
429 of inbreeding, which is measured by the probability of co-founding by females of the same
430 mother. Mathematical details are in Supporting Information 1.

431 *Statistical analysis*
432 All analyses were carried out in the R statistical environment[31]. Bioinformatic analyses and
433 figures are available and reproducible directly from the online supplementary data
434 (https://rpubs.com/Nurit_Eliash/1336154).
435

436 **Acknowledgments**
437 ASM was supported by a Future Fellowship from the Australian Research Council
438 (FT160100178). HZ was supported by the earmarked fund for CARS (CARS-44). We wish to
439 thank local beekeepers of Okinawa, Japan: Tsuto Arakaki, Takashi Ikemiya, and Ryo Kirino,
440 from the Agricultural Environment Coordinator in Onna-village, for their help in providing
441 Varroa-infested colonies, and Ms. Saori Chappell, OIST, for coordinating the sample
442 collection and administrative helping throughout. At OIST, we thank Jan Moran and the

443 HPC team for their support. We wish to thank Alison McAfee for providing the beautiful
444 illustrations of Varroa mites and honey bees incorporated in the manuscript figures. We
445 also thank Ms. Cristina de Morais Cianci from the Editorial Office of Genetics and Molecular
446 Biology journal, for kindly providing a copy of the fundamental research on Varroa
447 cytogenetics[31]. We also wish to thank Yoav Ram for commenting on the earlier draft of the
448 manuscript. Finally, we wish to thank Mikheyev and Economo laboratory members for
449 their continuous support and discussion throughout.
450

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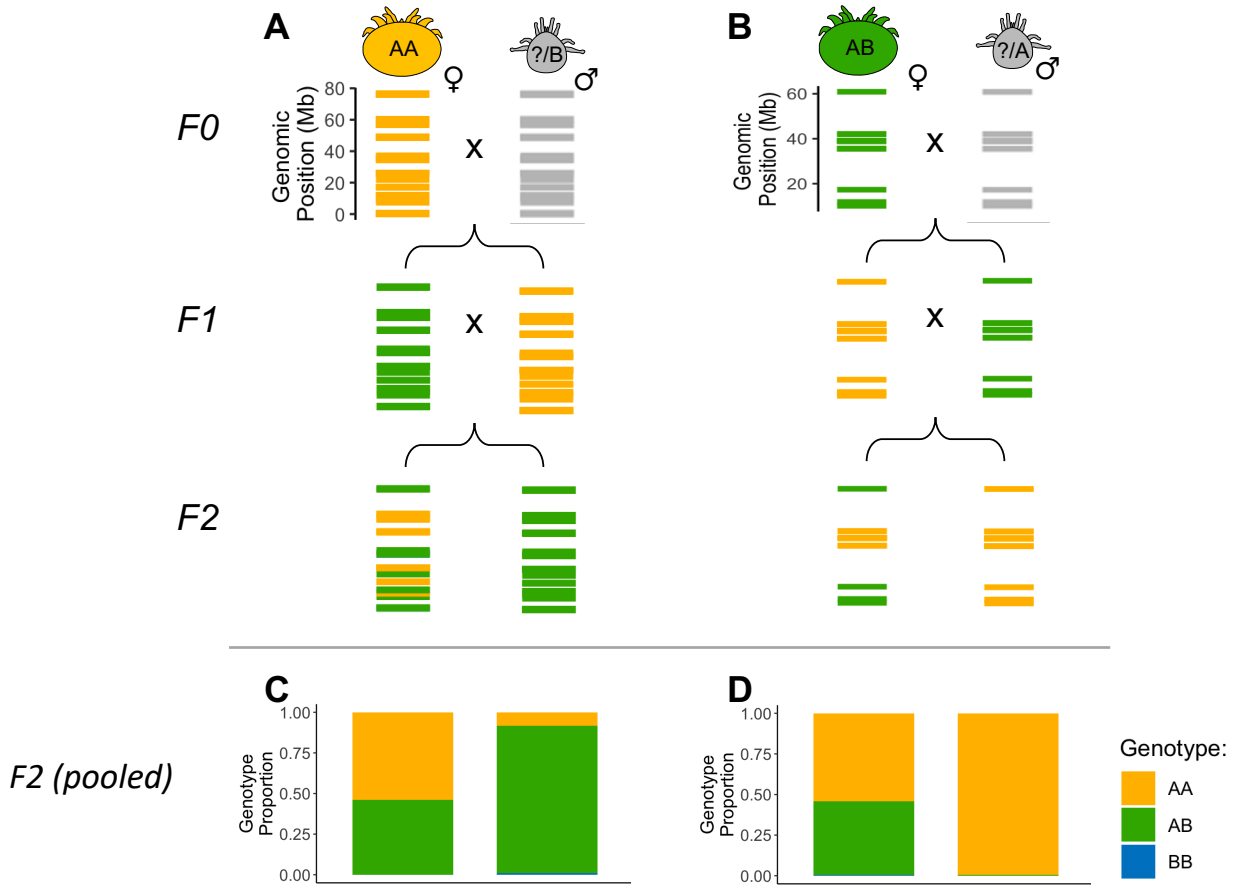
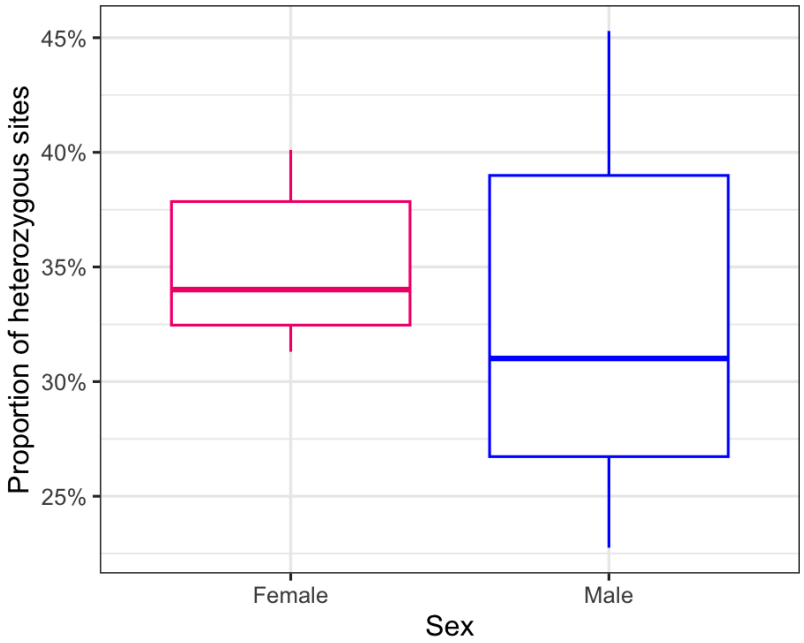


592

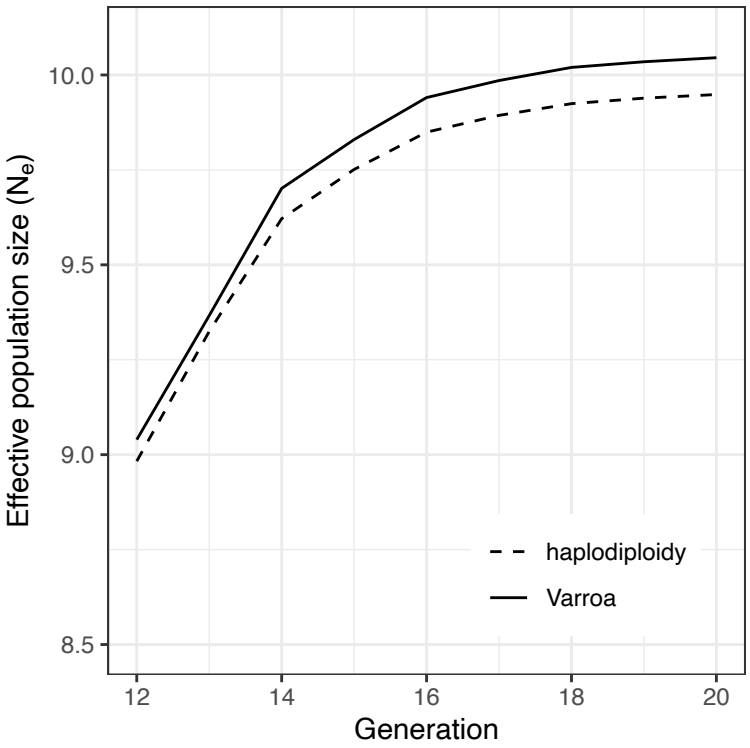
593 **Figure 1. Inheritance patterns in *Varroa destructor* mites shows that males inherit**
594 **two alleles from their mother and transmit both of them to their daughters with**
595 **equal probability. Females are produced sexually. Panels A and B show the genotype**
596 **flow of sites on the largest chromosome, NW_019211454, for a representative family**
597 **spanning three generations: the foundress grandmother (F0 female), her offspring**
598 **(F1 female and male), and their F2 offspring (F2 female and male).** [Panel A]: Family
599 478, crossing a heterozygous F1 female (AB) with a homozygous F1 male (AA), showing the
600 genotypes of their F2 female and male offspring across 33 sites on chromosome
601 NW_019211454. [Panel B]: Family 534, crossing a homozygous F1 female (AA) with a
602 heterozygous F1 male (AB), showing the genotypes of their F2 female and male offspring
603 across 14 sites on chromosome NW_019211454. **Panels C and D show the pooled**
604 **genotype proportions in F2 females and males from all families, across all sites on all**
605 **seven chromosomes, for the same cross types as in A and B, respectively.** [Panel C]:
606 Pooled data for the cross of heterozygous F1 female (AB) × homozygous F1 male (AA): 7 F2

607 females (11,207 sites) and 12 F2 males (16,515 sites). [Panel D]: Pooled data for the cross
608 of homozygous F1 female (AA) × heterozygous F1 male (AB): 11 F2 females (4,999 sites)
609 and 10 F2 males (2,536 sites). F2 females show a ~1:1 ratio of AA:AB genotypes, close to
610 sexual reproduction, while F2 males predominantly inherit their mother's genotype,
611 suggesting maternal inheritance. The data are filtered using strict quality thresholds (VAF <
612 0.01 for AA, 0.2-0.8 for AB, > 0.9 for BB; corresponding QAF thresholds). As expected from
613 AA × AB crosses, no BB genotypes were observed. F0 males were not collected for the
614 pedigree, as they remained in the cell when the honey bee emerges. However, based on the
615 genotypes of the F0 and F1 offspring, we could infer the F0 male genotype. For instance, in
616 the cross of F1 AB female × AA male, the F0 male must have had at least one B allele
617 (indicated as "?/B" in panel A). In the cross of F1 AA female × AB male, the F0 male must
618 have had at least one A allele (indicated as "?/A" in panel B). Colors: Orange = AA, Green =
619 AB, Blue = BB. Note: "?" indicates an inferred, but unobserved allele.
620



621

622 **Figure 2. RNA-seq analysis of heterozygous site proportions in male and female**
623 **Varroa mites.** Each point represents the mean proportion of heterozygous sites per mite,
624 with error bars showing 95% confidence intervals, and lines connecting mother-son pairs.
625 The two sexes show similar heterozygosity proportions in their RNA across five families
626 (mean difference = 0.022 ± 0.10, paired t-test: t = 0.46, df = 4, P-value = 0.672). The y-axis
627 shows the proportion of heterozygous sites as a percentage of total sites analyzed. Males
628 are much smaller than females, and result in lower-quality sequencing libraries, possibly
629 explaining their greater variability. Note that, while males and females are clones, we do
630 not expect identical heterozygosities in transcriptomic data because different genes are
631 expressed in the two sexes.



632

633 **Figure 3. Varroa diploid arrhenotoky results in a greater population size, compared**
634 **to ancestral haplodiploidy under identical population conditions.** This is a
635 representative plot simulated under the following conditions: 2 daughters per mother, a
636 carrying capacity of 100 mites, and 0.5 initial correlation between gametes. The reduced
637 correlation due to male diploidy produces a small but consistent increase in effective
638 population size under inbreeding conditions.

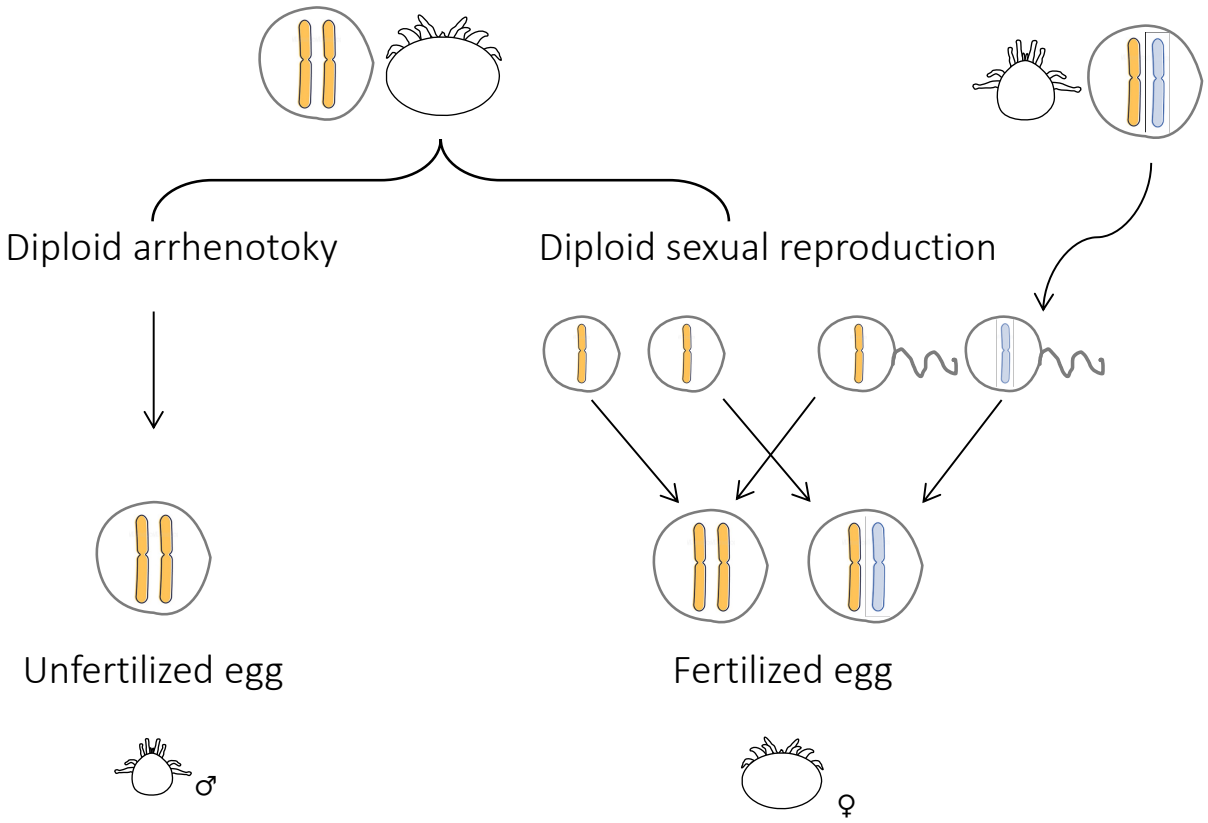


Figure 4. Hypothesized mode of genetic inheritance in Varroa male and female mites. Females are produced via diploid sexual reproduction, while males are produced via diploid arrhenotoky. To illustrate the inheritance of maternal and paternal alleles in the two sexes, we show an example of a cross between a homozygous female and a heterozygous male, and their possible female and male offspring (as in Figure 1).

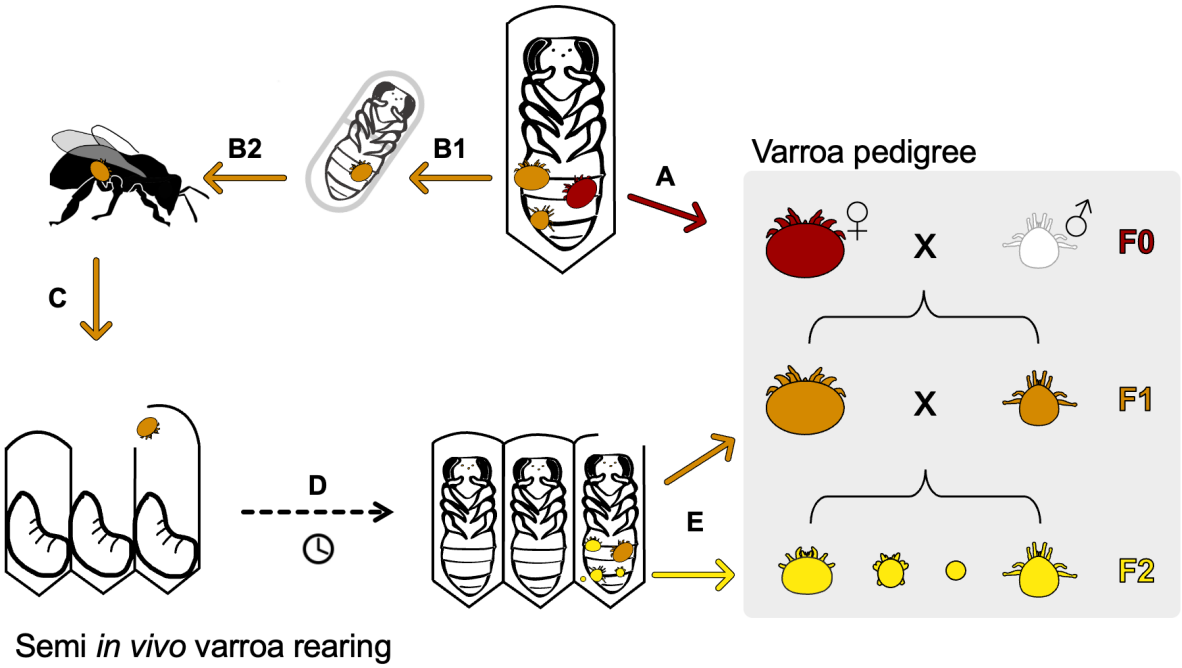


Figure 5. Pedigree experimental design. A three-generation Varroa pedigree (F0-in red, F1-in orange, and F2-in yellow) of 30 families was constructed via the following steps: **A.** Collecting a naturally infested family: foundress female mite (F0), and her two adult offspring: male and female F1 mites (F0 male's genotype was not possible to study because the F0 female was previously mated, typically by a single male); **B.** "Incubation phase": (B1) Transferring the young F1 female onto a bee pupa for 1-4 days, till cuticle sclerotized and darkened; "Dispersal phase": (B2) Transferring F1 female onto a nurse adult bee for another 3 days; **C.** Introducing the matured F1 female to a freshly capped 5th instar larva cell; **D.** F1 female reproducing inside the bee cell; **E.** 10 days post introduction, collecting the artificially infested family: F1 female and her F2 offspring. The F2 generation included a son and one or more daughter nymphs. This design provided known parents and genotypes for the F1 and F2 generations, allowing a detailed examination of inheritance.