Varroa mites escape the evolutionary trap of haplodiploidy

Nurit Eliash[1, 2, 3, 4], Tetsuya Endo[4], Xia Hua[5], J. Spencer Johnston[6], Maeva A. Techer[6, 7], Valerie R. Holmes[6], Juliana Rangel[6], Huoqing Zheng[7], Evan P. Economo[1,8] and Alexander S. Mikheyev[4, 9]

[1] Biodiversity and Biocomplexity Unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Okinawa, Japan
[2] Shamir Research Institute, Israel
[3] University of Haifa, Israel
[4] Ecology and Evolution Unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Okinawa, Japan
[5] Australian National University, Mathematical Sciences Institute, Canberra, Australia 2600
[7] Department of Entomology, Texas A&M University, College Station, Texas, USA
[6] Behavioral Plasticity Research Institute, NSF-BII, USA
[7] College of Animal Sciences, Zhejiang University, Hangzhou 310058, China
[8] Department of Entomology, University of Maryland, College Park, MD, USA
[9] Australian National University, Research School of Biology, Canberra, Australia 2600

*Corresponding author: Nurit Eliash, nurite@gri.org.il

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Abstract

Genetic diversity is essential for populations adapting to environmental changes. Due to genetic bottlenecks invasive species often have reduced genetic diversity. However, they must quickly adapt to changes in new environments, potentially including anthropogenic countermeasures. This paradox raises a fundamental question: how do species adapt to changes while having low genetic diversity? This tension is more pronounced for some species, for example parasites go through additional bottlenecks between hosts and haplodiploid species have a lower effective population size, as males can inherit and transmit only half of their mother's genome. To investigate how species with such traits reconcile diversity and adaptability, we examined inheritance in the Varroa mite (*Varroa destructor*), a well-studied invasive parasite of honey bees fitting all of the above criteria. By following the flow of alleles across three-generation pedigrees we found that Varroa, so far believed to be haplodiploid, is actually not. Rather, it has a unique reproductive system in which females clonally produce functionally diploid sons. While males lose somatic DNA during development, they can transmit either maternal allele to their daughters with equal probability. Modeling shows Varroa loses heterozygosity slower under typical sib-mating conditions, relative to haplodiploidy. This permits a greater effective population size relative to a purely haplodiploid system and increased evolutionary potential. This reversion to diploidy is a singular example of escaping the 'evolutionary trap' of haplodiploidy, which is believed to be an evolutionary stable end state. Plasticity in reproductive systems could be more common than assumed, and may potentially explain the remarkable resilience and high adaptivity of Varroa and other invasive parasites.

Significance

Varroa mites have driven the collapse of honey bee populations since their worldwide spread in the middle of the 20th century. Despite repeated genetic bottlenecks, Varroa has adapted to diverse environments and has overcome many pesticides. Using pedigree analysis, we found that Varroa re-evolved diplodiploid reproduction from an evolutionary history of haplodiploidy. Diplodiploidy permits a higher effective population size and evolutionary potential, likely facilitating Varroa's ongoing success. Females produce males clonally, passing on their entire genomes. Varroa is a singular exception to the theoretically

and empirically observed rule that, once evolved, haplodiploidy is an evolutionarily stable end state (an 'evolutionary trap'). Novel mechanistic studies of even well-known organisms can lead to surprising insights into the evolutionary plasticity of reproductive systems.

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). Selection dominates at higher population sizes, yet in small populations, beneficial alleles can be lost due to drift. 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 are highly adaptable 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 such mechanisms requires multi-generation-controlled crosses, which are often difficult if not impossible, 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. The mites 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 and three to four females) (19, 20). This mating behavior further increases the strength of genetic drift and makes natural selection less effective. Varroa is believed to be haplodiploid, which further reduces the effective population size relative to diplodiploid systems (21, 22). Yet, Varroa shows high adaptability: it efficiently switches between host species (23–25), and can rapidly evolve resistance to pesticides via various mechanisms (26). 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 with fertilized eggs developing into diploid females, while unfertilized eggs develop into haploid males (20, 27–29). Haplodiploidy was inferred from two karyotyping studies (30, 31). However, as those studies examined Varroa embryos, 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), which 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 (32, 33). Cruickshank and Thomas (34) used phylogenetic reconstruction to infer that Varroa evolved from diploid ancestors, via intermediate pseudo-arrhenotokous species Note that while Varroa was included in this study, its haplodiploidy was assumed based on the fact that all members of its family are haplodiploid. However, the taxon sampling in this study was sparse and until recently, the phylogenetic placement of Varroa was uncertain, opening conclusions to criticism (35). However, a recent phylogenetic analysis of Varroa placed it as a subfamily within Laelapidae allowing a more targeted comparison (36). Of

116 the 19 Laelapid sexually reproducing mites in the Tree of Sex database (37) only a single
117 one is not haplodiploid – *Dicrocheles phalaenodectes*, a parasite inhabiting the ear canal of
118 some Noctuid moths. However, the placement of *Dicrocheles* in Laelapidae is based solely
119 on morphological data, and has been debated (38); it is certainly only distantly related to
120 Varroa. Thus, based on the sum total of the evidence, haplodiploidy was ancestral to
121 Varroa.
122
123 Theory suggests that reversal to diploidy from arrhenotoky is unlikely (33), and, indeed, no
124 reversals of arrhenotokous sex determination are known to date (39). Yet, despite strong
125 circumstantial evidence suggesting the existence of arrhenotokous parthenogenesis in
126 Varroa, no study has examined Varroa's mode of genetic inheritance, making its success in
127 spite of genetic handicaps somewhat of a mystery. Here, we explicitly examine this species'
128 mode of genetic inheritance using multi-generational pedigrees.
129
130 **Results**
131 Three lines of evidence suggest that Varroa males are functionally diploid: [1] they inherit
132 two maternal alleles evident in their genotypes (Fig 1), [2] they randomly transmit these
133 genotypes to their daughters (Fig 1B), and [3] both alleles are expressed in somatic cells
134 (Fig 2). Interestingly, both flow cytometric and karyotyping investigations suggest that
135 adult females have twice as much genetic material and chromosomes as males (Fig S1 and
136 S2). This suggests that during development half the chromosomes are eliminated in males.
137
138 *Males are produced by diploid arrhenotoky preserving their mothers' genotype.*
139 Adult males can carry up to two alleles (Figure 1, Fig S3). Specifically, males inherit their
140 mothers' genotype, in contrast to their sisters, which are sexually produced (Fig 1).
141
142 *Males transmit their two alleles to their daughters with equal probability*
143 Consistently, males' maternal inheritance was also observed for the heterozygous variants.
144 Looking at the cross between a heterozygous F1 female (AB) and a homozygous F1 male
145 (AA), we found that 97% of the variants in F2 males maintained their mother's
146 heterozygosity (Fig 1A and 1C). Furthermore, males not only inherit two alleles, but they

147 also randomly transmit each allele to their daughters. Looking at the cross between a
148 homozygous female (AA) and a heterozygous F1 male (AB), we observed that about ~50%
149 of the variants in F2 females were homozygous (like mother) and the rest were
150 heterozygous (like father) (Fig 1B and 1D). F2 male offspring of the same cross were
151 homozygous (AA), just like their mother.
152
153 *Males carry and express two alleles*
154 Varroa males not only inherit and transmit two alleles, but also express both alleles, as
155 evident from RNA-seq data of adult female mites and their sons (Fig 2, Table S1). The two
156 sexes show similar heterozygosity proportions in their RNA, across five families (mean
157 difference = 0.022 ± 0.10, paired t-test: t = 0.46, df = 4, P = 0.672).
158
159 *Ploidy assessment suggest females have twice genetic material of males*
160 Flow cytometric and karyotyping investigations suggest that females have twice as much
161 genetic material as males (Fig S1 and S2). While karyotyping data were in general error-
162 prone, analysis of eight male and seven female immature mites shows that males had
163 significantly fewer chromosomes (mean = 8.79 ± 0.92 chromosomes) than the expected
164 diploid number of 14 (t-test: t = -6.59, df = 7, P = 0.0003; Fig S1). Flow cytometric studies of
165 75 samples showed that females had diploid peaks (2C) and males have the major peak at
166 1C, indicating that the bulk of the cells are haploid (Fig S2).
167
168 **Discussion**
169 In this study, we found that *V. destructor* has evolved from ancestral haplodiploidy (34) to a
170 novel reproductive strategy with diploid arrhenotokous males that are clones of their
171 mothers (for a scheme of the hypothesized mode of genetic inheritance see Fig 3). This strategy
172 has both short-term and long-term potential benefits for Varroa, each overcoming a
173 different evolutionary 'paradox'. In the short-term, the production of clonal males increases
174 the females' fitness by assuring transmission of both of her genetic variants. This strategy
175 can overcome the "paradox of sex" by blending the benefits of sexual and asexual
176 reproduction. Namely, we found that females can produce sons containing both copies of
177 their genes, but female eggs are produced meiotically. In the long term, functional

178 diplodiploidy can potentially increase the effective population size (vs. haplodiploidy) (Fig
179 S4). A higher effective population size imparts greater evolutionary potential, and indeed
180 Varroa has been remarkably adaptable despite repeated bottlenecks exemplifying the
181 "genetic paradox of invasion".
182 Another evolutionary surprise from this study is the effective loss of haploid arrhenotoky.
183 Once evolved, asymmetric inheritance appears to be in an evolutionarily stable state,
184 sometimes referred to as an 'evolutionary trap' (33). Obstacles to re-evolving Mendelian
185 inheritance are diverse. One obstacle is mechanistic, in that males would need to re-evolve
186 meiosis, which was always thought to be difficult (40). However, recent work from asexual
187 stick insects indicates that male meiotic regulatory mechanisms are preserved in the
188 females (41), so re-evolving male meiosis may be easier than expected. The other obstacle
189 is evolutionary, as examined in detail by Bull (42). Females reach a fitness optimum in
190 haplodiploid systems, being able to control male production and thus the sex ratio. Thus,
191 re-emergence of diploid males would have to be a male trait, and biparental diploid males
192 must carry mutations that allow them to produce diploid sons. However, given the level of
193 arrhenotokous female control over fertilization and sex ratios, these traits are theoretically
194 unlikely to spread (42). Indeed, the only evolutionary feasible transition from
195 haplodiploidy appears to be towards parthenogenesis, though these are short-lived (43).
196 Yet, Varroa overcame the constraint on the production of diploid sons by having females
197 solely control the sex ratio, as well as diploid male production. The production of meiotic
198 sperm and the evolution of diploid arrhenotokous male production illustrate how the
199 unforeseen complexity of biological mechanisms may challenge long-standing elegant
200 mathematical models.
201
202 The mechanism by which sex is determined in Varroa remains unclear, but other
203 facultatively parthenogenetic systems provide insights into how it may occur. For instance,
204 Cape honey bees (*Apis mellifera capensis*) store new eggs in their oviducts arrested in
205 metaphase I, while meiosis II takes place only after oviposition (44). If the egg is not
206 fertilized, it develops into a female by central fusion. If it is, a diploid female is sexually
207 produced. A similar mechanism may be at play in Varroa, except that unfertilized eggs
208 undergo central fusion to develop into males while preserving their mother's genotype (Fig

209 1). Unlike in honey bees, where sex is controlled genetically, an epigenetic factor would be
210 required for sex determination in Varroa.
211
212 While Varroa males are a mosaic of their mother's genotype, they eliminate one copy from
213 each cell during development (Fig S1 and S2). The mechanisms for genome elimination are
214 unclear, though genome elimination appears to take place rapidly, as early male nymphal
215 stages are already haploid. Indeed, elimination may happen during development in the
216 mother's ovaries, which would explain findings by two previous karyotyping studies that
217 found the presence of haploid embryos (30, 31). Haploidy in somatic tissues may maintain
218 the dosage of male-specific genes, which have evolved in a haploid context. Given that the
219 maternal genotype is preserved (Fig 1), the elimination process appears to be random, and
220 the male somatic genotype is likely chimeric. Unusually, spermatogenesis in Varroa is an
221 ongoing process during the adult stage (45, 46). This suggests that genome elimination
222 happens only in the male somatic tissues while germline remains diploid. Varroa genome
223 elimination differs from either symbiont-induced or paternal genome elimination
224 (reviewed in (47, 48)) as it does not arise from a reproductive conflict between the sexes
225 and thus does not favor the maternal genome.
226
227 *Conclusions*
228 Our work underscores the importance of comprehensive experimental inquiries into
229 diverse reproductive mechanisms, even in organisms that appeared to be well studied.
230 Recent investigations have unveiled a broader spectrum of reproductive strategies than
231 were previously recognized (40), exemplifying the dynamic nature of biological systems.
232 Yet, the discovery of a novel reproductive system, such as that in Varroa, can have wide-
233 ranging fundamental and applied consequences. For instance, Varroa's haplodiploidy is
234 assumed when considering how it evolves in response to countermeasures, such as
235 acaricides, breeding honey bees for parasite tolerance, and meiotic drive (49–51).
236 Furthermore, Varroa has emerged as a compelling model for the study of the evolutionary
237 dynamics of reproductive systems, offering insights into mechanisms for overcoming
238 evolutionary constraints and the intricacies of sex determination. These findings open

239 promising avenues for future research, contributing to a deeper understanding of
240 evolutionary innovation and reproductive plasticity in Varroa and beyond.
241
242 **Materials and methods**
243
244 *Honey bees and mites*
245 For the pedigree study: Mites and honey bees (*Apis mellifera* L.) were obtained from
246 colonies at the Onna village apiary and the experimental apiary of Okinawa Institute of
247 Science and Technology (OIST) in Okinawa, Japan. All colonies used in the experiment were
248 maintained without any treatment against Varroa and received sugar solution and 70%
249 pollen cakes as supplemental feed when necessary. Experiments were conducted between
250 July and November 2020.
251 For comparative transcriptomic analysis of Varroa gene expression heterozygosity
252 in mothers and sons: Mites were collected from *A. mellifera* colonies at the Zhejiang
253 University, China in November 2024. A total of ten mites were collected from five families:
254 a mother and her son from each family.
255 For the flow cytometry study: Mites were freshly collected from drones and worker
256 brood frames of *A. mellifera* colonies at the Texas A&M University Honey Bee Lab apiary
257 (Janice and John G. Thomas Honey Bee Facility). Mites were collected in June, July, and
258 December 2022. Capped cells containing red-eye pupae (day 15) to emerging adults (day
259 19-20) were carefully opened and inspected for single infestation, following standard
260 methods and procedures (52). The developmental stages and identification of vigorous
261 specimens were conducted using a soft and fine paintbrush within a petri dish under a
262 Leica macro-binocular.
263 For the karyotyping study: Varroa mites were collected from a drone brood frame
264 obtained from Onna village apiary, Okinawa, Japan, between January-February 2022. Mite
265 eggs and nymphs were collected from single-infested cells directly into a PBS buffer, to
266 preserve their vitality, and fixed within 3 hours after collection. First laid eggs, presumably
267 developing into males ((52) and personal observations), were collected from prepupa (~3
268 days post capping). Second and third laid eggs, presumably developing into female mites,
269 were collected from a pupa in later developmental stages. Protonymphs and deutonymphs

270 were collected from a pink eye pupa bee brood stage (~8 days post capping). The sex of
271 the nymphs was determined based on the shape of the end part of the ideosoma: males
272 have a more rectangular shape, while female nymphs have a perfect round shape (53).
273
274 *Artificial infestation for Varroa pedigree construction*
275 To obtain a three-generational mite pedigree (F0, F1, and F2), we used a semi-natural
276 infestation system. This started with the collection of a naturally infesting mated Varroa
277 female (F0). Her adult daughter (F1) was then artificially introduced into a honey bee pre-
278 pupal cell and allowed to commence the reproductive phase of its life cycle. The F1 mite
279 and her offspring (F2) were then collected, at the end of the reproductive cycle. For a
280 detailed description of the experimental design, see Fig 4 and the sections below. Using this
281 approach, we could genotype all members of the pedigree, except for the male that mated
282 with the F0 female.
283
284 Collection of naturally infesting families (F0 and F1 generations)
285 To obtain mites from the first two generations (F0 and F1), naturally infested families were
286 collected from within the cells of 18- to 20-day-old worker bee pupae. At this
287 developmental stage of the honey bee host, there is typically one dark brown female mite
288 (the foundress, F0), one adult male (the foundress's son, F1), at least one light-colored
289 female (the first foundress's daughter, F1), a few nymphs females, and an egg (27). We
290 determined each mite's sex and developmental stage according to external morphology and
291 cuticle coloring based on (53) for the color index, please see Fig S5. To ensure that all
292 offspring belonged to a single family, we thoroughly examined the cell content to confirm
293 that it was infested by a single foundress mite and that no other adults were present.
294 Except for the adult female daughters (F1), the rest of the family members were preserved
295 in a separate microcentrifuge tube kept at -20 °C for later DNA extraction. The adult F1
296 daughters were easily distinguished from the F0 foundress mite by their lighter cuticle
297 color (color index 0-2, see Fig S5), and were assumed to have already copulated with their
298 brother, the F1 male, by the time of the collection (20). Next, the F1 young adult females
299 were prepared to be artificially introduced into a freshly capped 5th instar larva cell to start
300 their first reproductive cycle.

301 Incubation ("phoretic" and "dispersal" phases) of F1 female mites

302 Based on our and others' observations (54), the survival and fecundity of young adult mites
303 increase if they stay for a few days on an adult nurse bee before starting their first
304 reproductive cycle. This incubation period is presumably imitating the "dispersal stage", in
305 which the adult female mite infests an adult honey bee (54, 55). Therefore, the collected
306 young adult females (F1 generation) spent three days on a nurse bee before infestation.
307 However, during the experiment, we noticed that the very young adult daughter's cuticle
308 was light and fragile, and most of the mites did not survive when they were transferred
309 directly from the pupae onto a nurse bee. Therefore, we let the young mites first feed on a
310 white-eyed pupa in a gelatin capsule for one to four days until their cuticle was sclerotized
311 and darker (color index 3-4, see Fig S5). After this intermediate period, the mites were
312 marked on their dorsal idiosoma (PC-1MR POSCA, Japan), and transferred onto an adult
313 nurse bee in a cage for another three days, for the above-mentioned "dispersal stage". In all
314 incubation periods, the mites were kept in a controlled dark environment, imitating hive
315 conditions (34-34.5 °C, 60-75% RH).

316

317 Artificial infestation of F1 female mites

318 After the artificial "dispersal phase", the mites were brushed off the bees using a fine paint-
319 brush following anesthesia by a 20 seconds exposure to CO_2, 0.2mPa at 10 L/hour, and
320 placed on a moist filter paper to avoid desiccating till infestation. The mites were then
321 introduced to a worker bee cell, one mite per cell, as described by (52). In detail, six hours
322 before infestation, cells containing a 5th instar larva (about to be sealed) were marked on
323 an acetate sheet attached to a frame from a mite-free hive. The frame was then placed back
324 into the hive so that the bees could seal the cells. Five hours later the frame was taken into
325 a warm room (25-28 °C) for the F1 females to be introduced into the freshly sealed cells.
326 For the artificial infestation, a precise incision (3-5 mm) was made at the margin of the cell-
327 capping using a surgical blade (N 11). After the incision was made, the cell-capping was
328 slightly lifted, just enough for the mite to crawl under the capping and into the cell. The
329 mite was then placed at the entrance of the cell using a moist fine paintbrush, with its head
330 facing the cell opening. After the mite had entered the cell, it moved under the larva to the

331 cell bottom. The cap was then resealed by gently pushing it down using the back end of the
332 paintbrush. The infested frame was then incubated in a dark controlled environment (34-
333 34.5 °C, 60-75% RH), and emerging bees were removed daily.

334 Collection of an artificially infested family (F2 generation)

335 Ten days after the F1 mites' infestation, the cells were opened, and their contents were
336 examined. The family members' sex, developmental stage (adult, nymph, or egg), and
337 viability were recorded. To assure the parenthood of the offspring (F2), double-infested
338 cells and unmarked mites were not included in the final analysis. In total, out of 78
339 successful infestations, 14 were double-infested by a non-marked mite. Each individual was
340 snap-frozen in a separate tube at -20 °C for later DNA extraction. In total, we collected 223
341 mites belonging to 30 families. Each family was composed of a foundress adult female (F0),
342 her male and female adult offspring (F1), and their F2 offspring (at least one male/female
343 mite in various developmental stages).
344
345 *Genomic analysis of the Varroa pedigree*

346 DNA extraction, library preparation, and sequencing

347 Genomic DNA was extracted from each single mite following the protocol described
348 previously (25). In brief, after surface sterilization using absolute ethanol, each mite was
349 crushed in a 1.5 mL microcentrifuge tube dipped in liquid nitrogen. The crushed mite was
350 then processed using QIAamp DNA Micro Kit (© Qiagen) following the manufacturer's
351 instructions and eluted in 20 µL of water. Total dsDNA quantity was measured using a
352 Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and DNA quality was
353 verified using a Bioanalyzer 2100 with high-sensitivity DNA kits (Agilent, Japan). Short-
354 inserts of 150-bp paired-end libraries were prepared for each individual using a Nextera
355 XT DNA Library Preparation Kit (Illumina, Japan) and 16 PCR cycles. cDNA was then
356 cleaned and size-selected using CA-magnetic beads (Dynabeads® MyOne Carboxylic Acid,
357 Invitrogen), and 11-11.5% PEG 6000 (Sigma-Aldrich, Japan). The library quality was
358 measured using a Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and its

359 size was assessed using a 4200 Tapestation with a high-sensitivity D5000 kit (Agilent,
360 Japan). Libraries were run on a NovaSeq6000 in 150 bp x 2 paired-end mode (Illumina,
361 Japan) at the OIST Sequencing Center. All biosamples are available in the Sequence Read
362 Archive (SRA) under the accession number PRJNA794941. Biosample details including sex,
363 developmental stage, pedigree information, and sequencing coverage, are provided in
364 Table S2.

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

366 We mapped all reads to the *V. destructor* reference genome [GCF_002443255.1] (18) using
367 the very sensitive mode of NextGenMap v0.5.0 (56). Reads were sorted and duplicates were
368 removed using SAMtools (57). We called variants against the reference genome using
369 FreeBayes v1.1.0 (58) with the following parameters: minimum alternative allele counting
370 = 5, minimum alternative allele fraction = 0.2, minimum mapping quality = 8, minimum
371 base quality = 5, and using the four best SNP alleles. The variant calling resulted in a total of
372 73,903,468 variated sites. As our population consisted of closely related individuals (mite
373 families from the same apiary, in a high-inbreeding system), we did not expect to detect
374 many variants. Therefore, we set the minor allele frequency threshold to 0.2, which
375 includes sites with a minor allele frequency of 20% or more, to exclude any rare variants
376 due to mutations or possible mapping errors. We included variants only for the seven
377 chromosomes (excluding the mitochondrial DNA and unscaffolded regions). In addition, we
378 filtered for the following site quality parameters: we included only bi-allelic sites, a depth
379 range between 16-40, and maximum missing data per site = 0.5. The variants' quality
380 distribution showed a clear bimodal distribution with a cutoff at ~10,000 (Fig S6). To
381 ensure the quality of the variants for the genetic inheritance analysis, we chose a strict
382 filter of minimum quality of 15,000. Filters were applied using VCFtools v0.1.12b (59)
383 resulting in 33,925 final variants in 223 individuals. The pipeline from fastq files down to
384 the VCF file was constructed using Snakemake (60), and codes are available and
385 reproducible on GitHub [https://github.com/nurit-eliash/Varroa-pedigree-study]. For
386 further security of site quality, we applied two additional site-quality parameters to
387 account for biases in alternate allele representation and to ensure high-confidence

388 genotype calls based on allele frequency and quality. Variant allele frequency (VAF) was
389 calculated as *VAF = AO/DP*, where AO (Alternate Observations) represents the number of
390 reads supporting the alternate allele, and DP (Depth) is the total read depth at the site.
391 Similarly, Quality Allele Frequency (QAF) was computed as *QAF = QA/QA+QO*, where QA
392 (Quality of Alternate Allele) is the sum of Phred-scaled base quality scores for reads
393 supporting the alternate allele, and QO (Quality of Reference Allele) is the sum of Phred-
394 scaled base quality scores for reads supporting the reference allele. To minimize errors in
395 genotyping due to sequencing artifacts or depth-related biases, we applied genotype-
396 specific thresholds for VAF and QAF based on observation of values from F2 females of
397 which parents and grandmother genotypes are fixed, and so their own genotype is
398 assumed with high confidence (Fig S7, S8 and S9 for QAF and VAF distribution, in
399 genotypes AA, BB and AB, in accordance). The thresholds (Table 1), ensure that
400 homozygous reference (AA) sites exhibit low alternate allele support (VAF and QAF < 0.01),
401 heterozygous (AB) sites maintain a balanced allele distribution (0.2< VAF < 0.8, 0.2 < QAF <
402 0.8), and homozygous alternate (BB) sites demonstrate high alternate allele support (VAF
403 and QAF > 0.9). This filtering approach enhances the accuracy of genotype classification
404 while reducing the inclusion of low-confidence variant calls.
405
406 **Table 1.** Quality Allele Frequency (QAF) and Variant allele frequency (VAF) threshold and
407 their ideal values used for sites' filtration in the genotype flow of mites' pedigree.

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

408
409 *Genotype validation using Sanger sequencing*
410 Several genotype calls obtained from whole genome sequencing were validated by Sanger
411 sequencing using the same genomic DNA extracted. For the validation, we randomly
412 selected three genes, located on different chromosomes, each containing at least one
413 variant site. In total, we re-sequenced 14 individual mites from four families on six variant

414 sites. The sequenced region was located on the exons of three genes: alpha-mannosidase
415 2x-like (LOC111243621 on chromosome NW_019211455.1), 5-oxoprolinase-like
416 (LOC111252938, on chromosome NW_019211459.1), and probable E3 ubiquitin-protein
417 ligase RNF144A-A (LOC111254119, on chromosome NW_019211460.1). Primers were
418 designed with the NCBI primer design tool (utilizing Primer3 and BLAST), with default
419 parameters and product size set to 300-1,000 bp. Primer sequence, product length, and
420 gene accession numbers are provided in Table S3. The size of the PCR product was checked
421 by running in 1% agarose gel (135V, 20 min), purified, and size selected using the solid
422 phase reverse immobilization (SPRI) method. The purified PCR products were Sanger
423 sequenced on a ThermoFisher SeqStudio Genetic Analyzer using the original reverse
424 primer and BigDye® Direct Cycle Sequencing kit (Thermo Fisher Scientific), following the
425 manufacturer's instructions. We then aligned the reverse complementary sequences to the
426 Varroa reference genome (GCF_002443255.1), to detect and validate the variant sites using
427 Geneious Prime 2022.1.1. (https://www.geneious.com). For sequence alignments and the
428 validated sites please see Fig S10.
429
430 *Ploidy assessment*

431 Flow cytometry

432 We used flow cytometry of the Varroa mite nuclei to assess the male and female mites'
433 ploidy during development from nymphs to adults. Live mites were freshly collected and
434 snap-frozen in liquid nitrogen before being processed. Samples were ground using 15
435 strokes of the "A" pestle in a 2 mL Kontes Dounce tissue grinder and filtered through a
436 40μm nylon mesh. Each sample consisted of an individual mite: for immature mites (larvae
437 and nymphs), a whole larval body, and for adult mites, dissected somatic or germinal
438 tissues. The DNA in the nuclei released by grinding was stained for 2 hr under dark
439 refrigeration with 25 μg/mL propidium iodide. The mean red PI fluorescence of the stained
440 nuclei was quantified using a Beckman-Coulter (Brea, CA) CytoFlex flow cytometer with a
441 solid-state laser emitting at 488 nm. As a standard, we used nuclei from the brain of
442 *Drosophila virilis,* 1C =328 Mbp (61). The total quantity of DNA in the sample was
443 calculated as the ratio of red fluorescence of the sample/standard (mean red fluorescence

444 of the 2C peak of the sample divided by the mean fluorescence of the 2C peak of the
445 standard), times the 1C amount of DNA in the standard. In total, 75 samples were evaluated
446 of different sex and developmental stages. We scored these samples to produce biological
447 replicates, to calculate the standard error of the mean genome size estimate. To identify the
448 ploidy level of a Cytometric DNA peak (62), genome size is reported as 1C, the mean
449 amount of DNA in Mb in one copy of a single complete genome.

450 Karyotyping

451 Sample fixation and staining: Procedure was based on the air-dry method by (63). In
452 brief, in a Colchicine-hypotonic solution (0.005 %), embryos were dissected from the eggs
453 while nymph mites were punctured to allow the solution to penetrate the inner tissues. The
454 samples were incubated in the Colchicine-hypotonic solution for 20-60 mins to inflate cells,
455 and increase mitosis events. The samples were then incubated in 60% 1:1 Acetic-ethanol
456 for 30-60 mins, quickly macerated then treated with 1:1 Acetic-ethanol for 1-2 mins
457 followed by 100% Glacial acetic acid. The samples were left to dry in room temperature for
458 one day then stained for 10 mins in 3% Giemsa solution in Sorensen's Phosphate buffer,
459 and covered with cover slip before visualization.
460 Karyotyping and data analysis: Chromosomes karyotyping was done by imaging the
461 fixed samples under a Nikon Ti2 fully motorized inverted microscope equipped with a
462 linear encoded motorized xy stage, controlled by NIS Elements software. A white LED was
463 used as a diascopic light source, and pictures were taken using a Nikon DS-Qi2 camera.
464 Cells undergoing mitosis (in both anaphase and metaphase) were used to determine the
465 chromosome count in each sample. All imaging was done at the OIST Imaging Section. For
466 examples of imaging of males and females please see Fig S11 and S12 in accordance.

467 K-mer distribution

468 We used the Smudgeplot method (64) to visualize and estimate the ploidy of male and
469 female genomes by analyzing heterozygous k-mer pair, from the raw reads of a male
470 Varroa mite (BioProject PRJDB6279, runs DRR121854 and DRR124352 for male and
471 females respectively).
472

473 *Transcriptomic analysis of Varroa mother and son*

474 RNA extraction, library preparation, and sequencing

475 Total RNA was extracted from each single mite and sequenced by Metware Biotechnology
476 Co., Ltd. (Wuhan, China) following an in-house protocol. RNA integrity was assessed using a
477 Qubit™ 4 Fluorometer (Invitrogen) and a Qsep400 Bioanalyzer. For mRNA enrichment,
478 polyadenylated RNA was isolated using Oligo(dT) beads, followed by fragmentation with
479 fragmentation buffer. First-strand cDNA was synthesized using random hexamers and
480 reverse transcriptase, while second-strand synthesis was carried out with dNTPs and DNA
481 polymerase I. The resulting double-stranded cDNA was purified, subjected to end-repair, A-
482 tailing, and adapter ligation, followed by PCR amplification. Library quality was assessed
483 using a Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and insert size
484 distribution was confirmed using a Bioanalyzer (Agilent, Japan). The final libraries were
485 pooled and sequenced on an Illumina platform in paired-end mode. All biosamples are
486 available in the Sequence Read Archive (SRA) under the accession number PRJNA1216898.
487 Biosample details including sex, family, and mapped reads, are provided in Table S1.

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

489 RNA-seq reads were mapped to the *V. destructor* reference genome
490 [GCF_002443255.2_Vdes_3.0] using HISAT2 v2.2.1 in default settings (65). The mapping
491 rate varied between samples, with uniquely mapped reads ranging from ~5.88% to
492 15.71% of the total reads. Clean reads were obtained after quality filtering using fastq
493 v0.23.2 (66), with adapters removed, reads containing more than 10% N bases discarded,
494 and bases with quality scores below 20 trimmed. Variant calling was performed using
495 GATK (67), and SNPs and InDels were annotated with ANNOVAR (68). To ensure high-
496 confidence variant sites, we filtered out low-quality SNPs and retained only sites with
497 adequate coverage and quality metrics, although the exact thresholds were not specified in
498 the company report. We observed thousands of exonic variants per sample, including both
499 synonymous and nonsynonymous SNVs, as well as stop-gain mutations. The resulting
500 variant dataset included annotations for exonic, 5' and 3' UTR regions.

Mathematical modeling

We followed the classical approach used by (69) to mathematically model declines in heterozygosity due to inbreeding within haplodiploid systems. This approach involves constructing path diagrams representing genetic transmission paths from ancestors to descendants and quantifying genetic relatedness through path coefficients. By enumerating paths and calculating their coefficients, we derived two mathematical relationships describing the expected declines in heterozygosity per generation under the reproductive life cycle of Varroa. One for the reproductive system with arrhenotokous diploid males and the other for haploid males. These two equations allowed a direct comparison between the two reproductive systems. Figure 4 plots the two equations in terms of how the rate of decline in heterozygosity changes with the degree of inbreeding, which is measured by the probability of co-founding by females of the same mother. Mathematical details are in Supporting Information 1.

Statistical analysis

All analyses were carried out in the R statistical environment (70). All bioinformatic analyses and figures are available and reproducible directly from the online supplementary data [https://rpubs.com/Nurit_Eliash/1288713].

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537

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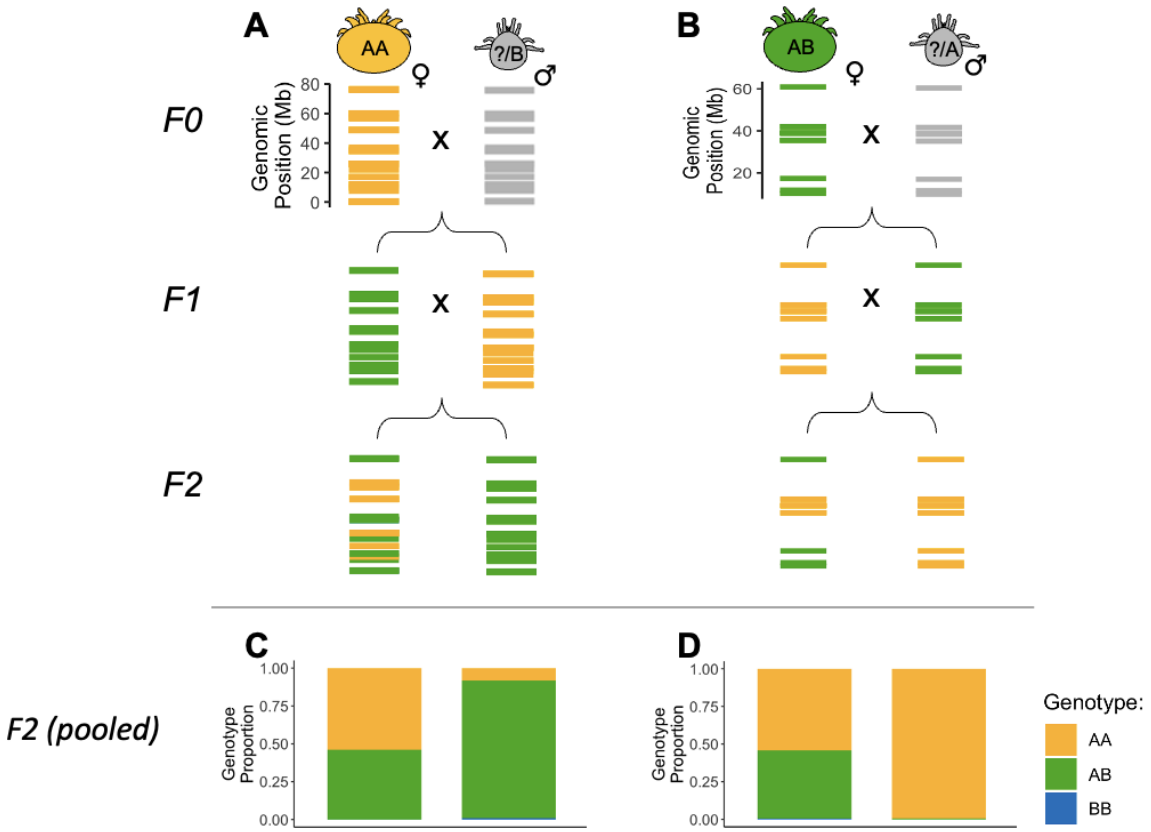
686

687 **Main figures:**



688

689 **Figure 1. Inheritance patterns in *Varroa destructor* mites shows that males inherit two**
690 **alleles from their mothers and transmit both of them to their daughters with equal**
691 **probability. Females are produced sexually.**
692 **Panels A and B show the genotype flow of sites on the largest chromosome, NW_019211454,**
693 **for a representative family spanning three generations: foundress grandmother (F0 female),**
694 **her offspring (F1 female and male), and their F2 offspring (F2 female and male).** A: Family
695 478, crossing a heterozygous F1 female (AB) with a homozygous F1 male (AA), showing the
696 genotypes of their F2 female and male offspring across 33 sites on chromosome NW_019211454. B:
697 Family 534, crossing a homozygous F1 female (AA) with a heterozygous F1 male (AB), showing the
698 genotypes of their F2 female and male offspring across 14 sites on chromosome NW_019211454.
699 **Panels C and D show the pooled genotype proportions in F2 females and males from all**
700 **families, across all sites on all seven chromosomes, for the same cross types as in A and B,**
701 **respectively.**

C: Pooled data for the cross of heterozygous F1 female (AB) × homozygous F1 male (AA): 7 F2 females (11,207 sites) and 12 F2 males (16,515 sites). D: Pooled data for the cross of homozygous F1 female (AA) × heterozygous F1 male (AB): 11 F2 females (4,999 sites) and 10 F2 males (2,536 sites).

F2 females show a ~1:1 ratio of AA:AB genotypes, close to sexual reproduction, while F2 males predominantly inherit their mother's genotype, suggesting maternal inheritance. The data is filtered using strict quality thresholds (VAF < 0.01 for AA, 0.2-0.8 for AB, > 0.9 for BB; corresponding QAF thresholds) to ensure high-confidence genotype calls.

As expected from AA × AB crosses, no BB genotypes were observed. F0 males were not collected for the pedigree, as they remain in the cell when the honey bee emerges. However, based on the genotypes of the F0 and F1 offspring, we can infer the F0 male genotype: In the cross of F1 AB female × AA male, the F0 male must have at least one B allele (indicated as “?/B” in panel A). In the cross of F1 AA female × AB male, the F0 male must have at least one A allele (indicated as “?/A” in panel B). Colors: Orange = AA, Green = AB, Blue = BB. Note: “?” indicates an inferred but unobserved allele.

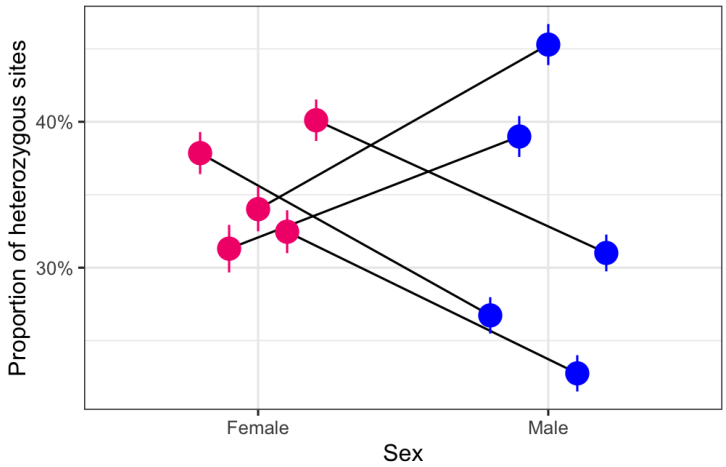
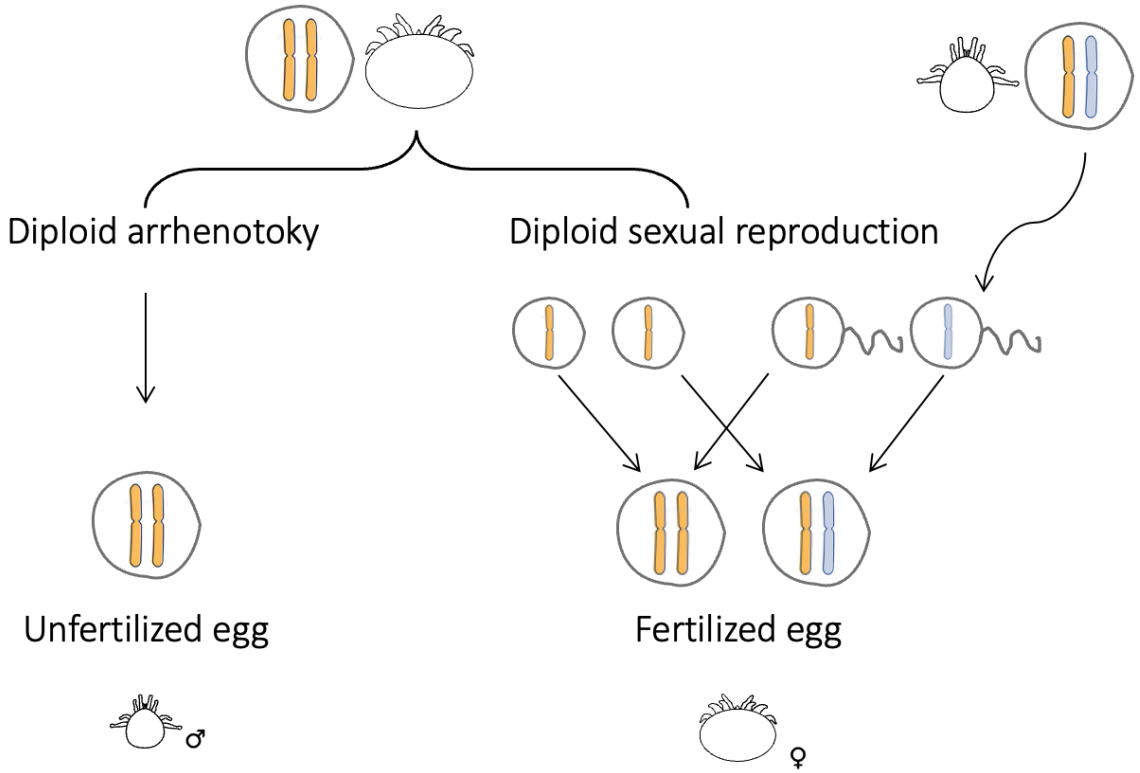


Figure 2. RNA-seq analysis of heterozygous site proportions in male and female mites. Each point represents the mean proportion of heterozygous sites per individual mite, with error bars showing 95% confidence intervals and lines connecting mother-son pairs. The two sexes show similar heterozygosity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



742 **Figure 3. Hypothesized mode of genetic inheritance in varroa male and female mites.**
743 Females are produced via diploid sexual reproduction, while males are produced via diploid
744 arrhenotoky. To illustrate the maternal and paternal alleles inheritance in the two sexes, we show
745 an example of a cross between a homozygous female and a heterozygous male, and their possible
746 female and male offspring (as in Figure 1). While the eggs start out as diploid in both sexes, males
747 lose half their genome during development (Figs S1 and S2).

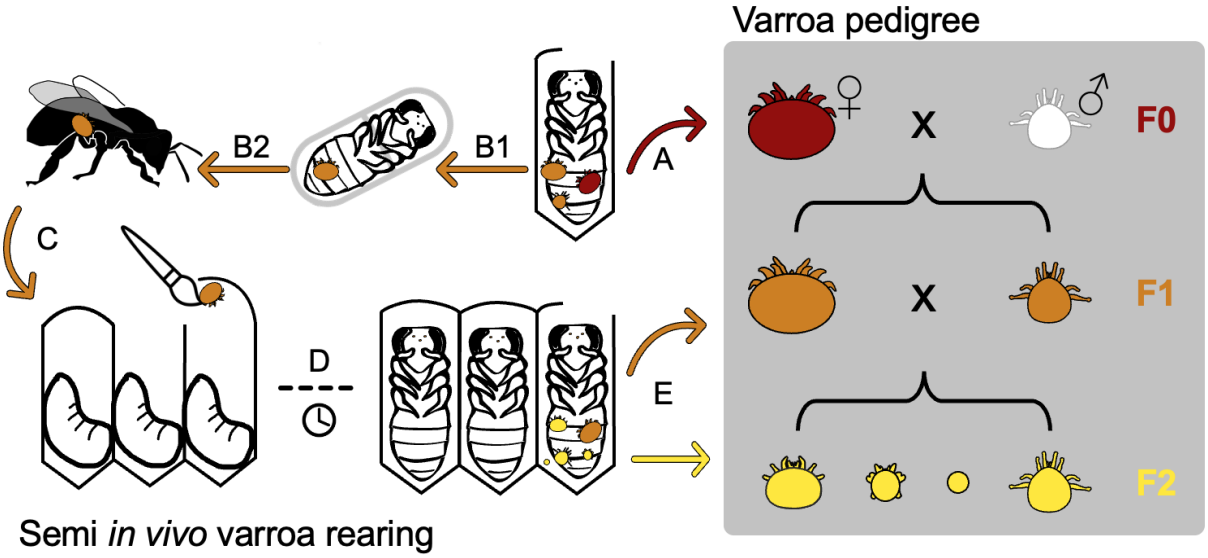


Figure 4. Pedigree experimental design. A three-generation Varroa pedigree (F0-in red, F1-in orange, and F2-in yellow) of 30 families, was constructed by the following steps: **A.** Collecting a naturally infested family: foundress female mite (F0), and her two adult offspring: male and female F1 mites (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; (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.