

*The ghost sex-life of the paedogenetic beetle *Micromalthus debilis**

Article

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1 **The ghost sex-life of the paedogenetic beetle *Micromalthus debilis***

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21 Genetic and sexual systems can be evolutionarily dynamic within and among
22 clades. However, identifying the processes responsible for switches between, for
23 instance, sexual and asexual reproduction, or cyclic and non-cyclic life histories
24 remains challenging. When animals evolve parthenogenetic reproduction,
25 information about the sexual mating system becomes lost. Here we report an
26 extraordinary case where we have been able to resurrect sexual adults in a
27 species of beetle that reproduces by parthenogenetic paedogenesis, without the
28 production of adults. Via heat treatment, we were able to artificially induce adult
29 beetles of *Micromalthus debilis* in order to describe its pre-paedogenetic mating
30 system. Adults showed a highly female biased sex ratio, out-breeding behaviour,
31 and sex-role reversal. Paedogenetic larvae of *Micromalthus* are infected with the
32 endosymbiotic bacteria *Rickettsia* and *Wolbachia*. Clear signs of vestigialization
33 in adults are concurrent with the loss of adults. Our data suggest an ancient
34 female sex ratio bias that predated the loss of adults, perhaps associated with
35 endosymbionts. We propose a model for the transition from a haplodiploid
36 cyclical parthenogenetic life history to parthenogenetic paedogenesis.
37 Paedogenetic development induces a new mechanism of sex ratio bias in midges,
38 wasps and beetles.

39

40

41

42 **Keywords**

43 Vestigialization, cyclical parthenogenesis, *Micromalthus*, *Wolbachia*, *Rickettsia*,
44 haplodiploidy, asexual

45

46 **Introduction**

47 Insects display an extraordinary richness of genetic and sexual systems . At least
48 eight genetic forms of thelytokous parthenogenesis have been described,
49 haplodiploidy is dominant in solitary and social Hymenoptera, hermaphroditism
50 in scale insects, androgenesis is widespread in stick insects, and no less than four
51 variations of hybridogenesis have been reported in ants^{1,2}. As such, we now have
52 a growing appreciation that genetic and sexual systems are far more dynamic
53 than once thought. Attempts to provide a theoretical framework for explaining
54 the evolution of genetic and sexual systems have revolved around the ideas of
55 genomic conflicts, including those arising from selfish genetic elements,
56 particularly between hosts and their endosymbionts ³⁻⁶.

57
58 However, testing such hypotheses remains a major challenge. First, many of the
59 species of interest share suites of characters that are often highly correlated
60 (such as inbreeding, skewed sex ratios, or the presence of endosymbionts),
61 making it difficult to identify cause and effect or eliminate third-variable
62 problems such as the combination of two or more selfish genetic elements in one
63 host ⁷. Second, many of these traits make the organisms cryptic or hard to study
64 by conventional means; they are typically not conventional “lab-rat” insects.
65 Third, some species are monotypic, they are the only species in a genus or even
66 family, making formal comparative analyses difficult or hard to interpret. Finally,
67 all three of these challenges together make reconstructing the order in which
68 traits evolved – crucial to disentangling cause and effect – extremely difficult.

69
70 Understanding the shifts to parthenogenesis can be particularly problematic, as
71 asexuality by definition removes many of the traits associated with the mating
72 system – including males – all at once. This can make reconstructing ancestral
73 states problematic, compounding the difficulties of assigning cause and effect.

74
75 Here we report a remarkable experiment in which we recreated the lost adult
76 stages in the beetle *Micromalthus debilis* LeConte, an insect with a bizarre sexual
77 system.

78

79 *Micromalthus* is considered native to North America ⁸, and is the only extant
80 species of the ancient family Micromalthidae. The larvae bore in wood and are
81 associated with fungal near-red-rot. It combines haplodiploidy, uni-oviparity for
82 males (one male egg is laid at a time), poly-viviparity for females (several female
83 larvae are delivered at a time), and internal and external matrophagy
84 (consumption of the mother by the offspring). Larvae display sexual
85 dimorphism. *Micromalthus* reproduces exclusively by larval thelytoky or
86 paedogenesis (parthenogenetic reproduction of the female larval stage) (Fig. 1),
87 and males are generally absent ⁹⁻¹³. Despite its extraordinary features, there is a
88 huge gap in research on *Micromalthus*. Only five experiments have been carried
89 out in the past 100 years ⁹⁻¹³ with the most recent on surveys of its associated
90 microflora ¹⁴. Constraints associated with working on this species range from its
91 microscopical size and the uncertainty in finding wild colonies, to the
92 dependency on micromanipulation of wood fragments to isolate living
93 specimens.

94
95 Some authors have based their understanding of the *Micromalthus* life cycle on
96 an opinion paper, assuming the adults are fertile ¹⁵. The entire experimental
97 literature, however, states that adults are sterile. Pollock and Normark, for
98 example, reported that adults mate based on an incorrect observation of Barber
99 ¹². Indeed, Barber observed two adults mounting, but after careful examination
100 of the specimens by an expert in aedeagi (male intromittent organ) of beetles,
101 both individuals were confirmed to be female. Barber was therefore the first
102 who observed female-female mounting in beetles ^{12,16}.

103
104 This is the first experiment aiming to quantify *Micromalthus* ghost adults and
105 their sex ratios and unravel clues on their past mating practices. To begin
106 piecing together the ancestral mating system of *Micromalthus*, we used heat-
107 treatment that led to the production of adults. We were interested in addressing
108 several questions. What are the sex ratios in paedogenetic larvae and in heat-
109 treated adults of *Micromalthus*? Sex ratio can be critical in hypothesizing the
110 ancestral mating system. Strongly female-biased adult - operational - sex ratios
111 are indicative of inbreeding and local mate competition ¹⁷. Is there any

112 observable mating behaviour expressed by the “resurrected” adults? Are heat-
113 induced adults fit to mate? Mating behaviour can inform us about the level of
114 mating competition, sex role observations and the extent of mating with kin.
115 Finally, to what degree is *Micromalthus* associated with intracellular sex ratio
116 distorters? Endosymbionts have been shown to play a role in shifts between
117 genetic and sexual systems and in shaping host behaviour.

118

119

120 **Results**

121 *Sex ratios of paedogenetic larvae*

122 One fully examined colony contained 5,140 mature cerambycid larvae and
123 occupied a volume of $\sim 25 \text{ cm}^3$. The sex ratio of paedogenetic larvae of the 2 cm^3
124 subsamples was highly female biased. Male producer larvae were rarely found
125 and only a single egg was recovered. In all instances, the sex ratios were
126 massively biased toward females (Binomial test, $P < 0.001$; Tables 1 and S1).
127 Heat treatment did not influence larval sex ratios. For all 36 subsamples of
128 paedogenetic populations not exposed to HT (including controls), the mean sex
129 ratio was 0.0043 (SD = 0.005) (generalized linear model: Wald $\chi^2_1 = 794.747$, $P <$
130 0.001). Sex ratios Before and After HT (Sets 1 and 2) were not significantly
131 different, neither by location (Table S1)(Wilcoxon Signed Rank test, $P = 0.678$;
132 and for normal distribution, Kolmogorov-Smirnov test, $Z_{11} = 0.941$, $P = 0.283$;
133 and $t_{11} = 0,313$, $P = 0.760$). However, there was a significant sex ratio difference
134 in Before and After HT between Locations 2 and 3 (generalized linear model:
135 Wald $\chi^2_1 = 159$, $P < 0.001$).

136 Heat treatment did have a very detrimental impact on the number of larvae,
137 killing the great majority of cerambycoids.

138

139 *Sex ratios of ghost adults*

140 Heat and drought treatment resulted in a slight decrease of the extremely female
141 bias: 1,000 females, 59 males (Table 2). Nine individually counted populations
142 yielded a maximum of 200 and a minimum of nine adults/population (patch).
143 The mean sex ratio across 75 adult-emergence days averaged 0.036 (SD = 0.14;
144 $N = 75$), clearly all highly significantly different to 0.5 (all $P < 0.001$). Analysis

145 from all four locations also showed a highly female bias, significantly different to
146 0.5 (Table 2), albeit with significant heterogeneity across the four locations ($\chi^2_3 =$
147 61.17, $LR_3 = 64.52$; $P < 0.001$). Comparisons of larval and adult sex ratios used
148 the only datasets available for paired comparisons across locations, and no
149 difference was found in sex ratio ($P = 0.109$; Table S2).

150

151 During the behavioural experiments it was found that females rejected 'related'
152 males - males from the same patch; therefore, the operational sex ratio was
153 corrected to an "outbreeding operational sex ratio" (OOSR). The resulting OOSR
154 was 0.057 (SD = 0.014), still highly female biased. Pre-mating dispersal involved
155 males flying a short distance towards another nearby population with 'unrelated'
156 (other patch) females (data not shown).

157

158 Each day, males and females emerged synchronously. The emergence period
159 never lasted more than 45 minutes, with adult production commencing only
160 when a peak temperature of 55 °C was reached. From a single colony, all
161 emerging females walked to the most exposed area. They moved as a group,
162 constantly walking and touching each other with their antennae (Movie S1).
163 Most males displayed their aedeagus as soon as they emerged from the wood.
164 They were unfit, sick or incapacitated; only 17 of 59 adult males matched the
165 fitness of the majority of females and these 17 were selected for the behaviour
166 experiments (Table S3).

167

168 *Mating behaviour of ghost adults*

169 Adult male and female *Micromalthus* exhibited a range of sexual behaviours.
170 Females preferred to mate with males from logs other than their natal log. Most
171 notably, in the mixed sex groups (Same Patch and Unrelated), the patterns of
172 sexual behaviour suggested sex-role reversal. Females initiated mating with
173 unrelated males, competed for access, and actively mounted males. Females
174 mounted the male, sometimes piling with up to five females at a time. In such
175 cases, females tried to dislodge the others with their mandibles, which are
176 otherwise used to bore tunnels in the wood. Damage to a male's genitalia was
177 observed once. Two females, larger than the male, everted a grasping organ

178 formed from a pair of sharp valves located at the edge of the muscular *bursa*
179 *copulatrix* (Fig. S1). The valves could move to grasp the aedeagus. Damage to
180 the male occurred after a failed copulation attempt when the female dismounted
181 the male while still holding on to part of his genitalia; consequently, the male lost
182 one of its parameres.

183

184 Sexual behaviour significantly varied amongst the three experimental settings:
185 females from the same patch, females from unrelated patches, and control
186 females (Fig. 2). When males were present (Experiment 1: Same Patch;
187 Experiment 2: Unrelated), females were far more likely to reject males
188 (behaviour A) coming from the Same Patch (likelihood ratio test: $LR_1 = 275.91$, P
189 < 0.001); females were also more likely to mount (behaviour D) an Unrelated
190 male ($LR_1 = 206.68$, $P < 0.001$). However, female dance (behaviour C) was more
191 likely to occur with males from the Same Patch ($LR_1 = 47.71$, $P < 0.001$). Across
192 all three experimental settings, the occurrence of female-female mounting
193 (behaviour B) did not vary with the presence or absence of males ($LR_2 = 1.74$, $P =$
194 0.42) whilst the occurrence of female-female fights (behaviour E) did increase if
195 males were present and those males were from a different patch (*i.e.* the most
196 favoured males; $LR_2 = 176.96$, $P < 0.001$; Fig. 2).

197 Female-male mounting (behaviour D) differed between localities 2 and 3 (GLMM,
198 $Z_1 = 4.46$, $P < 0.001$), while female-female fights (behaviour E) showed
199 significantly different performances between three localities (GLMM, L2 $Z_1 = -$
200 3.25 , $P < 0.001$; L3 $Z_1 = 3.94$, $P < 0.001$; L5, $Z_1 = 3.25$, $P < 0.001$).

201

202 Adults were unable to copulate, and none of the 1,000 adult females was able to
203 lay an egg or to produce progeny, either by sexual or parthenogenetic
204 reproduction.

205

206 *Life span, anatomy and physiology of ghost adults*

207 The average life span of adult females was 148.22 hours; approximately six days
208 (SD = 23.43, N = 23) and much shorter in males 12.92 hours (SD = 10.58, N = 12).

209

210 All adult females (N = 1,000) and males (N = 59) were investigated for any signs
211 of developmental abnormalities or vestigialization. Externally, deformed
212 antennomeres were observed in five individuals and widespread asymmetry
213 (supernumerary antennomeres) was observed in 20 of 60 females. Internally,
214 the majority of organs of 265 specimens ($\frac{1}{4}$ of adults) were reduced to not more
215 than one third of their original size when fully developed or were completely
216 missing, such that the thorax and abdomen looked nearly empty and transparent
217 under a stereomicroscope. Nine of the empty females looked disabled, showing
218 no movement and all died within 24 hours. When dissected, the thorax and
219 abdomen of empty individuals (N = 10) were virtually devoid of organs. Many
220 carried only a thin layer of what resembled fat body and the paired neural cord,
221 both attached to the integument.

222

223 In all dissected females that exhibited no apparent degeneration, the
224 spermatheca (receptacle where sperm is stored) was either reduced, vestigial, or
225 absent altogether. Oocytes did not reach maturation in any of the females,
226 including those that had mounted males.

227

228 *Endosymbiotic bacteria*

229 Two major endosymbionts were found and they varied in prevalence. Our rDNA
230 16S and *wspA* analyses identified *Wolbachia pipientis* in three of 62 larvae, while
231 rDNA 16S analysis identified *Rickettsia bellii* in all larvae examined. This was
232 confirmed by FISH analysis in cerambycoid larvae of *Micromalthus* (Fig. S2). No
233 endosymbiont was amplified by PCR or observed by FISH in the ovaries of the
234 dissected adult females, all of which were induced by the heat treatment.

235

236

237 **Discussion**

238 We were able to induce the production of adults in the paedogenetic beetle
239 *Micromalthus debilis*. Heat treatment can lead to the production of adults, which
240 are otherwise rarely seen in natural and laboratory populations. Heat treatment
241 was originally suggested by Barber ¹²; later Scott ^{11,18} experimented on colonies
242 and obtained only a few larval males and adult females. We were able to induce

243 both female and male adults. Heat treatment is a universally recognized method
244 to override mutations in viruses, prokaryotes and eukaryotes¹⁹⁻²¹. Phenotype
245 and sometimes life cycle attributes change between a normal low, permissive
246 temperature and a defined high, non-permissive temperature. The molecular
247 mechanism that regulates the formation of adults in *Micromalthus* might have
248 acquired missense mutation(s) that now prevent the formation of adults under
249 physiological or permissive temperatures. *Micromalthus* is the first
250 paedogenetic insect species that has been systematically exposed to defined high
251 temperature regimes. We think it plausible that the metamorphosis from larvae
252 to adults is controlled by a very limited number of genes that mutated. The
253 precocious activation of the ecdysone receptor and ultraspiracle in paedogenetic
254 gall midge larvae are sufficient to induce the development of ovaries within the
255 larval instars²². Metamorphosis being regulated by very few genes would
256 facilitate the selection for the paedogenetic phenotype. One could argue that
257 high temperature, for example at the height of summer, might have in the past
258 been a natural trigger for *Micromalthus* to change from the parthenogenetic part
259 of its cycle to the sexual part. Although this cannot be ruled out, increased
260 temperature is known to inhibit metamorphosis of butterfly and beetle larvae
261 rather than to induce or accelerate it^{23,24}. The temperature now required to
262 induce adult development is so high that it causes severe mortality in the larvae.
263 On average, only one in 315 cerambycid larvae survived the heat treatment. At
264 least 650 cerambycid larvae were required to obtain a single adult female,
265 while 10,000 triungulin larvae were estimated to be necessary for a single adult
266 male to develop. The temperature required for adult development is clearly non-
267 permissive and adults are no longer a physiological part of the life cycle (Fig 1).
268 Our studies support *Micromalthus* as a paedogenetic species in agreement with
269 earlier experimental work⁹⁻¹³. These findings are in conflict with Caillol, Pollock
270 and Normack, and Normack who proposed in opinion papers that it is a cyclically
271 parthenogenetic species^{15,25,26}.

272

273 *How informative are Micromalthus sex ratio biases?*

274 In untreated paedogenetic larvae, females vastly predominate and this is the case
275 for all locations. The sex ratio of larvae before and after heat treatment did not

276 change significantly. This shows that the heat treatment itself did not induce any
277 selective mortality with respect to sex of the larvae. In rescued ghost adults, sex
278 allocation is again extremely female biased, albeit less so than for larvae. This
279 suggests that the mechanisms governing sex allocation in larvae and adults are
280 different. The occasional male larva is a developmental relic, remnant of the
281 transition from cyclical parthenogenesis to paedogenesis. Under physiological
282 conditions, the rare male larvae do not develop into pupae or adults but die as
283 larvae. The sex ratio of larvae has no functional meaning and is an artefact,
284 whereas the sex ratio of the ghost adults might reflect the sex ratio that was
285 operational before the species became obligately paedogenetic. The observed
286 sex ratio variation in ghost adults across different locations, as opposed to larvae,
287 might be indicative of the genetic isolation of the paedogenetic populations and
288 of the consequential stochastic differences in the degree of vestigialization of the
289 ghost adults.

290

291 There are a number of explanations for biased sex ratios, especially for species
292 that live in rotting wood or other enclosed environments as is the case for
293 *Micromalthus* where interactions among kin may be important¹⁷.

294

295 First, local mate competition (LMC) selects for female-biased sex ratios when
296 related males (*i.e.* brothers or half-brothers) compete to fertilise their sisters²⁷.
297 By rescuing adults and observing their behaviour, it seems unlikely that LMC has
298 been a source of sex ratio selection in *Micromalthus*. Most importantly, females
299 have been observed to avoid mating with kin, exhibiting rejection behaviours
300 and being less likely to mount males from the same log, which would reduce LMC.
301 Males expose their aedeagus as soon as they emerge from the pupal stage, and
302 despite being surrounded by females from the same patch, to which they are
303 likely related, they are ignored; they then carry out a short flight towards a new
304 patch of unrelated females (thus supporting the hypothesis of females choice).
305 In addition, male pre-mating dispersal limits any effect of LMC to either partial
306 LMC or ameliorating it entirely if all matings are away from the natal log such
307 that kin would rarely be involved in competition.

308

309 Second, local resource competition (LRC) can also select for biased sex ratios,
310 although typically LRC involves competition amongst females for resources. LRC
311 should select for male-biased sex ratios, which is clearly not the pattern
312 observed in *Micromalthus* adults.

313

314 Third, local resource enhancement (LRE) can select for biased sex ratios if the
315 offspring of one sex increases the fitness of parents. By feeding on their mother,
316 there will probably be competition among female larvae, arguing against a LRC
317 interpretation. As such, the biology of *Micromalthus*, including our novel
318 observations of adult mating behaviour, does not offer strong support for LMC,
319 LRC or LRE shaping the patterns of sex allocation observed.

320

321 Most non-cyclically haplodiploid species have deviating sex ratios as adults.
322 Haplodiploid species with a social life history are almost all female biased. Often
323 females control sex allocation directly. Most non-social haplodiploid species are
324 parasitoids and have sex ratios ranging from highly male-biased ²⁸ to highly
325 female-biased ²⁹. In sexually reproducing diploid species, sex ratio biases of
326 intrinsic genetic origin (e.g., not due to manipulation by endosymbionts) are
327 extremely rare ³⁰. However, haplodiploid cyclically parthenogenetic species
328 have often been overlooked. West ¹⁷ suggested that haplodiploid cyclical
329 parthenogens in particular might prove useful for testing Düsing and Fisher's
330 theory of sex allocation. Monogonont rotifers, cecidomyid midges, cynipid wasps
331 and *Micromalthus* all fall into this special group. Despite their taxonomic
332 disparity, these groups exhibit similar life cycles. Exemplified by rotifers,
333 laboratory and field population studies revealed an even sex ratio for
334 haplodiploid cyclical parthenogens over time ³¹. Biases in haplodiploid cyclical
335 parthenogens might be more informative than generally assumed. The three
336 canonical explanations for sex ratio biases detailed above do not seem to be
337 applicable to haplodiploid cyclical parthenogens and *Micromalthus*. So what
338 might have caused the sex ratio deviation in *Micromalthus* adults?

339

340 *Mating behaviour of ghost adults is indicative of ancient sex-role reversal*

341 The mating behaviour of our ghost adults reveals clear patterns of sex-role
342 reversal, even when adults originate from different populations. Females
343 initiated interactions, competed for mates, and even grasped the male genitalia
344 with their own genitalia, leading in one case to male injury. Female-female
345 competition was also more common when preferred mates (*i.e.* males from
346 different patches) were available. Females are therefore both competitive over
347 mates and also selective, avoiding (presumed) kin. This is another clear sign of
348 choosy haplodiploid females, likely avoiding inbreeding³². However, female
349 dance, which we considered a sort of 'kin' signalling behaviour, was more likely
350 to occur with males from the same patch, suggesting that it is not courtship but
351 rather another form of rejection behaviour by females. Our results therefore not
352 only shed light on the sex ratio bias of *Micromalthus*, they also confirm that sex
353 roles are not necessarily fixed, and support mating systems theory that predicts
354 both males and females may combine choosiness and competitiveness over
355 mates to some extent^{33,34}.

356

357 *Vestigialization of adults and the loss of sexual reproduction*

358 Are the adults indeed ghosts and the observed behaviours ancient? Leaky
359 asexuality or rare sex in a predominant asexual species could upset any
360 interpretation of the results. Most cases of cryptic or occasional sexual
361 reproduction involve thelytokous species that have lost sexual reproduction. In
362 cyclically parthenogenetic species becoming paedogenetic, the loss is two-fold:
363 sex as well as the adult stage. In cyclical parthenogenesis the two are linked,
364 however, in neotenic species, the two phenomena are independent of each other.
365 For example, in ambystomid salamanders such as the Mexican axolotl, only the
366 imago stage has been lost for both sexes. Sexual reproduction continues at a
367 juvenile stage, in this case in an aquatic environment instead of in a terrestrial
368 one. In most twisted wing insects (Strepsiptera) or certain scale insects and
369 mealybugs (Hemiptera), the typical adult stage has been lost only for females but
370 not for males, and sexual reproduction has become asymmetrical in the sense
371 that adult males mate with females as larvae or pupae. In *Micromalthus*, both
372 sexual reproduction and (virtually) the adult stages have been lost for both sexes.

373

374 The mechanism by which sexual reproduction is lost is of great importance ³⁵.
375 Diplodiploid cyclically parthenogenetic aphids might lose sex through alterations
376 of periodicity genes or genes that regulate hormonal expression ³⁶. This can
377 happen so easily that a third of aphid species might be combinations of cyclically
378 parthenogenetic and obligate asexual populations ³⁷. Diplodiploid cyclically
379 parthenogenetic waterfleas (Cladocera) lose sex either through hybridogenesis
380 or through a meiosis suppressor gene. Especially in the case of a meiosis
381 suppressor gene, sex could return at any time and re-disappear thereafter. A
382 sex-dependent meiosis suppressor gene cannot maintain itself in haplodiploid
383 organisms ^{38,39}, which rules out any of these mechanisms for *Micromalthus*.

384

385 The dissection of ghost adults provides evidence that *Micromalthus* can no
386 longer return to sexual reproduction. The adults are physiologically incapable of
387 reproducing ^{10-12,18}. This has been experimentally confirmed and endorsed many
388 times ^{9,40,41}. In the original species description based on adults, LeConte referred
389 to the whole species as feeble and ill-developed ⁴². This strongly suggests that
390 *Micromalthus* lost the sexual part of its life cycle a long time ago, allowing for the
391 vast degeneration in the rare ghost adults. The retention of functionless males in
392 otherwise asexual species is well documented, but the retention of sterile
393 females has so far been overlooked ⁴⁰. Hebert ⁴⁰ would consider the production
394 of sterile females as "without precedent". Indeed, the adult females we produced
395 and rarely observed in the field (DKY) are ghost adults. It is well accepted that
396 adults have disappeared completely in many now obligate parthenogenetic
397 species ⁴³. It also indicates that rare episodes of sexual reproduction are
398 unlikely to contribute to the survival of the observed behavioural pattern or
399 *Micromalthus* as a species.

400

401 When sexually reproducing insect species become infected with
402 parthenogenesis-inducing *Wolbachia*, vestigialization of males has been
403 observed ⁴⁴⁻⁴⁶. A characteristic of decay of sexual functionality in *Wolbachia*-
404 induced thelytokous females of parasitoid wasps is the degeneration of the
405 spermathecae ⁴⁷⁻⁴⁹ as we found in the adults of *Micromalthus*. The extent of
406 vestigialization depends on the age of the association. When *Wolbachia*-infected

407 thelytokous females of various species are treated with antibiotics or heat to kill
408 *Wolbachia*, a continuum ranging from fully functional males to no male
409 production is observed⁵⁰. It seems that behavioural functions like mate
410 recognition are lost before physiological functions like male fertility disappear,
411 or male production ceases altogether. In *Micromalthus*, behavioural functions
412 including mate recognition are still strongly expressed whereas physiological
413 functions have severely degenerated or have been lost altogether. This suggests
414 that either the behaviour of ghost adults is more recent than we assume or that
415 the cost of retaining the behaviour is different in every species; we favour the
416 latter explanation. Nearly neutral behavioural traits might undergo very little
417 decay even after extended periods of relaxed or absent selection^{43,46}.

418

419 *Unifying features of Micromalthus and haplodiploid cyclical parthenogens*

420 Behaviour of lost adults can be reconstructed only in a very small time window.
421 According to fossil records (amber preserved triungulins), cyclical
422 parthenogenesis is at least 112 million years old in *Micromalthus*^{51,52}. The age of
423 paedogenesis in cecidomyid midges has been estimated to be at least 30 million
424 years based on Mexican amber and as old as 145 million years based on
425 Canadian amber⁵³. For *Micromalthus*, when functional adults were lost cannot
426 be said yet. In the cyclically parthenogenetic rotifer, *Branchionus calyciforus*,
427 loss of sex and adults can easily be induced experimentally in the laboratory
428 after 20-30 generations³⁹. For insects, it seems to be a much slower process.
429 Several species of the haplodiploid, cyclically parthenogenetic cecidomyid
430 midges living in decaying tree bark and mushrooms, and cynipid wasps living in
431 galls have lost sexual reproduction and the adult phenotype long ago. Two
432 species, the midge *Heteropeza pygmaea*⁵⁴⁻⁵⁶ and the gall wasp *Andricus*
433 *quadrilineatus* Hartig/*A. kiefferi* Pigeot^{57,58} seem to be at a similar junction in the
434 evolution of their life cycles. Both species exhibit some striking similarities to
435 the life cycle of *Micromalthus*. The two species produce adults only occasionally,
436 mainly after artificial induction. They exhibit hemocoelous development of the
437 larvae inside the hemocoel of the mother larvae, while *Micromalthus* exhibits
438 matriphagy. These two species have an extreme female bias and adults that are
439 increasingly sterile, while in *Micromalthus* sexual reproduction has disappeared

440 altogether. Both species also show large regional variation in the number and
441 relative sterility of adults produced. The midge, wasp and beetle (*Micromalthus*)
442 have already undergone a functional transition from a cyclically parthenogenetic
443 metamorphosis to a paedogenetic life cycle. Any adults are induced in
444 paedogenetic larvae and develop from paedogenetic larvae. We propose that the
445 sex ratio of these ghost adults is the result of the paedogenetic development. We
446 also propose that all haplodiploid cyclically parthenogenetic species that
447 transition to a paedogenetic life style will exhibit a female bias.

448

449 *Probable role of endosymbionts in Micromalthus.*

450 Perhaps the sex-role reversal we observed was the result of reproductive
451 parasites⁵⁹⁻⁶¹. Sex ratio distortion caused by bacteria or parasites, often referred
452 to as endosymbionts, is widespread in arthropods; examples of microbes that
453 manipulate the reproduction of their haplodiploid beetle hosts are well known
454^{62,63}.

455 *Rickettsia bellii* endosymbionts have been detected in all analysed larvae
456 suggesting they might serve an obligate beneficial role in the larvae which might
457 include restoring diploidy in oocytes during parthenogenetic reproduction as in
458 psocids⁶⁴. Assuming for the purpose of argument that the *Rickettsia* do restore
459 diploidy in *Micromalthus*, then the heat required to induce males must be high
460 enough to disable or kill the *Rickettsia* so that haploid male larvae can develop.
461 Indeed, *Rickettsia* cannot be detected in adults. However, this would also
462 postulate that the current form of paedogenesis of *Micromalthus* must be
463 different from the ancestral, holocyclical form of paedogenesis, which we assume
464 was not *Rickettsia* infected. Alternatively, these *Rickettsia* may provide a benefit
465 other than restoring diploidy. Interestingly, *R. bellii* has been associated with
466 male killing in a buprestid beetle⁶³.

467 *Wolbachia pipientis* endosymbionts were detected in only 5 % of the larvae,
468 which argues against a beneficial association or a still functional association. The
469 absence of the bacteria from the adults is likely a direct consequence of the heat
470 treatment and as such an epiphenomenon. The *Wolbachia* bacteria seem now to
471 be on a trajectory of being lost stochastically. It is possible that the *Wolbachia*
472 infection is a vestige of a sex ratio distortion event, although it does not rule out

473 that these *Wolbachia* might have been beneficial to the adult stage at one time
474 and are now superfluous. In *Acraea* butterflies, male-killing *Wolbachia* can exert
475 such selection pressure upon a species that the endosymbionts can cause sex-
476 role reversal ⁶⁵. Selection pressure can lead in some species to rapid suppression
477 of the male-killing effect ⁶⁶, while other species are unable to respond to the
478 male-killing effect ⁶⁷. The *Wolbachia*-induced extreme shortage of males in the
479 blue moon butterfly, *Hypolimnas bolina*, precipitates female promiscuity ⁶⁸. We
480 see in *Micromalthus* ghost adults the strong maintenance of mate choice that
481 discriminates on the basis of kin selection despite an extreme shortage of males.
482 This suggests that in a given species female response to male scarcity is not
483 predictable. Endosymbionts might explain a female bias and behaviour in
484 *Micromalthus* while it was a sexually reproducing species; endosymbionts cannot
485 explain the observed sex ratio in ghost adults.

486 Unlike the biases controlled by sex allocation mechanisms and selection, the
487 female bias of ghost adults is probable a temporary by-product of asexual
488 reproduction at a juvenile stage.

489
490 By reconstructing adults of an ancient haplodiploid cyclical parthenogen, we
491 have shown that they have strongly female-biased sex ratios in ghost adults. Our
492 behavioural observations of these ghost adults show remarkable sex-role
493 reversal behaviour. We hypothesize that the observed female-biased sex ratio is
494 a physiological consequence of paedogenetic difficulties of producing male
495 offspring, likely characteristic to all haplodiploid cyclical parthenogenetic species
496 transitioning to a paedogenetic life cycle. Unrelated to the female bias of
497 currently induced adults, the recovered behaviours are the consequence of an
498 ancient female-biased sex ratio. This ancient female bias predated the loss of
499 adults and might have been induced by a male-killing sex ratio distorter.

500

501

502 **Methods**

503 *Colonies of Micromalthus debilis*

504 Oak logs containing colonies of *M. debilis* were collected by DKY in 2002, 2004,
505 2005 and 2006 from two sites (43.19361°N, -90.23917°W; 43.19360°N, -
506 90.23844°W)
507 in Richland County, 46 miles west of Madison, Wisconsin (USA). Logs from these
508 two sites provided colonies from five different locations. Colonies are separate
509 patches on logs. Logs were reared for five years at Bangor University, kept under
510 constant environmental conditions inside styrofoam boxes (18 ± 2 °C and 90 %
511 RH). The location descriptors are: Location 1, 2002; Location 2, 2004; Location 3,
512 2006a; Location 4, 2005; and Location 5, 2006b.

513

514 *Manipulation of specimens*

515 The *Micromalthus*-containing wood is tunnelled by larvae parallel to the trunk
516 and up to 2.5 cm deep into the bark. It allows fragmentation using
517 microdissection forceps. Although this method is disruptive it is the only way to
518 reach, observe and collect the small, fragile specimens (legged triungulins reach
519 up to 1 mm, while mature cerambycoids attain a length of up to 3.3 mm)⁵². Due
520 to their small size, all procedures were performed under magnification lenses
521 and stereomicroscopes. Whole colonies were isolated in insect containers.

522

523 *Heat treatment and the induction of ghost adults*

524 In February 2005, five, 2004 colonies were exposed to a high temperature
525 treatment (HT¹²: with the photoperiod fixed at 16 h light. The treatment
526 consisted of progressively raising the temperature inside the cages containing
527 *Micromalthus* over the course of a month, to a daily maximum of 55 °C at mid-
528 afternoon and a night minimum of 20 °C for 3 weeks (55 % RH). The colonies
529 were daily monitored every two hours for the presence of adults. Once adults
530 started emerging (March 2005), they were monitored every hour and counted.
531 Sexing was performed *in situ* and controlled later by examining preserved
532 specimens under the stereomicroscope. The treatment was repeated in March
533 2007 with two colonies from each of the 2004, 2005 and 2006a and b collections
534 (*i.e.* N = 8 colonies). Adults emerged from only five of these eight colonies; the
535 other three colonies never produced adults as a result of HT.

536

537 *Sex ratio of larvae*

538 To estimate the sex ratio, samples of wood were disaggregated and the larvae
539 counted and sexed in two sets of 24 samples of approximately 2 cm³ wood. One
540 of the two sets was exposed to a heat treatment (named After-HT), while the
541 other set was called Before-HT. The controls consisted of populations kept at lab
542 environmental conditions, not exposed to HT (named Controls). Controls
543 allowed testing for natural variation, *i.e.* whether larvae died of natural causes, or
544 other environmental modifications, other than heat and drought. Larval
545 counting was destructive, because once the larvae are exposed to the elements,
546 they can no longer be reintroduced into the tunnels and they die. The moment
547 adults started emerging from the HT populations the wood pieces were opened
548 and the larvae were counted.

549

550 *Behavioural assays*

551 Three hundred and sixty seven adults (350 females and 17 males) were used in
552 experiments of sexual behaviour. They were grouped in 17 sets of mixed sex,
553 with experimental group named Same Patch (as the patch of the male) and
554 Unrelated (different to the patch of the male) and six sets of single sex, female-
555 only groups (experiments) named Controls. The grouping in sets was only
556 governed by the patch and did not alter the sex ratio. All sets contained 6-21
557 individuals each (21 individuals was the maximum number that the observer
558 could handle). Each session took place 30 min post-emergence of the adults,
559 once adults were located inside the experimental area. Observations of
560 behaviors lasted a maximum of 55 min because the beetles disappeared inside
561 the wood chips, becoming inactive for the rest of their life span; males did not
562 move anymore, some females flew away in the direction of the light. Several
563 populations did not produce males, therefore the number of experiments on
564 sexual behaviour were limited to the availability of non-impaired males (N=17).

565

566 The behavioural experiments were conducted with pieces of wood containing
567 *Micromalthus* from three of the five locations. Observations took place under
568 two dissecting microscopes, using up to 40 x (Leica ZOOM 2000) and 100 x (Leitz,
569 Wetzlar) magnification. Once adults commenced emerging, they did so daily,

570 between 11:30 (earliest) and 17:00 (latest). Behaviours were studied on a
571 specially designed arena consisting of an open glass-disc containing a 2 cm³-size
572 fresh wood piece in the centre.

573 In each experiment, the male was carefully transferred on the tip of a smooth
574 brush to the wood surface of another female-producing colony (patch of a log).
575 Behaviours were scored by presence/absence of behaviour per female. Females
576 were easily identified not only by size: some kept the wings extended in
577 particular positions, others could not close the elytra, and most had different
578 loads of nymphal phoretic Astigmata mites (these mites were seen solely on
579 females).

580

581 *Behaviour descriptors*

582 **A** - Male discrimination: Females ignored the presence of a male emerging from
583 the same patch of wood. Females ran away from the male; on occasions they
584 faced the males head on and moved their mandibles against the male.

585 **B** - Female-Female mounting: The females attempted to mount each other at a
586 rate of one per minute. A mounting lasted 10 to 20 seconds (N=4) (longer when
587 a larger female was on top).

588 **C** - Female dance (Movie S1): Immediately after emergence females produced an
589 up-down and sideways shaking of their abdomens while beating their wings.

590 **D** - Female-Male mounting: Females mounted the male.

591 **E** - Female combat: Females fought with each other, trying to dislodge the
592 female on top of the male, even piling on the male.

593 **F** - Females injuring males: Rare behaviour where two females were observed to
594 grasp and the male genitalia leading to male injury in one case.

595

596 Images and movies were obtained by attaching with a tube adaptor (Brunel
597 Microscopes) a digital camera (SONY Cyber-shot MP, 3 x optical zoom) to one of
598 the oculars.

599

600 *Life span, anatomy and physiology of ghost adults*

601 All emerging adults used in the behavioural experiment were followed until
602 death. The life span of 23 females and 12 males was measured; males were the
603 limiting factor in these experiments.

604 The external morphology of all 1,059 adults was examined. The abdomen was
605 screened with inverted light to detect signs of gross vestigialization, and 10 of
606 these were subsequently dissected.

607 In addition, the abdomen of 50 females involved in the behavioural experiments
608 (plus 10 clearly physically impaired individuals) were dissected and the internal
609 organs, particularly the ovaries, examined.

610

611 *Analysis for endosymbionts*

612 *Micromalthus* larvae were screened for endosymbiosis: 10 individuals from
613 location 2 were investigated by transmission electron microscopy, 60 by
614 molecular analysis (PCR, sequencing), and 22 by fluorescent in situ hybridization
615 (FISH) with species-specific probes (for confirmation of localization of relevant
616 species). The 60 and 22 specimens were randomly picked from locations 2, 3, 4
617 and 5.

618 For FISH, whole mature cerambycid larvae were fixed; fixation, hybridization,
619 mounting and confocal microscopy analyses followed methods described in
620 Perotti, et al. ⁶⁴ for whole-specimen FISH on insects.

621 The 60 above described larvae and 60 non-impaired adult beetles (randomly
622 picked from all adult producing colonies) were screened by PCR amplification
623 using rDNA16S primers for sex ratio distorting bacteria and *wspA* primers for
624 *Wolbachia* following previously described protocols ^{30,64}.

625

626 *Statistical analyses*

627 A binomial test checked for deviation from expected adult sex ratio (*i.e.* 0.5).
628 For paired sex ratios before and after HT, frequencies were transformed into
629 proportions (male/(female + male) and compared with a non-parametric
630 Wilcoxon Signed Rank test; to check for normality we used the Kolmogorov-
631 Smirnov test.

632 To test for the effect of locality on the sex ratios Before and After HT, a
633 Generalized Linear Model was fitted to a Poisson distribution with link log

634 criteria, analysis type Wald and full likelihood ratios. Localities were analysed as
635 main factors. The analyses were two-tailed; we used SPSS v. 22.

636 For adults, a Likelihood Ratio (LR) was calculated to measure the effect of
637 location on sex ratio between different populations, and the effect of relatedness
638 (Unrelated and Same Patch) between different behaviours. Generalised Linear
639 Mixed Models were used for each of the six behaviours using a Poisson
640 regression with link log to explore random effects of location and behaviour. A
641 Zero-Inflated Poisson regression was initially run for overdispersed zero-
642 inflated data, however, the Vuong test indicated no significant difference with
643 the standard Poisson regression (STATA v. 13.1).

644

645

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651

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655 analysis, carried out statistical analyses and participated in the drafting of the
656 manuscript. MAP conceived of the study, designed the study, collected data,
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660

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662

663

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839

840

841

842 **Figure Legends**

843

844 **FIGURE 1**

845 Evolution of the life cycle of *M. debilis*. Bottom left: green arrows show the
846 present time obligate paedogenetic life cycle, where viviparous 1st instar female
847 larvae or triungulins are the legged, migratory stage. Bottom right: yellow
848 arrows indicate rare, relic development of a male larva, where the oviparous 1st
849 instar male larva is legless. The male larva is under present conditions a
850 developmental dead end. Top: red arrows show the rare development of adults
851 induced through exposure to extreme heat. Adults are no longer reproductively
852 functional; they were part of the ancestral cycle of sexual and asexual
853 reproduction.

854

855 **FIGURE 2**

856 Normalized boxplot showing the portion of females of the three groups, Same
857 Patch (red), Unrelated (blue), and Control (green) performing each behaviour.
858 No female in the group Same Patch displayed Female dance, Female-male
859 mounting, Female-female combat or Female injuring males. Outliers are
860 identified as small circles (1.5 x interquartile range), whereas extreme values (3
861 x interquartile range) are marked with a star.

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874 **Table 1:** Sex ratio of paedogens. Total number of females FF, total number of males
 875 MM ($N_{\text{samples}} = 24$ for Total and 12 for Treats and Controls; Median indicated between
 876 brackets), and averages of sex ratio $[MM/(FF+MM)]$ obtained from each set of sub-
 877 samples ($N_{\text{samples}} = 24$ and 12; STD indicated between brackets).

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HT Treatment and Controls

	Before			After		
	FF	MM	SEX RATIO	FF	MM	SEX RATIO
Grand Total	5,007 (213)	24 (1)	0.0046 (± 0.005)	2,521 (64)	10 (0)	0.002 (± 0.004)
Treatments	2,502 (226)	11(1)	0.0044 (± 0.005)	8 (0)	0	0
Controls	2,505 (210)	13 (1)	0.0046 (± 0.006)	2,513 (214)	10 (1)	0.004 (± 0.006)

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883 **Table 2:** Adults that emerged from the wood of four locations after the heat and
 884 drought treatment (HT), except for the control. There were four Locations from
 885 which adults were counted [Number of emergencies are indicated in square brackets].
 886 Males and females were counted (Median indicated between brackets). Averages of
 887 sex ratio [MM/(FF+MM)] were obtained for each location or origin of the wood
 888 (STD indicated between brackets)

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Location/Origin	FF	MM	SEX RATIO
2 [n=46]	483 (7)	4 (0)	0.005 (\pm 0.02)
3 [n=14]	224 (11)	37 (0)	0.135 (\pm 0.28)
4 [n=2]	47 (24)	0	0
5 [n=13]	246 (12)	18 (0)	0.049 (\pm 0.11)
Grand Total	1,000 (9)	59 (0)	0.037 (\pm 0.13)

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