Spontaneous parthenogenesis in the parasitoid wasp *Cotesia typhae*: low frequency anomaly or evolving process?

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Abstract

Hymenopterans are haplodiploids and unlike most other Arthropods they do not possess sexual chromosomes. Sex determination typically happens via the ploidy of individuals: haploids become males and diploids become females. Arrhenotoky is believed to be the ancestral reproduction mode in Hymenopterans, with haploid males produced parthenogenetically, and diploid females produced sexually. However, a number of transitions towards thelytoky (diploid females produced parthenogenetically) have appeared in Hymenopterans, and in most cases populations or species are either totally arrhenotokous or totally thelytokous. Here we present the case of *Cotesia typhae* (Fernandez-Triana), a Braconidae that produces parthenogenetic females at a low frequency. The phenotyping of two laboratory strains and one natural population showed that this frequency is variable, and that this rare thelytokous phenomenon also happens in the wild. Moreover, mated females from one of the laboratory strains produce a few parthenogenetic daughters among a majority of sexual daughters. The analysis of daughters of heterozygous virgin females allowed us to show that a mechanism similar to automixis with central fusion is very likely at play in *C. typhae*. This mechanism allows some parts of the genome to remain heterozygous, especially at the chromosomes' centromeres, which can be advantageous depending on the sex determination system involved. Lastly, in most species, the origin of thelytoky is either bacterial or genetic, and an antibiotic treatment as well as PCR experiments did not demonstrate a bacterial cause in *C. typhae*. The unusual case of low parthenogenetic frequency described in this species constitutes another example of the fascinating diversity of sex determination systems in Arthropods.

Introduction

Sexual reproduction is the most widespread reproductive strategy among multicellular organisms and especially in animals. In contrast with its predominance, this reproductive mode appears costly due, for instance, to the necessity to detect and attract a partner, escape sexually transmitted diseases or avoid predation during mating. Because they share parenthood with their mate, sexual individuals transmit two-fold less of their genetic material to their progeny compared to asexual counterparts. The ubiquity of sex despite such disadvantages led to the definition of the so-called "paradox of sex" (Meirmans et al., 2012; Otto, 2009).

Numerous cases of evolution toward asexual reproduction or parthenogenesis have been reported, notably within arthropod taxa (The Tree of Sex Consortium, 2014). Parthenogenesis can produce either males (arrhenotoky) or females (thelytoky) from unfertilized eggs, but only the last case strictly coincides with asexual reproduction. It is also referred to as parthenogenesis *sensu stricto*.

Thelytoky has been observed in almost all basal Hexapoda and non-holometabolous insect taxa (Vershinina and Kuznetsova, 2016) as well as in many holometabolous insect species (Gokhman and Kuznetsova, 2018). This wide taxonomic range illustrates the frequent transition from sexual to asexual taxa that arose independently in various lineages. This scattered distribution hides a global low percentage of parthenogenesis: thelytokous species represent less than 1% of the Hexapoda (Gokhman and Kuznetsova, 2018). The proportion of asexual lineages is also highly heterogeneous among taxa. Liegeois et al., (2021) detected frequencies between 0 and 6.7% among families of mayflies. Van der Kooi et al. (2017) reported frequencies ranging from 0 to 38% among genera of haplodiploid arthropods.

Transition from a sexual to asexual reproductive mode requires bypassing genetic and developmental constraints, a challenge that may be easier to face in some taxa. In most species with a haplodiploid sex determination system, males develop from unfertilized eggs and are haploid while females develop from fertilized eggs leading to a diploid state. In such cases, embryonic development is initiated independently from egg fertilization, a trait probably favouring an evolution toward thelytoky (Vorburger, 2014). The variable frequency of asexual reproduction even among haplodiploid lineages indicates that other factors allowing the transition toward this reproductive mode remain to be identified (van der Kooi et al., 2017).

The multiple and independent acquisitions of asexual reproduction are associated with numerous mechanisms that maintain or restore diploid state and produce females (Rabeling and Kronauer, 2013; Vorburger, 2014), as illustrated in Figure 1A. The figure focuses on genetic consequences in terms of heterozygosity, but each described situation may result from different cytological mechanisms. Apomixis induces clonal reproduction and allows the complete preservation of heterozygosity. It may arise from mitosis, but also from endoreplication preceding meiosis with sister chromosome pairing, resulting in recombination between identical chromosomes (Archetti, 2010; Ma and Schwander, 2017). In automixis, meiosis occurs and is followed by different diploid restoration processes. Two meiosis products may assemble to generate a diploid cell: i) fusion of non-sister products separated during the first *reductional* division in central fusion or ii) fusion of sister cells produced during the second *equational* division in terminal fusion. Note that similar patterns are obtained when one meiotic division is suppressed to ensure the maintenance of a diploid state: the lack of first division is equivalent to central fusion while the lack of second division equates to terminal fusion. The restoration of diploidy may also result from gamete duplication involving either fusion of mitosis products or chromosomal replication without cellular division. In some lineages, the restoration of diploidy may operate during embryogenesis *via* endomitosis (Little et al., 2017; Pardo et al., 1995). Other mechanisms not illustrated here involve endomitosis followed by meiosis with non-sister chromosomes pairing or inverted meiosis with central or terminal fusion (Archetti, 2022, 2010). The consequences of thelytoky in terms of heterozygosity are variable depending on the mechanism: from complete homozygosity in one generation under gamete duplication to completely preserved heterozygosity in apomixis, with intermediate levels of homozygosity in terminal and central fusion. According to the biology of a given species and the degree of necessity for maintaining heterozygosity, one or other mechanism may be favoured.

Three main origins of thelytoky have been described: hybridization, bacterial endosymbiosis and genetic mutation (Tvedte et al., 2019). Hybridization, joining genomes from two distinct species, leads to improper chromosome pairing and dysfunctional meiosis that may promote asexuality (Morgan-Richards and Trewick, 2005). Endosymbiotic origin is the most widely studied cause of parthenogenesis (Ma and Schwander, 2017). To date, only bacteria have been shown to act as parthenogenesis inducers, but it is likely that other microorganisms could be involved. Most of the

described causative agents belong to the genera *Wolbachia*, *Rickettsia* and *Cardinium*, endosymbionts also known to induce cytoplasmic incompatibility or feminization of male embryos. The particularity of endosymbiont induced parthenogenesis resides in its partial or total reversibility. Thelytokous species treated with antibiotics or heat may revert to sexual reproduction, although often performing less well than true sexual counterparts (Stouthamer et al., 1990). The genetic origin of thelytoky has often been suggested when antibiotic or heat treatment had no effect, but the precise identification of loci responsible for parthenogenesis has only been conducted in a few species (Chapman et al., 2015; Jarosch et al., 2011; Lattorff et al., 2005; Sandrock and Vorburger, 2011).

The relative frequency of thelytoky and sexual reproduction within species also varies according to taxa (Gokhman and Kuznetsova, 2018; Vershinina and Kuznetsova, 2016). Some species are described as obligate thelytokous when this mode of reproduction is the only one observed. Alternatively, thelytoky appears cyclic in some species where asexual generations alternate with sexual ones (Neiman et al., 2014). In other cases, polymorphism in the reproductive mode is observed either between populations (Foray et al., 2013; Leach et al., 2009) or within populations (Liu et al., 2019). Even in such polymorphic situations, thelytokous females usually produce female only progeny, albeit with a very low frequency of males in some cases, allowing rare events of sexual reproduction (Pijls et al., 1996).

Spontaneous occurrence of parthenogenesis has also been described in species reproducing via a sexual mode and qualified as tychoparthenogenesis (Ball, 2001; Pardo et al., 1995). Tychoparthenogenesis is characterized by a low hatching rate and a weak survival probability of the offspring (Little et al., 2017). It is typically considered as a dead-end accidental phenomenon in species adapted to sexual reproduction, although it may also correspond to an intermediate state in the evolution toward asexuality (van der Kooi and Schwander, 2015).

Cotesia typhae (Fernandez-Triana; Hymenoptera, Braconidae) is a gregarious endoparasitoid wasp native to Eastern Africa (Kaiser et al., 2017, 2015). It is specialized to one host, the corn stemborer *Sesamia nonagrioides* (Lefèbvre, Lepidoptera, Noctuidae). *Cotesia typhae* reproduces sexually and fertilized females typically lay 70-100 eggs in the first host encountered, among which about 70% develop into females and 30% into males (Benoist et al., 2020b). At least in laboratory conditions, sister-brother mating (sib-mating) frequently occurs indicating that inbreeding is not detrimental to this species. A genetic survey was conducted on this parasitoid wasp to compare two laboratory strains initiated from wild individuals sampled in two distant Kenyan localities, Kobodo and Makindu (Benoist et al., 2020a). The study led to the construction of a genetic map, based on crosses between the two strains. The phenotyping of the progenies obtained from these controlled crosses revealed an extremely variable sex-ratio, ranging from 100% to as low as 5% of females. Such a phenomenon could result from poorly mated females but also from rare thelytokous events in the progeny of unfertilized females. This last hypothesis was validated in a preliminary experiment allowing virgin females to oviposit. Among the numerous males emerging from the parasitized hosts, a few females were detected.

The aim of this study is to describe the low frequency thelytoky phenomenon in *Cotesia typhae* as a possible case of asexuality emergence. We first confirmed that this low frequency phenomenon is not restricted to artificial breeding but is also observed in natural conditions. We then tested whether thelytoky is induced by environmental conditions, that is lack of fertilisation, or observed in the progeny of mated as well as virgin females. Finally, we addressed the question of the mechanisms allowing the asexual production of females, because these mechanisms are tightly linked to the loss or conservation of heterozygosity and to the evolvability of asexual lineages.

137

138 ## Material and Methods

139 ### Biological material

140 Two separate *Cotesia typhae* parasitoid strains were obtained from adults that emerged from
141 naturally parasitized *Sesamia nonagrioides* caterpillars collected in the field at two localities in Kenya
142 (Kobodo: 0.679S, 34.412E; West Kenya; 3 caterpillars collected in 2013 and Makindu: 2.278S,
143 37.825E; South-East Kenya; 10 caterpillars collected in 2010-2011). Isofemale lines were initiated in
144 2016 and inbred rearings have been subsequently kept for more than 80 generations at the
145 Evolution, Génomes, Comportement et Ecologie laboratory (EGCE, Gif-sur-Yvette, France), where
146 cross experiments and phenotyping were performed. The phenotyping of the wild population was
147 performed on individuals that emerged from naturally parasitized *Sesamia nonagrioides* caterpillars
148 collected in the field in 2020 at Kobodo (see above). The *S. nonagrioides* host strain used was
149 initiated from caterpillars collected at Makindu (see above) and Kabaa (1.24S, 37.44E). The rearing
150 protocol of *C. typhae* and *S. nonagrioides* is detailed in Benoist et al. (2020b).

151

152 ### DNA extraction and genotyping methods

153 For our different experiments, DNA was extracted from *C. typhae* individuals using the NucleoSpin®
154 Tissue from Macherey Nagel, following the manufacturer's instructions. For the experiment analysing
155 the offspring of mated females, a direct PCR method was used instead of a classic DNA extraction
156 because of the high number of individuals to be genotyped. In this case, for each individual, the
157 abdomen was removed (because the presence of gametes could hinder the genotyping) and the rest
158 of the body was placed in 20µL of Dilution Buffer and 0.5µL of DNA Release Additive
159 (Thermoscientific). The tubes were kept at room temperature for 5 minutes then placed at 98°C for 2
160 minutes. One microliter of this mix or of DNA was then used as template for the following PCR.

161 Two different methods were used to genotype the chosen SNP markers, either HRM (High Resolution
162 Melt), or allele specific PCR. HRM is based on the analysis of melt curves of DNA fragments after
163 amplification by PCR. The melt curves are different according to the nucleotide composition of the
164 DNA fragments, and therefore allow discrimination between homozygotes and heterozygotes at a
165 given SNP. For each SNP marker, a 10µL mix was made with about 1ng of DNA, 0.2µM of each
166 primer, and 5µL of Precision Melt Supermix (Bio-Rad), completed with water. The PCR protocol was
167 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 30
168 seconds, followed by a complete denaturation of 30 seconds at 95°C before performing the melt
169 curve. The melt curve was performed on a CFX96™ Real-Time System (Bio-Rad), and was started by
170 an initial step of 1 minute at 60°C, followed by 10 seconds of every 0.2°C increment between 65°C
171 and 95°C. The raw data resulting from the melt curves were analysed with the uAnalyze v2.1
172 software (Dwight et al., 2012) in order to infer the individuals' genotypes at each SNP marker.

173 Two SNP markers (8225nov and 21770nov) were genotyped using allele specific PCR. For this
174 method, two parallel amplifications were performed on individuals, one of the primers of the couple
175 being a common primer, and the other one being a specific primer, either to the Makindu, or Kobodo
176 allele. About 1ng of DNA was mixed with 1X buffer, 3mM $MgCl_2$, 0.4mM dNTP, 0.4µM of each primer,
177 1U GoTaq® Flexi DNA Polymerase (Promega), and completed with water. The PCR programme was 5
178 minutes at 95°C, followed by 40 cycles of 95°C for 1 minute, 50°C or 55°C for 1 minute (50°C for
179 8225nov and 55°C for 21770nov), 72°C for one minute and a final elongation of 5 minutes at 72°C.

180 The PCR products were then run on a 2% agarose gel to check which PCR were positive and therefore
181 infer the genotypes.

182 All the primers used were designed for this study and their sequences are given in Supplementary
183 Table 2.

184

185 **Phenotyping the strains/populations for the thelytokous character**

186 In this study, the phenotyping consists of counting the number of males and females in the offspring
187 of virgin females, to quantify the thelytoky phenomenon. To obtain virgin females, individual
188 cocoons were isolated from cocoon masses and kept in tubes with a moistened cotton wool ball and
189 a drop of honey at 27°C until the emergence of the adults. The virgin females were then each
190 allowed to oviposit in one *S. nonagrioides* caterpillar, and the number of males and females in their
191 offspring was counted after the development of the new *C. typhae* generation.

192

193 **Flow cytometry for ploidy analysis**

194 Flow cytometry analysis was performed on one control female from a mixed cocoon mass (produced
195 by a fertilized female), two control males, and five parthenogenetic females (produced by a virgin
196 female), all coming from the Makindu laboratory strain, to determine their ploidy. The individuals
197 were frozen in liquid nitrogen and processed in the Imagerie-Gif Platform of Institute for Integrative
198 Biology of the Cell (I2BC), CNRS, Gif-sur-Yvette according to the protocol in (Bourge et al., 2018).

199

200 **Fecundity assessment of parthenogenetic females**

201 Parthenogenetic females were tested for their fecundity and their ability to perform thelytoky. To
202 test whether mated parthenogenetic females had the same fecundity as mated control females,
203 cocoon masses resulting from the eggs laid by *C. typhae* Makindu virgin females in *S. nonagrioides*
204 caterpillars were divided in smaller cocoon packs to spot the few parthenogenetic females more
205 easily among the males after the emergence of the adults. Adults were left together for one day with
206 water and honey to allow mating, and eleven parthenogenetic females were then allowed to oviposit
207 in *S. nonagrioides* caterpillars. After the emergence of the resulting offspring, the number of males
208 and females was counted for each one of them and the sex-ratio was calculated for comparison with
209 that obtained from fertilized females from the control Makindu laboratory strain. To test whether
210 virgin parthenogenetic females were able to produce parthenogenetic daughters, all the cocoons
211 from 5 virgin females' progenies were isolated and the virgin parthenogenetic females emerging
212 from these cocoons were allowed to oviposit in *S. nonagrioides* caterpillars. The number of males
213 and females was then counted in each resulting offspring.

214

215 **Identifying the thelytoky mechanism in *Cotesia typhae***

216 To find out which thelytoky mechanism is at play in the *C. typhae* Makindu laboratory strain, virgin
217 heterozygous females are needed to analyse the recombination patterns of their offspring. Indeed,
218 according to the mechanism, the female offspring will be more or less heterozygous, as explained in
219 introduction (Figure 1A). To obtain virgin heterozygous females, six controlled crosses were
220 performed between the Makindu and Kobodo laboratory strains, 3 in each direction (Figure 1B). Prior

221 to this, cocoons had been isolated from masses of each strain, in order to obtain virgin males and
222 females for the crosses. Cocoons were then isolated from the masses resulting from the crosses,
223 leading to the emergence of virgin F1 heterozygous females. 57 of these females were allowed to
224 oviposit in *S. nonagrioides* caterpillars, and the offspring of the 57 resulting cocoon masses were
225 sexed and counted. Six females and four males from the two parental strains (including the
226 individuals used for the initial crosses), five F1 heterozygous females and the nine parthenogenetic
227 females obtained through this experiment were kept for DNA extraction and genotyping, in order to
228 analyse the recombination patterns resulting from parthenogenesis.

229 To analyse the recombination patterns of the nine parthenogenetic females, we genotyped 63 SNP
230 (Single Nucleotide Polymorphism) markers, having different alleles between the Makindu and
231 Kobodo strains, and being distributed along the 10 chromosomes of the genetic map of *Cotesia*
232 *typhae*, (Benoist et al., 2020a). Four chromosomes contained more markers than the others to
233 investigate the recombination patterns along chromosomes. For these four chromosomes, the
234 markers were chosen to have about 10cM between two successive markers when possible. The
235 markers were genotyped according to the protocol described above, and their genetic position is
236 given in Supplementary Table 1.

237

238 **Identifying the thelytoky phenomenon in mated females' progenies**

239 To see if mated females produce parthenogenetic daughters as well as sexual daughters, 40 crosses
240 between Makindu virgin females and Kobodo males were performed, according to the protocol
241 described in the previous section. By genotyping the daughters of these crosses with a SNP marker
242 differing between the Makindu and Kobodo strains, we can deduce if they were produced sexually (if
243 heterozygous at the marker) or through parthenogenesis (if homozygous for the Makindu allele at
244 the marker).

245 Out of the 40 crosses, five resulted in male only offspring and were removed from our analysis. The
246 35 remaining crosses lead to a mix of male and female offspring, for a total of 1861 daughters and
247 1803 sons. The 1861 daughters were genotyped at one SNP marker, 27068nov, according to the
248 protocol described in the dedicated section. All the females that had a clear Makindu homozygote
249 genotype and all the females presenting an uncertain genotype were then genotyped at 2 more
250 markers (8225nov and 21770nov) to confirm their status.

251

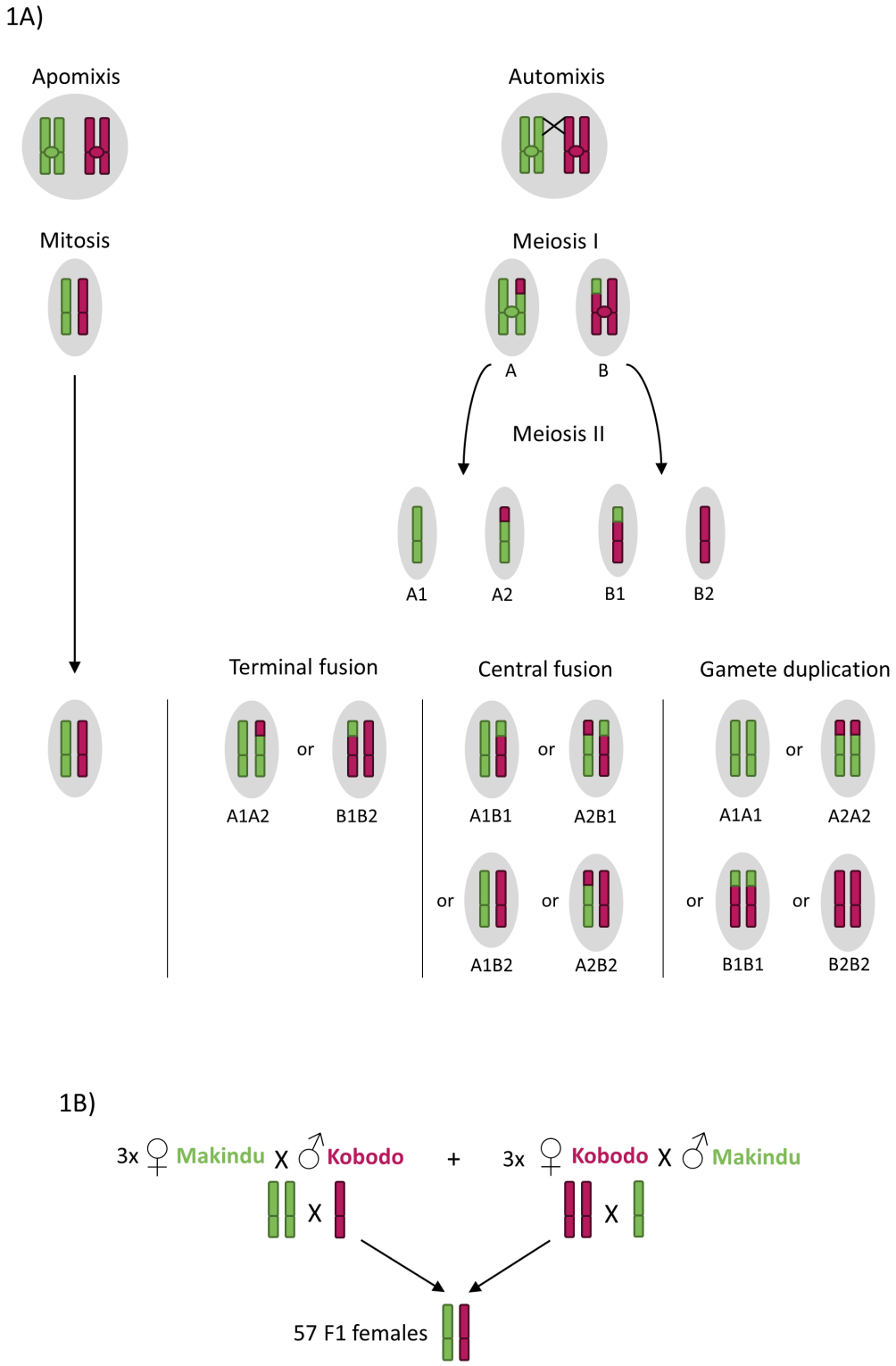


Figure 1: A) Expected genotype patterns of parthenogenetic daughters according to the main known thelytoky mechanisms. The cytological mechanisms are simplified because the figure focuses on the

genetic consequences that can be achieved in different ways (see introduction for further details). The patterns can vary from complete loss of heterozygosity (under gamete duplication) to complete maintenance of heterozygosity (under apomixis). B) Crosses performed in this study in order to obtain virgin heterozygous females. The analysis of recombination patterns of their daughters can give clues on the thelytoky mechanism involved.

Search for a bacterial cause of thelytoky in *Cotesia typhae*

To find out if the cause of thelytoky in *C. typhae* could be bacterial, we first performed PCR with primers designed to amplify DNA sequences from several micro-organisms known to manipulate sex in insects. Ten virgin Makindu females that produced parthenogenetic daughters and two virgin Makindu females that didn't produce daughters were tested with 8 primer sets, taken from Foray et al., (2013), except for one primer set, specific to *Wolbachia*, taken from Casiraghi et al., (2005). The primer sequences, Tm used for PCR, and their original publication are given in Supplementary Table 3. About 1ng of DNA was mixed with 1X buffer, 3mM $MgCl_2$, 0.4mM dNTP, 0.4µM of each primer, 1U GoTaq® Flexi DNA Polymerase (Promega), and completed with water. The PCR programme was 5 minutes at 95°C, followed by 40 cycles of 95°C for 1 minute, Tm for 1 minute, 72°C for one minute and a final elongation of 5 minutes at 72°C. The PCR products were then run on a 1% agarose gel to check for positive amplification.

The amplified fragments obtained with the *Arsenophonus* primer set were sequenced with the BigDye™ Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific), following the manufacturer's protocol. After the identification of the bacteria *Pantoea dispersa* through sequencing, 8 virgin Kobodo females that didn't produce any parthenogenetic daughters were also tested with this primer set. Since our Kobodo laboratory strain doesn't undergo thelytoky, this test was performed to check if *Pantoea dispersa* could be the causative agent of thelytoky in *C. typhae*.

To complete this experiment, we performed an antibiotic treatment on the Makindu laboratory strain to remove any potential sex manipulating bacteria in *C. typhae* females. Rifampicin was added in the host caterpillars' artificial diet, at a final concentration of 2g/L, for 4 *C. typhae* generations. This treatment has previously been shown to eliminate *Wolbachia* bacteria in a close species, *Cotesia sesamiae* (Mochiah et al., 2002). The phenotyping results after this first treatment being ambiguous, it was continued for 7 more generations, with tetracyclin added for the 4 last generations, also at a final concentration of 2g/L. At that point, 79 virgin *C. typhae* females were phenotyped, according to the phenotyping protocol previously described.

Results

Phenotypes of the different strains/populations

We phenotyped two laboratory strains for the thelytokous character, Makindu and Kobodo, and a wild population, coming from the Kobodo locality. The numbers of available virgin females obtained for phenotyping were as follows: 99 from 13 different cocoon masses for the Makindu strain, 40 from 7 cocoon masses for the Kobodo strain, and 29 from 6 cocoon masses for the Kobodo wild population. The results (Table 1) are very contrasted between these three populations, since the number of parthenogenetically produced females (hereafter referred as parthenogenetic females) is null in the Kobodo isofemale strain, intermediate in the Kobodo wild population (28% of virgin females produced daughters), and high in the Makindu isofemale strain (68% of virgin females

298 produced daughters). The thelytokous phenotype is therefore present in the wild and is not a
299 laboratory artefact, but was apparently lost in the Kobodo laboratory strain (likely due to genetic
300 drift), or present at a frequency too low to be detected. Unfortunately, the wild Makindu population
301 does not exist anymore and could not be tested in this study.

302

		N	Number of virgin females that produced female offspring (parthenogenetically) Percentage [95% confidence interval]	Number of males produced by all virgin females	Number of females produced by all virgin females Percentage [95% confidence interval]	Mean number [min ; max] of parthenogenetic females in the offspring presenting females
Makindu isofemale strain		99	67 68% [57.4-76.5]	10657	225 2% [1.81-2.36]	3.4 [1;8]
Kobodo isofemale strain		40	0	5405	0	-
Kobodo wild population		29	8 28% [13.4-47.5]	2132	8 0.4% [0.17-0.77]	1 [1;1]
Makindu strain after antibiotic treatment	After 4 generations	55	15 27% [16.5-41.2]	5886	17 0.3% [0.17-0.47]	1.13 [1;2]
	After 11 generations	79	13 16% [9.4-26.9]	8272	16 0.2% [0.11-0.32]	1.23 [1;4]

303 Table 1: Results of the thelytoky phenotyping of the different strains/populations. The phenotyping is
304 based on the number and frequency of daughters produced parthenogenetically (parthenogenetic
305 females) in the offspring of virgin *C. typhae* females. "N" is the total number of virgin females.

306

307 **Ploidy of the daughters of virgin females**

308 One daughter from the progeny of a fertilized female and 2 males, all belonging to the Makindu
309 laboratory strain, were processed by flow cytometry as respective controls for diploid and haploid
310 *Cotesia typhae* genomes. Five parthenogenetic daughters of virgin females were then processed,
311 resulting in an estimated genome size identical to the control female and twice that of the control

312 males (Table 2). We can therefore conclude that *C. typhae* parthenogenetic females are diploid and
313 not the result of feminization of haploid eggs.

Sample	Size (pg)	Size (Mpb)	Ploidy
Control female	0.47	458.27	Diploid
Control male 1	0.26	251.84	Haploid
Control male 2	0.25	241.12	Haploid
Parthenogenetic female 1	0.48	467.51	Diploid
Parthenogenetic female 2	0.48	473.24	Diploid
Parthenogenetic female 3	0.49	475.28	Diploid
Parthenogenetic female 4	0.49	475.91	Diploid
Parthenogenetic female 5	0.5	484.26	Diploid

314 Table 2: Genome size estimated by flow cytometry. Parthenogenetic females have the same genome
315 size as the control female, corresponding to about twice the males' haploid genome size.

316

317 **Fecundity of parthenogenetic females**

318 Eleven parthenogenetic females (issued from virgin Makindu mothers) were randomly allowed to
319 mate with their brothers and were used to parasitize eleven caterpillars. Out of these eleven
320 females, 4 had male only offspring and 7 had a mixed offspring. The number of offspring per female
321 and the sex-ratio are indicated in Table 3. No significant difference of the offspring size and sex ratio
322 was observed between the control and parthenogenetic female datasets (p-value obtained following
323 Mann-Whitney rank test was 0.559 for offspring number and 0.07 for sex-ratio). The fecundity of
324 parthenogenetic females is therefore equivalent to that of the control females.

	N	Mean number of offspring per mated female ± Standard Error	Mean female sex-ratio (when mated) ± Standard Error
Control Makindu strain[a]	41	59 ± 4.2	0.78 ± 0.03
Parthenogenetic females	7	54.3 ± 9.2	0.65 ± 0.12

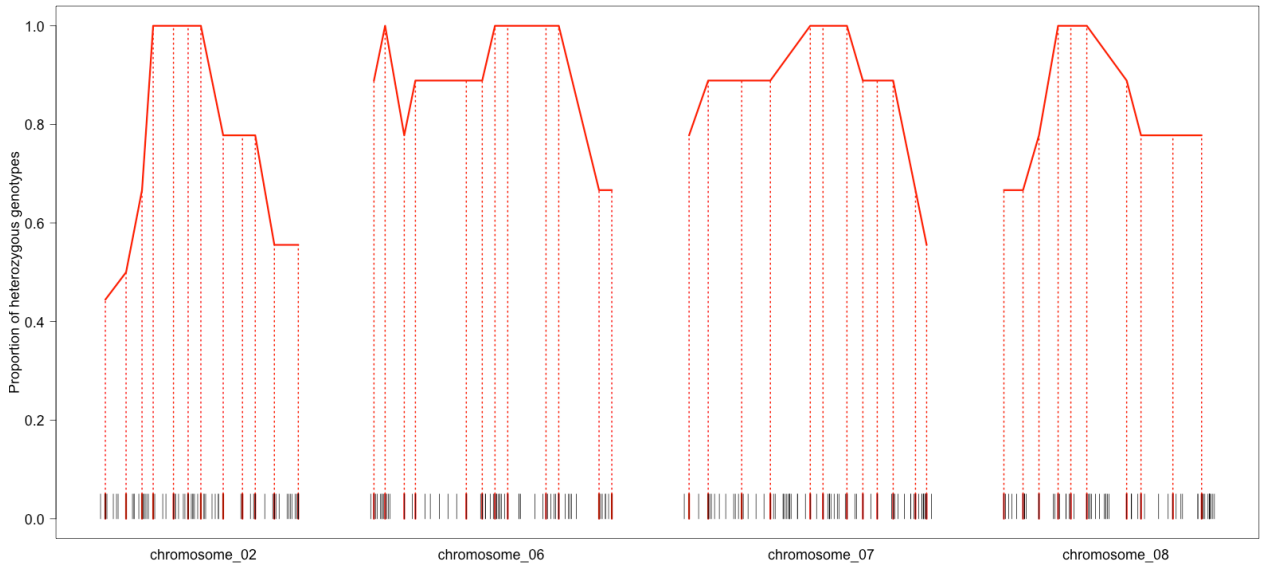
325 Table 3: Comparison of the fecundity between Makindu parthenogenetic females and control
326 females of the same laboratory strain. a: data from (Benoist et al., 2017).

327 All the cocoons resulting from the egg-laying of five virgin females (385 cocoons in total) were
328 isolated in order to obtain virgin parthenogenetic females. Fourteen females were thus obtained, out
329 of which ten produced an offspring. Three out of these 10 offspring (30%) contained parthenogenetic
330 females, for a total of 6 females and 773 males. Virgin parthenogenetic females are therefore able to
331 produce parthenogenetic females themselves.

332 **Thelytoky mechanism occurring in *Cotesia typhae***

333 The genotyping of the 63 SNP markers first confirmed that fathers and mothers of the initial crosses
334 between the Makindu and Kobodo laboratory strains were homozygotes for their strain's alleles. The
335 57 virgin F1 daughters resulting from these crosses were thus heterozygous at the SNP markers,
336 which was confirmed for the 5 F1 daughters that were genotyped. Each of these females successfully
337 parasitized a host larva, and from the 57 resulting offspring, six contained parthenogenetic females
338 (originating from 4 of the initial 6 crosses, 2 in each cross direction), corresponding to a total of 9 F2
339 parthenogenetic females for 6653 males. These 9 females were genotyped for the 63 SNP. The
340 genotypes and the deduced recombination events are presented in Supplementary Table 1. The
341 recombination patterns of the 4 chromosomes genotyped with a higher density of markers are
342 shown in Figure 2.

343 For six of the females, a mixture of heterozygous and homozygous markers was observed, with a
344 surplus of heterozygotes (280 heterozygous genotypes for 94 homozygous genotypes). The number
345 and pattern of heterozygous markers for these females indicates a mechanism similar to automixis
346 with central fusion (it could either result from the lack of first meiotic division or from the fusion of
347 two non-sister meiotic products). Indeed, the central parts of the chromosomes maintain a
348 heterozygous state while there is a recombination gradient leading to more homozygous genotypes
349 towards the extremities of the chromosomes (Fig. 2). On average, nine recombination events per
350 genome were detected for these six females with a minimum value of five events and a maximum of
351 16 events detected. Based on the density of the markers characterized, these results are consistent
352 with the genetic length measured by Benoist et al. (2020a).



353

354 Figure 2: Proportion of heterozygous females (out of nine) for each genotyped SNP marker. The
355 results are only shown for the 4 chromosomes for which a higher number of markers were
356 genotyped. The black segments on the x axis are indicative of the genetic position of all the markers
357 of the genetic map (Benoist et al., 2020a) and the red segments with the dotted lines correspond to
358 the positions of the markers genotyped in this study. The occurrence of homozygous and
359 heterozygous states along the chromosomes is congruent with a mechanism similar to automixis.
360 Given the metacentric nature of *C. typhae* chromosomes (C. Bressac, personal communication), the
361 observation of 100% heterozygosity in the central part of the chromosomes suggests that diploidy is

362 either maintained by the suppression of the first meiotic division or restored through central fusion
363 and is indicative of the position of each chromosome's centromere.

364 For the other 3 parthenogenetic females, all the 63 markers were heterozygous, revealing no
365 detection of recombination event on the 10 chromosomes. We estimated the probability of such an
366 observation under the hypothesis that a unique mechanism such as central fusion occurs. For each
367 chromosome, we calculated a mean number of recombinations based on the nine parthenogenetic
368 females. Assuming that the number of recombinations on a chromosome follows a Poisson
369 distribution, we can estimate the probability of zero recombination for each chromosome based on
370 the mean number estimate. It varies according to the genetic length of the chromosome and to the
371 density of markers genotyped: it was estimated between 0.29 for chromosome 2 and 0.8 for
372 chromosome 9. Multiplying the probability over the ten chromosomes, we calculated a probability of
373 0.0025 to observe an entirely heterozygous parthenogenetic daughter. Using this individual
374 probability, we estimated that the probability to detect three out of nine parthenogenetic daughters
375 showing no recombination events on the 10 chromosomes was 1.76×10^{-6}. This hypothesis is very
376 unlikely, therefore we suspect another mechanism could also be at play in causing thelytoky in
377 *Cotesia typhae*. It is interesting to note that we observed both patterns (partial homozygosity and
378 complete heterozygosity) in the offspring resulting from initial crosses of both directions. Moreover,
379 one of the F1 heterozygous females displayed both patterns in her progeny. The raw data and the R
380 script used to estimate the given probability are available at https://zenodo.org/record/6420801.

381

382 **Presence of parthenogenetic females among the daughters of mated females**

383 Among the 40 crosses between Makindu females and Kobodo males, five gave male-only offspring
384 and were excluded. The 35 remaining crosses that presented offspring comprising both males and
385 females were kept in our analysis, leading to a total of 1861 females and 1803 males. All 1861
386 females were genotyped for one SNP marker. Females resulting from fecundation should be
387 heterozygous while parthenogenetic females should be homozygous for the Makindu allele. In total,
388 we found 14 homozygous females, which were confirmed by the genotyping of 2 other markers.
389 These 14 females correspond to 0.77% of the parthenogenetic offspring (males resulting from
390 arrhenotoky representing 99.33%) and originate from 10 different mothers (29% of the 35 mothers)
391 (Table 4). Even though the percentage of parthenogenetic females found is much smaller than in the
392 offspring of virgin females, this finding shows that the female progeny of mated females can come
393 from a mixture of parthenogenesis and sexual reproduction.

	N	Number of females that produced parthenogenetic daughters Percentage [95% confidence interval]	Number of parthenogenetic offspring (males plus parthenogenetic females)	Number of parthenogenetic females among the parthenogenetic offspring Percentage [95% confidence interval]	Mean number [min ; max] of parthenogenetic females in the offspring presenting females
Makindu virgin	99	67 68% [57.4-76.5]	10882	225 2% [1.81-2.36]	3.4 [1;8]

females					
Makindu mated females	35	10 29% [15.2-46.5]	1817	14 0.77% [0.44-1.32]	1.4 [1;3]

Table 4: Comparison of the frequency of the thelytokous character between virgin and mated Makindu females. In each case, the number of parthenogenetic daughters is presented as a percentage of the total number of parthenogenetic offspring, mainly composed of males obtained from arrhenotoky. "N" is the number of offspring analysed.

Origin of thelytoky in *Cotesia typhae*

In order to find out if thelytoky in *Cotesia typhae* has a bacterial origin, we extracted DNA from Makindu virgin mothers that produced daughters and used primers to try to amplify the DNA of six different micro-organisms known for sex manipulation in insects: *Wolbachia, Ricketssia, Cardinium, Arsenophonus, Spiroplasma* and *Microsporidia* (Foray et al., 2013). Only one primer set led to a solid amplification, the one designed to amplify *Arsenophonus* 23S. After sequencing the amplified fragment, the bacterium was identified not as *Arsenophonus* but as *Pantoea dispersa*, for which no mention in relation to thelytoky was found in the literature. We then tried to amplify this same bacterium from the DNA of Kobodo virgin mothers, who don't produce any daughters: *Pantoea dispersa* was present in all the samples tested. This makes it unlikely for this bacterium to be responsible for thelytoky in *C. typhae*.

After rearing parasitized caterpillars for four generations on a rifampicin diet, we phenotyped the Makindu strain again. 55 virgin females, coming from 5 different cocoon masses, were allowed to parasitize *Sesamia nonagrioides* caterpillars. Fifteen of these virgin females produced daughters, leading to a total of 17 daughters for 5886 sons (Table 1). Another phenotyping was performed on 79 females from 10 different cocoon masses, after 11 generations of a rifampicin diet (with tetracyclin added for the last 4 generations). Thirteen of these virgin females produced daughters, leading to a total of 16 daughters for 8272 sons (Table 1). The percentage of thelytokous females is thus smaller than the one observed before antibiotic treatment but not null.

Discussion

The phenotypic survey presented here confirms the biological reality of low frequency asexual production of females in the haplo-diploid Hymenoptera *Cotesia typhae*. The process has been observed in a significant number of progenies from both an inbred laboratory strain and a natural population. It has been shown to occur in the progeny of virgin as well as fertilized females, despite concerning only a small fraction of the individuals from a cocoon mass.

This configuration of low frequency thelytoky is poorly illustrated in the literature, except in the well-studied species *Apis mellifera* for which the phenomenon has been described for a long time (Mackensen, 1943; Tucker, 1958). In the honey bee, both workers and virgin queens are able to produce a small proportion of females among unfertilized progeny (Gloag et al., 2019). However, in a common acceptation, the expression thelytoky is rather defined as a "parthenogenetic mode where females produce only females from unfertilized eggs" (Vershinina and Kuznetsova, 2016). Among most illustrated examples of parthenogenesis *sensu stricto*, even when facultative, asexual

432 production of females involves the whole progeny. The case described here is somewhat closer to
433 what is called tychoparthenogenesis, based on the frequency of birth of parthenogenetic female eggs
434 (Whiting, 1945). Tychoparthenogenesis is defined as "kind of occasional thelytoky characterized by
435 the spontaneous hatching of a small proportion of eggs laid by virgin females" (Pardo et al., 1995). It
436 has been mainly described in diplodiploid species where embryonic development is induced by
437 sperm fertilization. In such species, developmental constraints and inbreeding depression prevent
438 successful hatching of unfertilized eggs in most of the cases (Little et al., 2017). In haplodiploid
439 species, unfertilized eggs hatch with a high frequency because they naturally produce males in
440 species reproducing sexually. It is thus difficult to classify *C. typhae* as a tychoparthenogenetic
441 species. Moreover, because the daughters produced parthenogenetically turned out to be viable and
442 fertile in *C. typhae*, low frequency thelytoky may be either neutral or beneficial but not
443 disadvantageous as in most cases of tychoparthenogenesis described.

444 The question remains as to whether the phenomenon is accidental or an ongoing evolutionary
445 process due to its adaptive benefit (van der Kooi and Schwander, 2015). In the eusocial species *Apis*
446 *mellifera*, low frequency thelytoky is clearly beneficial to workers when confronted to a queen-less
447 colony (Gloag et al., 2019). This advantage led to an increased frequency of workers' reproduction in
448 an honey bee subspecies (*Apis m. capensis*) and to the development of social parasitism where laying
449 workers get adopted by a colony and compete the local queen (Neumann, 2001). Studying the
450 occurrence of parthenogenesis among Ephemeroptera, Liegeois et al. (2021) suggested that asexual
451 reproduction was selectively advantageous in many species from this insect order despite its
452 associated low hatching success. The benefit derives from the short adult life and the low dispersal
453 ability that reduce the probability of encountering a reproductive partner. The fitness of sexually
454 reproducing individuals may consequently be reduced under certain circumstances. As in mayflies, *C.*
455 *typhae* has an adult life limited to a few days (between two and three days in laboratory conditions,
456 Kaiser et al., 2017). However, it is gregarious and mating between sisters and brothers emerging
457 from the same cocoon mass is observed in rearing conditions. Female access to male fertilization
458 should thus be facilitated unless sib-mating is avoided in natural conditions as demonstrated for its
459 relative species *Cotesia glomerata* (Gu and Dorn, 2003) or *Venturia canescens* (Collet et al., 2020).
460 Even in the presence of sexual partners, limited access to fertilization may also derive from
461 ineffective copulation in cases of highly female biased sex ratios for example (Boivin, 2013). Whether
462 it results from restricted access to males or to sperm itself, sperm limitation may favour expansion of
463 asexual reproduction. Further experiments are needed to estimate mating and fertilization success of
464 *Cotesia typhae* in natural conditions.

465 Beyond reproductive strategy itself, parthenogenesis has been shown to be associated with
466 ecological characteristics that may favour or prevent its evolution. Two opposite ecological trends
467 have been described co-occurring with asexual reproduction expansion: the "general purpose
468 genotype" (GPG) where asexual lineages are observed on broader ecological niches than their sexual
469 counterparts and the "frozen niche variation" (FNV) where parthenogenetic species or populations
470 have far more restricted niches than sexual ones (Tvedte et al., 2019). Exploring a wide dataset of
471 haplodiploid arthropods reproducing exclusively parthenogenetically (obligate parthenogenesis), van
472 der Kooi et al. (2017) concluded that GPG was the most common situation. They showed that most
473 parthenogenetic species have broader ecological and geographical range than close relative sexual
474 species but also that transition toward parthenogenesis was more likely for species exhibiting a wide
475 distribution. Studying the relative advantage of sexual and asexual reproduction, Song et al., (2012)
476 developed a model based on resource availability, in spatial and temporal heterogeneous situations.
477 They showed that sexual reproduction prevails in most of the cases but that asexual reproduction
478 may be favoured when resource diversity is low and resource remains abundant over generations.

Such a model corroborates numerous cases of ecological specialization of asexual lineages that have been described, such as *Venturia canescens*. In this polymorphic species, two kinds of populations live in sympatry: parthenogenetic populations found in stable anthropic habitats (bakeries and granaries) and sexual ones associated with natural and more instable resources (Schneider et al., 2002). Interestingly, before being characterized as a new species, *Cotesia typhae* was first identified as a specialized clade (only one host insect, *Sesamia nonagrioides,* mainly found on one host plant, *Typha domingiensis*) of the parasitoid species *Cotesia sesamiae*. According to (Branca et al., 2019), some populations of *C. sesamiae* are less specialized than others. Studying the existence of thelytokous reproduction in those populations would be informative about the possible link between emerging parthenogenesis and specialization.

Regarding the mechanism involved in thelytokous reproduction, we faced an unexpected result as data strongly suggest that two different processes may co-occur: automixis with central fusion (or a similar cytological mechanism) and apomixis. More surprisingly, the two supposed mechanisms were observed to co-occur in the progeny of a single female (K4M1) and independently of the cross direction to obtain F1 virgin mothers (Kobodo female x Makindu male or Makindu female x Kobodo male). Unfortunately, this result is supported by small sample size due to the scarcity of the phenomenon. We may wonder whether a unique mechanism, distinct from those already described, could explain such a result. Ma and Schwander (2017) describe for example an unusual process where meiosis is inverted (sister chromatids separate before homologs) followed by terminal fusion. However, the resulting progeny of such a process is 100% heterozygous, a result that does not differ from apomixis. Another mechanism presented in the same review implies an endoreplication preceding meiosis. Assuming such a process occurs in *C. typhae*, and hypothesizing that recombination, and consequently segregation during the first division, may arise either between identical or between homologous chromosomes, some intermediate situations are expected. Once again, it does not reconcile the clear-cut figure we observe with individuals entirely heterozygous suggesting zero recombination between homologs and individuals for which recombination is observed for almost all homologs. If different mechanisms truly co-occur to produce females in an asexual way, it could reflect an accidental phenomenon arising from the relaxed control of sexual reproduction as observed in the honey bee (Aamidor et al., 2018). To better understand the mechanism underlying thelytoky in *C. typhae*, a cytological approach of meiosis and parthenogenesis would be necessary.

Despite the lack of a unified mechanism to explain the genotypic profile observed in the F2 progenies obtained, we can confirm that recombination occurred in at least 6 out of 9 cases, and that these recombination events were as frequent as those observed in sexual reproduction (Benoist et al., 2020a). By contrast, severe reductions of recombination rates were observed associated with parthenogenetic reproduction in the literature. For example, recombination in thelytokous workers is reduced by up to 10-fold in comparison to their sexually reproducing mothers in the Cape bee, *Apis mellifera capensis*, the social parasite of honeybee which reproduces parthenogenetically *via* automixis with central fusion (Baudry et al., 2004). In the little fire ant *Wasmannia auropunctata*, sexual populations coexist with asexual populations in which reproductive queens are produced by automictic parthenogenesis with central fusion. In asexual populations, recombination rate is reduced by a factor of 45 compared to the sexual populations (Rey et al., 2011). The reduction of recombination rate is assumed to mitigate the potential deleterious impact of thelytoky: under automixis with central fusion, heterozygosity is preserved unless recombination occurs (Figure 1). In species affected by inbreeding depression, a homozygosity increase would be detrimental and could be advantageously limited by a low recombination rate. As the molecular mechanisms involved in thelytoky and recombination are probably distinct, the situation observed in the Cape bee and little

526 fire ant may result from a long-term evolutionary process. If the phenomenon described in *C. typhae*
527 is recent, it may explain the unchanged recombination rate.

528 Otherwise, the inbreeding impact could be meaningless in *C. typhae*. Hymenoptera are haplodiploid
529 and could thus be less sensitive to inbreeding because most of the deleterious alleles are purged at
530 the haploid state in males (Hedrick and Parker, 1997; Henter, 2003). However, their sex
531 determination system may be highly compelling regarding homozygosity and ability to reproduce *via*
532 thelytoky (Vorburger, 2014). The most common, and likely ancestral, sex determination system is
533 governed by the genotype at one (sl-CSD: single locus Complementary Sex Determination) or few loci
534 (ml-CSD: multi locus CSD) (Heimpel and de Boer, 2008). Under such a determinism, individuals that
535 are heterozygous at least at one of these CSD loci develop as diploid females while hemizygous or
536 homozygous individuals at all CSD loci develop as haploid or diploid males respectively. In most
537 hymenopteran species, diploid males have a low survival rate and/or are often sterile. Enhanced
538 homozygosity due to thelytoky may be very costly when it results in diploid male production (de Boer
539 et al., 2015, 2012; van Wilgenburg et al., 2006; Zhou et al., 2007). Other sex determination systems
540 that are less sensitive to homozygosity have been described in Hymenoptera, such as Paternal
541 Genome Elimination (Heimpel and de Boer, 2008) or genome imprinting, described in *Nasonia*
542 *vitripennis* (van de Zande and Verhulst, 2014). The mechanism of sex determination is unknown in *C.*
543 *typhae*. However, it has been reared for more than 80 generations in laboratory conditions starting
544 from an isofemale line and seems poorly sensitive to homozygosity increase.

545 The bacterial origin of thelytoky in *C. typhae* could not be either confirmed or completely discarded
546 in the present study as an intermediate state (in terms of frequency of parthenogenesis) was
547 observed following antibiotic treatment. The knowledge of the genetic mechanism could give some
548 clues about the origin of parthenogenesis as endosymbionts have been mainly shown to favour
549 gamete duplication. However, detailing specific interactions reveals a more complex picture.
550 *Cardinium* is able to feminize diploid males (Giorgini et al., 2009) but also to induce automixis with
551 central fusion (Zchori-Fein and Perlman, 2004). *Wolbachia* is mainly known to induce gamete
552 duplication (Leach et al., 2009; Ma and Schwander, 2017) but it has also been described to promote
553 apomixis (Weeks and Breeuwer, 2001). *Rickettsia* has also been shown to trigger functional apomixis
554 (Adachi-Hagimori et al., 2008). Furthermore, the list of endosymbionts is probably partial and in most
555 of the documented examples of parthenogenesis endosymbiotically determined, the cytological
556 mechanism remains unknown. Evidence that microorganisms can promote all processes of
557 parthenogenesis will probably arise from future research. The examples of demonstrated genetic
558 determinism of thelytoky are rare and only concern automixis with central fusion. This is the case for
559 the Cape honey bee (Verma and Ruttner, 1983) and for the wasp *Venturia canescens* (Beukeboom
560 and Pijnacker, 2000). However, Tsutsui et al., (2014) described an apomixis mechanism in the
561 parasitoid wasp *Meteorus pulchricornis* for which they proposed a genetic origin of thelytoky. Even
562 more than for endosymbiont origin, genetic determinism of parthenogenesis requires thorough
563 investigations to determine whether it is restricted to a few cytological mechanisms. Anyway, the
564 clearly evidenced mechanism of autoximis with central fusion in *C. typhae* does not allow to settle
565 between genetic and endosymbiont origin as this mechanism is common to both situations.

566

567 **Conclusion**

568 In this study, we described an unusual example of low frequency thelytokous reproduction within a
569 sexually reproducing species. We demonstrated that this asexual reproduction is likely the result of
570 different mechanisms and occurs even in the progeny of fertilized females, an undescribed

phenomenon to our knowledge. As most studies on asexual reproduction focus on obligate or cyclical situations, we may wonder whether such low frequency and probably accidental thelytoky is common but until now mostly undetected among sexually reproductive species. It is well known that asexuality has emerged many times in numerous lineages. If the occurrence of accidental parthenogenesis turns out to be usual, it could represent the first step of evolutionary trajectories favoured either by reproductive or ecological advantages of asexual lineages.

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Data availability

Raw data for Figure 2, and Tables 1, 3 and 4 are available at https://zenodo.org/record/6420801

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