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 of 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 pluricellular 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 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 heterogenous 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 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 favoring the 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 to restore diploid state and produce females (Rabeling and Kronauer, 2013; Vorburger, 2014), illustrated in figure 1B. Apomixis is based on mitosis and induces clonal reproduction. The same clonal issue may also occur when endoreplication precedes meiosis, resulting in recombination between identical chromosomes (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. 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). 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 species and to the necessity to maintain heterozygosity, each mechanism may be favored or not.

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). Up to date, only bacteria have been evidenced 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 frequency of thelytoky within species also varies between 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 produce female only progeny, albeit low frequency of males may be reported allowing to maintain 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) currently 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*. We measured the percentage of females able to produce daughters parthenogenetically as well as the proportion of thelytokous daughters to arrhenotokous sons in their progeny. These ratios were obtained for both laboratory strains but also for the wild population from Kobodo. We measured the ploidy level of the thelytokous daughters to test whether they result from male feminization or from diploid restoration. Taking advantage of the available genetic map, we investigated the mechanism responsible for thelytoky. The identified markers were also used to detect the presence of thelytokous daughters in the progeny of fertilized females. Finally, we tested the endosymbiotic origin of thelytoky and its reversibility by supplying wasps with antibiotics for successive generations and testing for the presence of well-known endosymbionts using barcoding markers.

Material and Methods

Biological material

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

Phenotyping the strains/populations for the thelytokous character

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

Flow cytometry for ploidy analysis

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

Fecundity assessment of parthenogenetic females

Cocoon masses resulting from the eggs laid by *C. typhae* Makindu virgin females in *S. nonagrioides* caterpillars were divided in smaller cocoon packs to spot parthenogenetic females more easily among the males after the emergence of the adults. Adults were left together for one day with water and honey to allow mating, and eleven parthenogenetic females were then allowed to oviposit in *S. nonagrioides* caterpillars. After the emergence of the resulting offspring, the number of males and females was counted for each one of them and the sex-ratio was calculated for comparison with that obtained from fertilized females from the control Makindu laboratory strain.

Identifying the thelytoky mechanism in *Cotesia typhae*

To find out which thelytoky mechanism is at play in the *C. typhae* Makindu laboratory strain, virgin heterozygous females are needed to analyse the recombination patterns of their offspring. Indeed, according to the mechanism, the female offspring will be more or less heterozygous, as explained in introduction (Figure 1B). To obtain virgin heterozygous females, six controlled crosses were

176 performed between the Makindu and Kobodo laboratory strains, 3 in each direction (Figure 1A).
177 Prior to this, cocoons had been isolated from masses of each strain, in order to obtain virgin males
178 and females for the crosses. Cocoons were then isolated from the masses resulting from the crosses,
179 leading to the emergence of virgin F1 heterozygous females. 57 of these females were allowed to
180 oviposit in *S. nonagrioides* caterpillars, and the offspring of the 57 resulting cocoon masses were
181 sexed and counted. Six females and four males from the two parental strains (including the
182 individuals used for the initial crosses), five F1 heterozygous females and the nine parthenogenetic
183 females obtained through this experiment were kept for DNA extraction and genotyping, in order to
184 analyse the recombination patterns resulting from parthenogenesis.

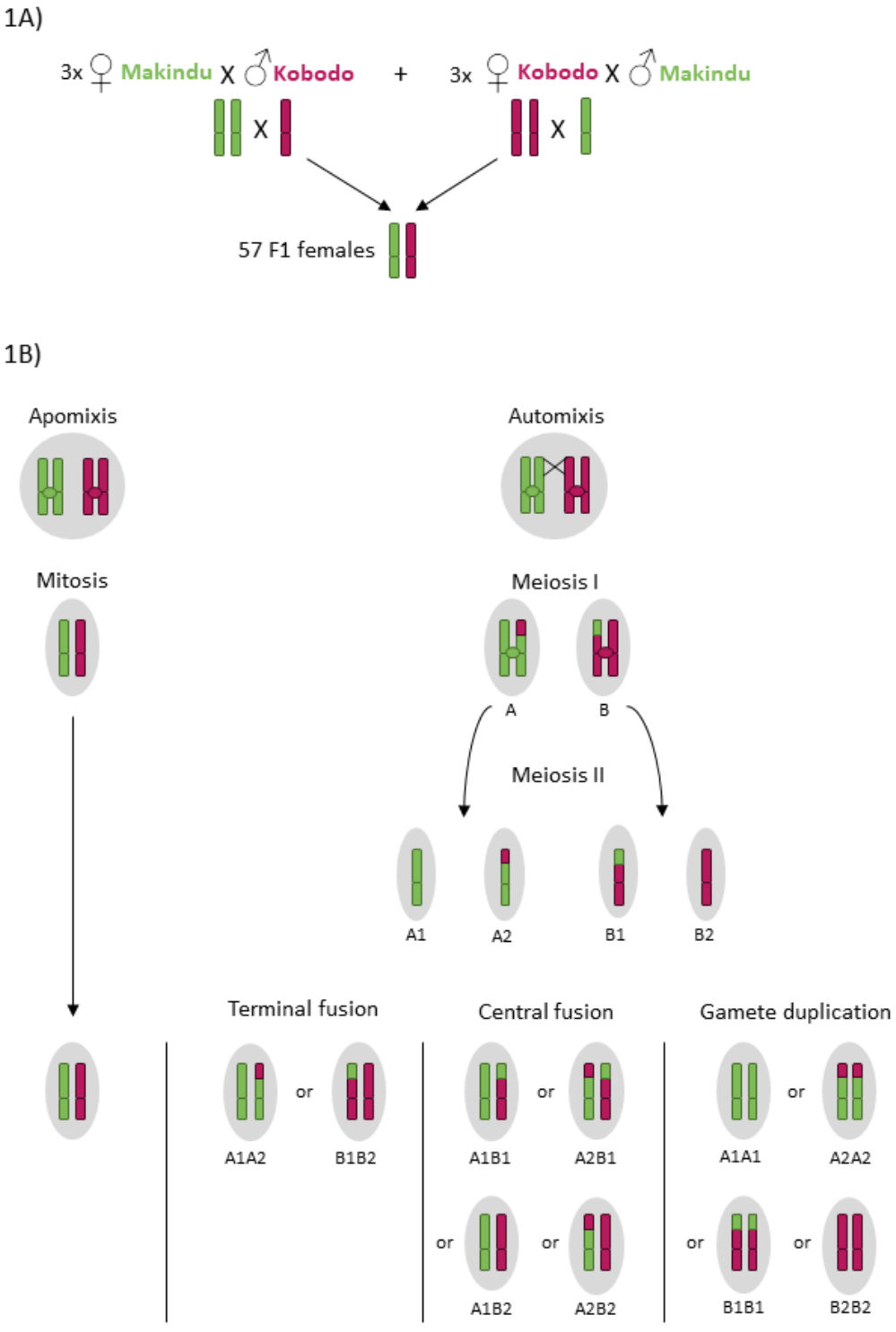
185 DNA was extracted from the 24 individuals using the NucleoSpin® Tissue from Macherey Nagel,
186 following the manufacturer's instructions.

187 To analyse the recombination patterns of the nine parthenogenetic females, we genotyped 63 SNP
188 (Single Nucleotide Polymorphism) markers, having different alleles between the Makindu and
189 Kobodo strains, and being distributed along the 10 chromosomes of the genetic map of *Cotesia*
190 *typhae*, (Benoist et al., 2020a). Four chromosomes contained more markers than the others to
191 investigate the recombination patterns along chromosomes. For these four chromosomes, the
192 markers were chosen to have about 10cM between two successive markers when possible. The
193 genetic position of all the markers used in this present study are given in Supplementary Table 1.

194 The HRM (High Resolution Melt) technique was used to genotype 61 of the markers. This technique
195 is based on the analysis of melt curves of DNA fragments after amplification by PCR. The melt curves
196 are different according to the nucleotide composition of the DNA fragments, and therefore allow to
197 discriminate between homozygotes and heterozygotes at a given SNP. For each SNP marker, a 10µL
198 mix was made with about 1ng of DNA, 0.2µM of each primer, and 5µL of Precision Melt Supermix
199 (Bio-Rad), completed with water. The PCR protocol was 95°C for 2 minutes, followed by 40 cycles of
200 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a complete denaturation
201 of 30 seconds at 95°C before performing the melt curve. The melt curve was performed on a CFX96™
202 Real-Time System (Bio-Rad), and was started by an initial step of 1 minute at 60°C, followed by 10
203 seconds of every 0.2°C increment between 65°C and 95°C. The raw data resulting from the melt
204 curves were analysed with the uAnalyze v2.1 software (Dwight et al., 2012) in order to infer the
205 females' genotypes at each SNP marker.

206 The two remaining SNP markers (8225nov and 21770nov) were genotyped using allele specific PCR.
207 For this method, two parallel amplifications were performed on individuals, one of the primers of the
208 couple being a common primer, and the other one being a specific primer, either to the Makindu, or
209 Kobodo allele. About 1ng of DNA was mixed with 1X buffer, 3mM $MgCl_2$, 0.4mM dNTP, 0.4µM of
210 each primer, 1U GoTaq® Flexi DNA Polymerase (Promega), and completed with water. The PCR
211 programme was 5 minutes at 95°C, followed by 40 cycles of 95°C for 1 minute, 50°C or 55°C for 1
212 minute (50°C for 8225nov and 55°C for 21770nov), 72°C for one minute and a final elongation of 5
213 minutes at 72°C. The PCR products were then run on a 2% agarose gel to check which PCR were
214 positive and therefore infer the genotypes.

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



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218 Figure 1: A) Crosses performed in this study in order to obtain virgin heterozygous females. B)
219 Expected genotypes' patterns of parthenogenetic daughters according to the thelytoky mechanism

involved. The patterns can vary from complete loss of heterozygosity (under gamete duplication) to complete maintenance of heterozygosity (under apomixis).

Looking for thelytoky in mated females

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

Out of the 40 crosses, only the ones leading to a mix of male and female offspring were kept in our analysis, leading to a total of 1861 daughters and 1803 sons. To genotype the 1861 daughters, a direct PCR method was used, instead of a classic DNA extraction. For each female, the abdomen was removed (because the presence of gametes could hinder the genotyping) and the rest of the body was placed in 20µL of Dilution Buffer and 0.5µL of DNA Release Additive (Thermoscientific). The tubes were kept at room temperature for 5 minutes then placed at 98°C for 2 minutes. One microliter of this mix was used as template for the PCR performed for the HRM genotyping (see above) of one SNP marker, 27068nov. All the females that had a clear Makindu homozygote genotype and all the females presenting an uncertain genotype were withdrawn from their buffer and their DNA was properly extracted with the NucleoSpin® Tissue kit from Macherey Nagel. They were then genotyped at 2 more markers (8225nov and 21770nov) to confirm their status, by an allele-specific PCR method. The three markers used here for genotyping were used previously for the identification of the thelytoky mechanism, and the protocol is the same as described above.

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 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 primers sets, taken from (Foray et al., 2013), except for one primer set, specific to *Wolbachia*, taken from (Casiraghi et al., 2005). The primers' 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 the following: 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 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 produced daughters). The thelytokous phenotype is therefore present in the wild and is not a laboratory artefact, but was apparently lost in the Kobodo laboratory strain, or present at a frequency too low to be detected. Unfortunately, the wild Makindu population does not exist anymore and could not be tested in this study.

		N	Number of offspring with parthenogenetic females	Total number of males in the N offspring	Total number of parthenogenetic females in the N offspring	Mean number [min ; max] of parthenogenetic females per offspring
Makindu isofemale strain		99	67 (68%)	10657	225 (2%)	2.3 [0;8]
Kobodo isofemale strain		40	0	5405	0	0
Kobodo wild population		29	8 (28%)	2132	8 (0.4%)	0.3 [0;1]
Makindu strain after antibiotic treatment	After 4 generations	55	15 (27%)	5886	17 (0.3%)	0.3 [0;2]
	After 11 generations	79	13 (16%)	8272	16 (0.2%)	0.2 [0;4]

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

288 **Ploidy of the daughters of virgin females**

289 One daughter from the progeny of a fertilized female and 2 males, all belonging to the Makindu
290 laboratory strain, were processed by flow cytometry as respective controls for diploid and haploid
291 *Cotesia typhae* genomes. Five parthenogenetic daughters of virgin females were then processed,
292 resulting in an estimated genome size identical to the control female and twice that of the control
293 males (Table 2). We can therefore conclude that *C. typhae* parthenogenetic females are diploid and
294 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

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

297

298 **Fecundity of parthenogenetic females**

299 Eleven parthenogenetic females (issue from virgin Makindu mothers) were randomly allowed to
300 mate with their brothers and were used to parasitize eleven caterpillars. Out of these eleven
301 females, 4 had male only offspring and 7 had a mixed offspring. The number of offspring per female
302 and the sex-ratio are indicated in Table 3. No significant difference of the offspring size and sex ratio
303 was observed between the control and parthenogenetic female datasets (p-value obtained following
304 Mann-Whitney rank test was 0.559 for offspring number and 0.07 for sex-ratio). The fecundity of
305 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

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

Thelytoky mechanism occurring in *Cotesia typhae*

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

For six of the females, a mixture of heterozygous and homozygous markers was observed, with a surplus of heterozygotes (280 heterozygous genotypes for 94 homozygous genotypes). The number and pattern of heterozygous markers for these females indicates a mechanism of automixis with central fusion. Indeed, the central parts of the chromosomes maintain a heterozygous state while there is a recombination gradient leading to more homozygous genotypes towards the extremities of the chromosomes (Fig. 2). On average, nine recombination events per genome were detected for these six females with a minimum value of five events and a maximum of 16 events detected. Based on the density of the markers characterized, these results are consistent with the genetic length measured by Benoist et al. (2020a).

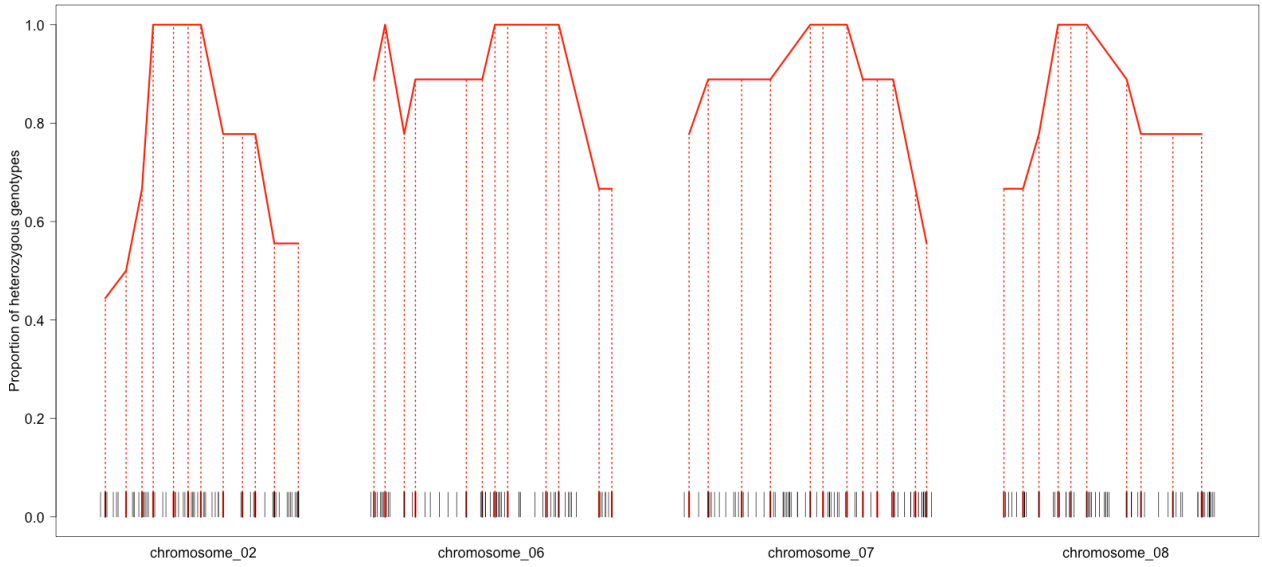

Figure 2: Proportion of heterozygous females (out of nine) for each genotyped SNP marker. The results are only shown for the 4 chromosomes for which a higher number of markers were genotyped. The black segments on the x axis are indicative of the genetic position of all the markers of the genetic map (Benoist et al., 2020a) and the red segments with the dotted lines correspond to the positions of the markers genotyped in this study. The occurrence of homozygous and heterozygous states along the chromosomes is congruent with a mechanism of automixis. The observation of 100% heterozygosity in the central part of the chromosomes suggests that diploidy is restored through central fusion and is indicative of the position of each chromosome's centromere.

For the other 3 parthenogenetic females, all the 63 markers were heterozygous, revealing no detection of recombination event on the 10 chromosomes. We estimated the probability of such an observation under the hypothesis that a unique mechanism of central fusion occurs. For each chromosome, we calculated a mean number of recombinations based on the nine parthenogenetic females. Assuming that the number of recombinations on a chromosome follows a Poisson distribution, we can estimate the probability of zero recombination for each chromosome based on the mean number estimate. It varies according to the genetic length of the chromosome and to the density of markers genotyped: it was estimated between 0.29 for chromosome 2 and 0.8 for chromosome 9. Multiplying the probability over the ten chromosomes, we calculated a probability of 0.0025 to observe an entirely heterozygous parthenogenetic daughter. Using this individual probability, we estimated that the probability to detect three out of nine parthenogenetic daughters showing no recombination events on the 10 chromosomes was 1.6×10^{-6}. This hypothesis is very unlikely, therefore we suspect another mechanism could also be at play in causing thelytoky in *Cotesia typhae*. It is interesting to note that we observed both patterns (partial homozygosity and complete heterozygosity) in the offspring resulting from initial crosses of both directions. Moreover, one of the F1 heterozygous females displayed both patterns in her progeny.

Presence of parthenogenetic females among the daughters of mated females

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

	N	Number of parthenogenetic offspring (males plus parthenogenetic females)	Number of parthenogenetic females among the parthenogenetic offspring	Mean number [min ; max] of parthenogenetic females in offspring when present
Makindu virgin females	99	10882	225 (2%)	2.3 [1;8]
Makindu mated females	35	1817	14 (0.77%)	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 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 as well. 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 unusual. As a matter of fact, the expression thelytoky is even defined by some authors as a "parthenogenetic mode where females produce only females from unfertilized eggs" (Vershinina and Kuznetsova, 2016). Among illustrated examples of parthenogenesis *sensu stricto*, even when facultative, asexual production of females involves the whole progeny. The case described here is somewhat closer to what is called tychoparthenogenesis, based on the frequency of birth of parthenogenetic female eggs (Whiting, 1945). Tychoparthenogenesis is defined as "kind of occasional thelytoky characterized by the spontaneous hatching of a small proportion of eggs laid by virgin females" (Pardo et al., 1995). It has been mainly described in diplodiploid species where embryonic development is induced by sperm fertilization. In such species, developmental constraints and inbreeding depression prevent successful hatching of unfertilized eggs in most of the cases (Little et al., 2017). In haplodiploid species, unfertilized eggs hatch with a high frequency because they naturally produce males in species reproducing sexually. It is thus difficult to classify *C. typhae* as a tychoparthenogenetic species.

A better description of its asexual reproductive mode would require the comparative study of the hatching success of fertilized diploid eggs, unfertilized diploid eggs and unfertilized haploid eggs. As *C. typhae* is an endoparasitoid species, such estimates are difficult to obtain. The low proportion of asexual daughters seems not however to be detrimental to the wasp as progeny numbers were similar between those without, and those including thelytokous daughters. Benoist et al. (2020b)

showed that the number of oviposited eggs was close to the number of emerging adults. The survival ratio deduced from these two estimates is similar between the two laboratory strains Kobodo and Makindu, however thelytoky was demonstrated only in the Makindu strain. Taken together, these data suggest that the phenomenon most likely results from rare events rather than from low developmental success. Furthermore, the daughters produced parthenogenetically turned out to be viable and fertile, indicating that low frequency thelytoky may be either neutral or beneficial but not disadvantageous.

The question remains as to whether the phenomenon is accidental or an ongoing evolutionary process due to its adaptive benefit (van der Kooi and Schwander, 2015). Studying the occurrence of parthenogenesis among Ephemeroptera, Liegeois et al. (2021) suggested that asexual reproduction was selectively advantageous in many species from this insect order despite its associated low hatching success. The benefit derives from the short adult life and the low dispersal ability that reduce the probability of encountering a reproductive partner. The fitness of sexually reproducing individuals may consequently be reduced under certain circumstances. As in mayflies, *C. typhae* has an adult life limited to a few days (between two and three days in laboratory conditions, Kaiser et al., 2017). However, it is gregarious and mating between sisters and brothers emerging from the same cocoon mass is observed in rearing conditions. Female access to male fertilization should thus be facilitated. Whether sib-mating occurs in natural conditions is actually unknown but it may be favoured by the endophytic mode of life of its larval host, because cocoon masses are formed inside plant stems. The dispersal behaviour of males and females is also unknown but is likely to be limited to short distances, as it was estimated to be at most 64m for the close relative *Cotesia flavipes* (Dinardo-Miranda et al., 2014; Sallam et al., 2001). Sib-mating has been shown to be avoided in several studies. In *Cotesia glomerata*, it was demonstrated that females mated with males from the same cocoon mass in only 27.5% of the cases (Gu and Dorn, 2003). In *Venturia canescens*, studies of mating behaviour showed that sib-mating was limited by a male-biased dispersal (Collet et al., 2020). If *C. typhae* males behave similarly in nature, females may face limited access to copulation. Another potential restricted access to fertilization concerns sperm limitation. A mated female may meet some difficulties due to sperm incompatibility or a delay post-mating where sperm is not available. When the sex ratio is highly biased, males have to mate with numerous females and may undergo temporarily or definitively sperm depletion (Boivin, 2013). Preliminary experiments revealed that *C. typhae* males were able to fertilize ten females successively with no detectable impact on the progeny's sex-ratio (data not shown). However, some of the mated females (whatever their mating rank was in the protocol) produced male-only offspring, suggesting that sperm transfer and/or usage is sometimes difficult. Whether it results from restricted access to males or to sperm itself, sperm limitation may favour expansion of asexual reproduction. Further experiments are needed to estimate mating and fertilization success of *Cotesia typhae* in natural conditions.

Beyond reproductive strategy itself, parthenogenesis has been shown to be associated with ecological characteristics that may favour or prevent its evolution. Two opposite ecological trends have been described co-occurring with asexual reproduction expansion: the "general purpose genotype" (GPG) where asexual lineages are observed on broader ecological niches than their sexual counterparts and the "frozen niche variation" (FNV) where parthenogenetic species or populations have far more restricted niches than sexual ones (Tvedte et al., 2019). Exploring a wide dataset of haplodiploid arthropods reproducing exclusively parthenogenetically (obligate parthenogenesis), van der Kooi et al. (2017) concluded that GPG was the most common situation. They showed that most parthenogenetic species have broader ecological and geographical range than close relative sexual species but also that transition toward parthenogenesis was more likely for species exhibiting a wide distribution. Nevertheless, numerous cases of ecological specialization of asexual lineages have been

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

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

488 Despite the lack of a unified mechanism to explain the genotypic profile observed in the F2 progenies
489 obtained, we can confirm that recombination occurred, at least in cases attributable to central
490 fusion, and that these recombination events were as frequent as those observed in sexual
491 reproduction (Benoist et al., 2020a). By contrast, severe reductions of recombination rates were
492 observed associated with parthenogenetic reproduction in the literature. For example,
493 recombination is reduced by up to 10-fold in the Cap bee, *Apis mellifera capensis*, a social parasite of
494 honeybee which reproduces parthenogenetically *via* automixis with central fusion (Baudry et al.,
495 2004). In the little fire ant *Wasmannia auropunctata*, sexual populations coexist with asexual
496 populations in which reproductive queens are produced by automictic parthenogenesis with central
497 fusion. In the asexual populations, recombination rate is reduced by a factor 45 compared to the
498 sexual populations (Rey et al., 2011). The reduction of recombination rate is assumed to mitigate the
499 potential deleterious impact of thelytoky: under automixis with central fusion, heterozygosity is
500 preserved unless recombination occurs (Figure 1). In species affected by inbreeding depression, a
501 homozygosity increase would be detrimental and could be advantageously limited by a low
502 recombination rate. As the molecular mechanism involved in restoration of diploidy and
503 recombination are probably distinct, the situation observed in Cap bee and little fire ant may result
504 from a long-term evolutionary process. If the phenomenon described in *C. typhae* is recent, it may
505 explain the unchanged recombination rate.

Otherwise, the inbreeding impact could be meaningless in *C. typhae*. Hymenoptera are haplodiploid and could thus be less sensitive to inbreeding because most of the deleterious alleles are purged at the haploid state in males. However, their sex determination system may be highly compelling regarding homozygosity and ability to reproduce *via* thelytoky (Vorburger, 2014). The most common, and likely ancestral, sex determination system is governed by the genotype at one (sl-CSD: single locus Complementary Sex Determination) or few loci (ml-CSD: multi locus CSD) (Heimpel and de Boer, 2008). Under such a determinism, individuals that are heterozygous at least at one of these CSD loci develop as diploid females while hemizygous or homozygous individuals at all CSD loci develop as haploid or diploid males respectively. In most hymenopteran species, diploid males have a low survival rate and/or are often sterile. Enhanced homozygosity due to thelytoky may be very costly when it results in diploid male production (de Boer et al., 2015, 2012; van Wilgenburg et al., 2006; Zhou et al., 2007). Other sex determination systems have been described in Hymenoptera that are less sensitive to homozygosity. In Paternal Genome Elimination, haploid males initially develop from a fertilized egg from which the paternal genome is expulsed secondarily (Heimpel and de Boer, 2008). This mechanism can be discarded for *Cotesia typhae* as virgin females are able to produce haploid sons. Genome imprinting has been studied in *Nasonia vitripennis* and relies on epigenetic landmarks deposited by the mother in the oocyte that will be modified by paternal genome expression to allow female development (van de Zande and Verhulst, 2014).

Within the *Cotesia* genus, sex determination has been investigated in a few species. The CSD system has been demonstrated in three species: *C. glomerata* where sex is determined by a single locus (Zhou et al., 2006), *C. vestalis* and *C. rubecula* where sex determination is encoded by at least two different loci (de Boer et al., 2012, 2008). The sl-CSD system was discarded for *C. flavipes* and *C. sesamiae* (Niyibigira et al., 2004), the closest relative to *C. typhae*. The two strains of *C. typhae* studied here have been reared for more than 80 generations in laboratory conditions starting from an isofemale line, using full-sisters mated with their brothers to generate each generation. Each new generation has subsequently been produced from at most 20 different females, leading to highly inbred strains. Despite this, the sex-ratio in the rearing strains remains female biased, indicating that either several CSD loci or another mechanism, such as genome imprinting, are involved in sex determination. Whatever the process responsible for sexual identity, it is less sensitive to homozygosity increase than sl-CSD and should induce a lower selective pressure on recombination rate than observed in *Apis mellifera capensis* or the ant *Wasmannia auropunctata*.

The bacterial origin of thelytoky in *C. typhae* could not be either confirmed or completely discarded in the present study as an intermediate state (in terms of frequency of parthenogenesis) was observed following antibiotic treatment. The knowledge of the genetic mechanism could give some clues about the origin of parthenogenesis as endosymbionts have been mainly shown to favour gamete duplication. However, detailing specific interactions reveals a more complex picture. *Cardinium* is able to feminize diploid males (Giorgini et al., 2009) but also to induce automixis with central fusion (Zchori-Fein and Perlman, 2004). *Wolbachia* is mainly known to induce gamete duplication (Leach et al., 2009; Ma and Schwander, 2017) but it has also been described to promote apomixis (Weeks and Breeuwer, 2001). *Rickettsia* has also been shown to trigger functional apomixis (Adachi-Hagimori et al., 2008). Furthermore, the list of endosymbionts is probably partial and in most of the documented examples of parthenogenesis endosymbiotically determined, the cytological mechanism remains unknown. Evidence that microorganisms can promote all processes of parthenogenesis will probably arise from future research. The examples of demonstrated genetic determinism of thelytoky are rare and only concern automixis with central fusion. This is the case for the Cape honeybee (Verma and Ruttner, 1983) and for the wasp *Venturia canescens* (Beukeboom and Pijnacker, 2000). However, Tsutsui et al. (2014) described an apomixis mechanism in the

parasitoid wasp *Meteorus pulchricornis* for which they proposed a genetic origin of thelytoky. Even more than for endosymbiont origin, genetic determinism of parthenogenesis requires thorough investigations to determine whether it is restricted to a few cytological mechanisms. Anyway, the clearly evidenced mechanism of autoximis with central fusion in *C. typhae* does not allow to settle between genetic and endosymbiont origin as this mechanism is common to both situations.

Conclusion

In this study, we described an unusual example of low frequency thelytokous reproduction within a sexually reproducing species. We showed that, despite its scarcity, this phenomenon is biologically significant, occurring in a laboratory strain but also in natural populations, and concerning virgin but also mated females that produced mixed progenies composed of arrhenotokous males, rare parthenogenetic females and mostly sexually determined females. Two distinct mechanisms were described to produce parthenogenetic females, apomixis and automixis with central fusion. This unfixed mechanism associated with the low frequency of the phenomenon could reflect an accidental process, but may also be consistent with an emerging and evolving property of this parasitoid species.

The results concerning the origin (genetic or endosymbiotic) need to be consolidated, notably through the inspection of ovaries and search for the presence of microorganisms. Those further investigations would benefit from an increased frequency of thelytoky, therefore we will try in the future to select strains showing higher frequency of asexual reproduction.

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Data availability

Raw data for Figure 2, and Tables 1, 3 and 4 are available at https://zenodo.org/record/5785373

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