Automictic parthenogenesis in the parasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae) revisited

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Abstract: Both arrhenotokous and thelytokous reproduction are known to occur in the parasitoid wasp *Venturia canescens*. The cytological mechanism of thelytoky was previously reported to involve the formation of a restitution metaphase after the reduction division, but the exact nature of the subsequent divisions, whether reductional or equational, remained unclear. We reinvestigated the cytological mechanisms in a thelytokous strain collected in France. Our observations confirm previous results, but an equational and not a reduction division was observed after restitution. This type of reproduction can be classified as central fusion automictic parthenogenesis. In two arrhenotokous strains the normal pattern of oogenesis and syngamy of Hymenoptera was observed. In addition, we used PCR amplification to show that thelytoky in *V. canescens* is not caused by *Wolbachia* bacteria. The results are discussed in relation to maintenance of heterozygosity and female sex.

Key words: automictic parthenogenesis, central fusion, genetic variation, restitution, *Venturia canescens*, *Wolbachia* bacteria.

Résumé : Les modes de reproduction arrhénotoque et thélytoque sont tous deux connus chez la guêpe parasitoïde *Venturia canescens*. Il a été précédemment rapporté que le mécanisme cytotologique de la thélytoque impliquait la formation d'une métaphase restitutionnelle après la division réductionnelle. Cependant, la nature exacte des divisions subséquentes, réductionnelles ou équationnelles, demeurait inconnue. Les auteurs ont réexaminé les mécanismes cytotologiques chez une souche thélytoque provenant de France. Leurs observations confirment les résultats antérieurs mais ont permis de déterminer qu'une division équationnelle, et non réductionnelle, suivait la restitution. Ce type de reproduction peut être qualifiée de parthénogenèse à fusion centrale automictique. Chez deux souches arrhénotoques, le déroulement de l'oogenèse et de la syngamie était typique de ce qui se produit chez les hyménoptères. De plus, les auteurs ont employé l'amplification PCR afin de montrer que la thélytoque chez le *V. canescens* n'est pas causée par des bactéries du genre *Wolbachia*. Ces résultats sont discutés en relation avec le maintien de l'hétérozygotie et du sexe femelle.

Mots clés : parthénogenèse automictique, fusion centrale, variation génétique, restitution, *Venturia canescens*, bactéries du genre *Wolbachia*.

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Introduction

Most Hymenoptera reproduce by arrhenotokous parthenogenesis. However, many independent cases of thelytokous reproduction have arisen in several groups. Although apomixis has been reported, automixis is the predominant

mode, including premeiotic doubling, terminal fusion, central fusion, and gamete duplication (reviewed in Suomalainen et al. 1987). In some species, thelytoky is induced through infection with *Wolbachia* bacteria, and these cases always involve gamete duplication (Legner 1985; Stouthamer and Kazmer 1994). The different mechanisms by which diploidy is restored in the haploid egg have very different genetic consequences (Lamb and Wiley 1987; Suomalainen et al. 1987). For example, premeiotic doubling results in offspring genetically identical to the mother, whereas gamete duplication leads to complete homozygosity within a single generation.

Venturia canescens with former generic names *Devorgilla* or *Nemeritis* (Hymenoptera: Ichneumonidae) is a solitary endoparasitoid of pyralid moth larvae (Salt 1976; Beling 1932). These wasps have been used for many years in numerous laboratory studies on insect physiology, genetics, behaviour, life-history, and population dynamics (e.g., Ahmad 1936; Simmonds 1943; Corbet and Rotherham 1965; Rogers 1972; Salt 1975; Harvey et al. 1993; Begon et al. 1995;

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Driessen et al. 1995; Hellers et al. 1996; Marris et al. 1996). In the temperate zones, where *V. canescens* seemed to occur only in and around grain storage areas where it overwinters, thelytoky was thought to be the only mode of reproduction. J. Daumal (INRA, Antibes, personal communication) reported on a sexual strain near Nice in southern France, and recently we discovered that sexual individuals are more abundant than thelytokous ones in that area (Beukeboom et al. 1999). In order to understand how these two reproductive modes can co-exist, we started a detailed analysis of the population genetic structure and life-history characteristics of thelytokous and arrhenotokous populations. As part of these investigations we here present a study of whether and to what extent genetic variation may be maintained and generated in thelytokous lines.

Speicher (1937) performed a detailed cytological study of oogenesis in a thelytokous strain of *V. canescens* ($2n = 22$). He describes an aberrant meiosis consisting of a fusion of the two groups of first telophase chromosomes, formation of a restitution metaphase from these chromosomes, and then a further reduction division. The somatic number of chromosomes is apparently restored by an endomitosis (cf. Suomalainen et al. 1987). However, in a subsequent paper using genetic experiments (Speicher et al. 1965), it is shown that after the restitution, the genetic factors are divided according to an equational division. This result has been used by several authors to state that heterozygosity in *V. canescens* can be maintained for loci close to the centromere (Crozier 1971; Rössler and DeBach 1973) and that a proximal locus determines the female sex (Crozier 1971). In contrast, if the second division were reductional, homozygosity of proximal loci would be maintained. Because of this apparent paradox between cytological and genetic data, we reinvestigated the meiotic divisions in the eggs of a thelytokous strain of *V. canescens* in order to correctly interpret genetic variation within and among clones. Further, for the first time, we present meiotic divisions of two arrhenotokous strains of *V. canescens*, which we used as a reference. Finally, using *Wolbachia*-specific PCR primers, we test whether thelytoky is *Wolbachia*-induced.

Materials and methods

We used a thelytokous laboratory strain of *V. canescens* (coded as Golfe) established from a collection near Juan les Pins in 1996, and two arrhenotokous strains, one established from Mont Boron near Nice in 1998 and one from Antibes in 1999 (all at the Mediterranean coast of southern France), all cultured on *Ephesia kuehniella*. For investigation of meiotic stages in eggs before oviposition, we offered adult *Venturia* females *E. kuehniella* larvae to stimulate egg development in ovaries. Females were fixed 24 h later in Carnoy's fixative (ethanol : acetic acid, 3:1) for at least 24 h. To look at meiotic stages in deposited eggs, eggs were dissected from *Ephesia* larvae that had been exposed to a large number of ovipositing wasps for 30 min, resulting in numerous eggs per larva due to superparasitism. Larvae were fixed at 30-min time intervals after oviposition. Dissected eggs were stained with 2 µg DAPI (4',6-diamidino-2-phenylindole) in 1 mL Vectashield (Vector Laboratories) on a microscopic slide and covered with a cover glass. They were stored at room temperature for at least one day to allow sufficient penetration of stain into the egg and then were squashed whenever necessary. They were examined under a Zeiss Axiophot

fluorescence microscope and photographed with Kodak Elite chrome 400 colour slide film. Photos were made from these slides.

Presence of *Wolbachia* bacteria was determined through PCR amplification using primers wsp 81F and wsp 619R according to Braig et al. (1998) as well as FtsZ primers according to Holden et al. (1993). Two thelytokous strains (collected in Antibes and Valbonne in 1999) and one arrhenotokous strain (collected at Mont Boron 1999) were investigated. Total genomic DNA was extracted by pooling five wasps of each strain using the Nucleon BACC Kit (Amersham) according to the manufacturer's instructions. The Leiden LabII strain of the parasitoid wasp *Nasonia vitripennis*, which is known to carry *Wolbachia*, was used as a positive control. PCRs and amplifications were performed as described in Zhou et al. (1998), except that $MgCl_2$ was left out because it resulted in better amplifications.

Results

The thelytokous Golfe strain

The cytological analysis of adult females showed that mature eggs in ovaries are in first metaphase. The bivalents are close together and cannot be counted exactly, even after squashing (Fig. 1a). Univalents were not found. The spindle is in one tip of the egg close to the periphery and stands a little skew from or perpendicular to the cell surface outside the long axis of the egg. The bivalents remain in first metaphase, until 30 min after oviposition, when a set with the haploid number of 11 two-chromatid chromosomes can be seen moving towards each pole, evidencing a reduction division (Fig. 1b–c). The chromosomes are in first telophase after one hour and demonstrate some despiralization from late anaphase onwards.

During the next 30 min, a diploid restitution metaphase is formed from the two groups of telophase chromosomes (Fig. 1d). Interkinesis nuclei are not formed. The chromosomes spiralize again for a short period just before anaphase (Fig. 1e–g). The spindle is skew from or perpendicular to the cell surface and was situated in the long axis of 11 out of 36 eggs (31%). Mitosis proceeded, and the diploid number of daughter chromosomes could be counted only during early anaphase because the chromosomes despiralized as in the first division (Fig. 1h–j). Two large interphase nuclei containing fine-structured chromatin are formed between 2 and 3 h after oviposition (Fig. 1k). One nucleus remains close to the cell surface; the other one is found at various distances from this nucleus towards the centre of the egg, indicating active migration. The fates of these two nuclei differ. The more centrally located nucleus, the pronucleus, starts divisions synchronously to form the diploid embryo. Four nuclei are found spread over one quarter of the egg-cell length, 8 nuclei over one half, 16 nuclei over three quarters, and 32 nuclei may be found over the whole length. Sixty-four nuclei are present after 7 h. Nuclei situated furthest from the tip may lag during mitosis, but all nuclei appear in interphase simultaneously.

The peripheral nucleus may divide simultaneously with the pronucleus. The diploid number of chromosomes is observed. The spindle is skew or, rarely, perpendicular to the cell surface. The resulting nuclei remain close to the surface and enter abortive divisions. These nuclei, together with the two formed by the pronucleus, form a row of four in the long axis of the egg in 4 out of 14 eggs (29%). The periph-

Fig. 1. Meiosis in a thelytokous strain of *Venturia canescens* ($2n = 22$). (a) Compact first metaphase. (b, c) First anaphase. (d) Restitution metaphase with somewhat despiralized chromosomes. (e, f, g) Restitution metaphase chromosomes, different levels and drawing. (h, i, j) Anaphase after restitution, different levels and drawing with not all chromosomes visible through overlapping. (k) Polar body in interphase and pronucleus in interphase (at right). (l) Polar body in prophase and first cleavage metaphase. Egg periphery on the left; bar represents 3 μm for each row.

eral nucleus may also directly start an abortive mitosis, which in general occurs later than the division of the pronucleus. The peripheral nucleus can thus be considered as a polar body and its products, one to several pycnotic (or sticky) masses of chromatin after 7 h, remain in the surface plasm of the tip. The polar body retains the ability to enter at least one mitotic cycle (Fig. 1l).

Anomalous developments were observed in 6 out of 80 eggs (8%) that were 4–7 h old. They showed: 3 telophases, 6 interphases, 10 interphases, 12 interphases, 14 interphases, and about 46 prophases, each with some phase of polar body development.

The arrhenotokous Mont Boron and Antibes strains

Both strains have a diploid complement of 22 chromosomes and a similar meiosis. The chromosome configurations and spindle positions do not differ principally from those of the thelytokous strain. In short, the first metaphase shows a compact group of bivalents (Fig. 2a) and during late first anaphase, the chromosomes of both separating sets start to despiralize (Fig. 2b). Interkinesis nuclei are not formed. The inner-located haploid set spirals and enters the second meiotic division (Fig. 2c–e). Thereafter, the inner set of chromosomes forms a relatively large interphase pronucleus. In the absence of a spermatozoan, a haploid embryonic development follows. If a spermatozoan is present, it adopts a similar pronuclear structure during the meiotic period (Fig. 2e). Both nuclei come to lie together, then demonstrate

a prophase simultaneously and start the first diploid cleavage division. The outer located haploid sets of chromosomes form the first and second polar body, respectively, in the surface plasm of the egg. Their chromosomes remain spirals, generally lie close together and do not show signs of degeneration up to at least six hours after oviposition. The first polar body may enter either an abortive or a normal mitosis. The latter mitosis takes place relatively in concert with the second meiotic division and the spindles are then found lengthwise (Fig. 2d). In both strains, the first telophase was found about one hour after oviposition and the second one after about 2.5 h. Fusion of the pronuclei occurred after about 3 h, the first cleavage division after about 4.5 h, and the second one 1 h later. Unfertilized eggs showed the cleavage divisions at similar times (Fig. 2f). Embryonic development thus begins later than in the thelytokous strain.

Screen for *Wolbachia* bacteria

Two thelytokous strains (collected in Antibes and Valbonne in 1999) and one arrhenotokous strain (Mont Boron 1999) were screened for presence of *Wolbachia* bacteria using PCR and two sets of specific primers. A *Wolbachia*-harbouring strain of *Nasonia vitripennis* was used as positive control. No *Wolbachia* bacteria were found in any of the *V. canescens* strains with either primer set, whereas the positive control showed a clear band in both cases. Both the *wsp* and *FtsZ* primer pairs showed a single band at approximately 600 and 800 bp respectively (Fig. 3, only *wsp* data

Fig. 2. Meiosis in arrhenotokous strains of *Venturia canescens* ($2n = 22$). (a) Compact first metaphase. (b) First telophase with despiralizing chromosomes. (c) First polar body in metaphase and second metaphase (at right). (d) First polar body in anaphase and second anaphase. (e) First polar body (light spot, out of focus), second anaphase and sperm in interphase. (f) First polar body (bottom) and second polar body both with metaphase chromosomes and, at a distance, haploid first cleavage anaphase, different levels. Egg periphery on the left; bar represents 3 μm .

shown). These results confirm previous unpublished data (R. Stouthamer, Wageningen University, and R. Butcher, University of Dundee, personal communications).

Discussion

Meiosis in the thelytokous Golfe strain is largely similar to the strain described by Speicher (1937). The first meiotic division enters a restitution during telophase and is followed by a mitosis of the restituted number of diploid chromosomes. One nucleus becomes a polar body and degenerates, whereas the other starts developing the diploid embryo. This type of thelytokous reproduction is best classified as central fusion automictic parthenogenesis and occurs, for example, also in the cape honey bee *Apis mellifera capensis* (Tucker 1958; Verma and Ruttner 1983; Moritz and Haberl 1994). A reduction division after restitution as reported by Speicher (1937) was not observed. It may be that Speicher missed this mitotic anaphase and considered a delayed first meiotic division as a second one (cf. Figs. 31–33, p. 471, Speicher 1937). Note that for studying fine-structured chromatin contemporaneously, DAPI has a higher resolution than the Feulgen reaction, especially in yolky eggs. Meiosis in the arrhenotokous strains Mont Boron and Antibes is normal and follows the observed cytological pattern of other hymenopterans (Crozier 1975). The structural phases of the chromosomes and localizations of the spindles in the Golfe strain do not differ principally from the arrhenotokous strains, and consequently are not indicative of the origin of parthenogenesis.

Recently, Belshaw et al. (1999) reported evidence for occasional sexual processes in the thelytokous parasitoid *Lysiphlebus*. We have preliminary data for *Venturia* that thelytokous females can mate with arrhenotokous males (M.V. Schneider, unpublished data). The cytological mechanism of thelytoky would possibly allow a change towards a normal meiosis and syngamy after entrance of sperm, and we are currently investigating this.

Fig. 3. PCR analysis showing absence of *Wolbachia* bacteria in *Venturia canescens*. DNA from two thelytokous and one arrhenotokous strain was amplified with *Wolbachia*-specific wsp primers. PCR products were electrophorised on 1% agarose gel and stained with ethidium bromide. mm, molecular marker; T1, thelytokous strain Antibes; T2, thelytokous strain Valbonne; A, arrhenotokous strain Mont Boron; +, *Wolbachia*-harbouring strain of *Nasonia vitripennis* (positive control); and –, no DNA (negative control).

Our observations are consistent with the genetic data of Speicher et al. (1965). In the thelytokous strains of *V. canescens*, loci closely linked to the centromere will maintain their respective original heterozygous or homozygous states. Heterozygous loci distal from a possible chiasma will have a 50% chance of becoming homozygous. Over generations, crossing-over will increase homozygosity in thelytokous lineages. These results also imply that offspring of thelytokous females are not identical to their mother and may be genetically different from each other, which is consistent with the high intracolon variability in wing characteristics observed in laboratory populations (Slobodchikoff 1983; see also Greeff and Villet 1993). In other words, thelytokous lineages may have some potential for evolutionary change and adaptation to changing environments (Slobodchikoff 1983). In addition, if particular traits

rely on heterozygosity, such as, for example, femaleness under single-locus sex determination (Whiting 1943; Cook 1993), these loci need to be located close to the centromere, as previously stated (Speicher et al. 1965; Crozier 1971).

Occasionally (7.5% of the time), we observed odd numbers of nuclei in embryos, suggesting that in eggs with 3, 6, 12, and about 46 nuclei, a division product of the simultaneously divided first polar body sometimes becomes involved in embryonic development. This might occur when the additional nucleus remains at a distance from the cell surface, i.e., cases in which four nuclei form a row. The resulting individual (if it reaches adulthood) will probably be a genetic mosaic and may produce two genetic types of gametes.

It is generally believed that thelytoky has evolved from arrhenotoky in Hymenoptera (Cook 1993). We have shown that both thelytokous and arrhenotokous strains of *V. canescens* are free of *Wolbachia* bacteria, which can induce parthenogenesis (e.g., Stouthamer et al. 1993). Thus, thelytoky in *V. canescens* appears to be genetically determined, although the genetic basis of it remains unknown. In this context, it is interesting to note that virgin sexual females produce daughters at very low frequencies in the laboratory (Beukeboom et al. 1999). This means that if sex determination is based on heterozygosity, such females are not the result of a simple chromosome duplication (endomitosis) leading to complete homozygosity, but rather originate through a mechanism that maintains (some) heterozygosity. This suggests that a tendency to establish thelytoky by altering the female meiosis to include the above described restitution stage of the Golfe strain is present in the investigated sexual strain.

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