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Spermatogenesis in the parasitoid *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae)

Leonela Carabajal Paladino,[1,*] Fabiana Lo Nostro,[2,3] Alba Papeschi,[4] Jorge Cladera[1] and María José Bressa[4,5]

[1]Instituto de Genética "Ewald A. Favret", Instituto Nacional de Tecnología Agropecuaria (INTA-Castelar), Nicolás Repetto y de los Reseros s/n (1686), Hurlingham, Buenos Aires, Argentina; [2]Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Pabellón II Ciudad Universitaria (C1428EGA), Ciudad Autónoma de Buenos Aires, Argentina; [3]Instituto de Biodiversidad y Biología Experimental y Aplicada, Consejo Nacional de Investigaciones Científicas y Técnicas – Universidad de Buenos Aires, Pabellón II Ciudad Universitaria, Intendente Güiraldes 2160 (C1428EGA),, Ciudad Autónoma de Buenos Aires, Argentina; [4]Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II Ciudad Universitaria Intendente Göiraldes 2160, (C1428EGA), Ciudad Autónoma de Buenos Aires, Argentina; [5]Instituto de Ecología, Genética y Evolución de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas – Universidad de Buenos Aires, Intendente Güiraldes 2160, Pabellón II Ciudad Universitaria (C1428EGA), Ciudad Autónoma de Buenos Aires, Argentina ; *Present address: Institute of Entomology, Biology Centre CAS, v. v. i., Ceske Budejovice, Czech Republic

Abstract

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The study of spermatogenesis in Hymenoptera is limited to few taxa due to limitations to access the material in the right stage for analysis. The information available up to now states that the gametogenesis in males involves a modified meiosis, similar to mitosis. This is due to the haplodiploid sex determination system present in the order, where females are diploid and males are haploid. In the present work, we examined the spermatogenesis process in the parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae). We performed squash preparations from testes of individuals from the third larval instar until the adult stage. Some cytological traits, such as the presence of a monopolar spindle, were analysed in detail by means of histological semi-thin sections. The spermatogenesis in this species comprises an abortive first division with the formation of an anucleated cytoplasmic bud and a nucleated cell, and an equal second division of the nucleated cell, leading to the generation of two mature spermatozoa. The results presented herein provide more information about the process of spermatogenesis in Hymenoptera, which has not been studied in depth in parasitic species.

Leonela Carabajal Paladino, Laboratory of Molecular Cytogenetics, Institute of Entomology, Biology Centre CAS, Branisovska 31/1160, 370 05 Ceske Budejovice, Czech Republic. E-mail: carabajal@entu.cas.cz

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Introduction

In Hymenoptera, the process of gametogenesis involves different steps depending on the sex of the individual (Crozier 1975). Due to the characteristic haplodiploid sex determination system, females are diploid and males are haploid. Gametes are therefore produced by normal meiosis in females, and by an altered meiosis, similar to mitosis, in males (Patterson and Porter 1917; Dreyfus and Breuer 1944; Crozier 1975; Palomeque *et al.* 1990).

Regarding the spermatogenesis process, three systems have been described in different groups (Fig. 1):

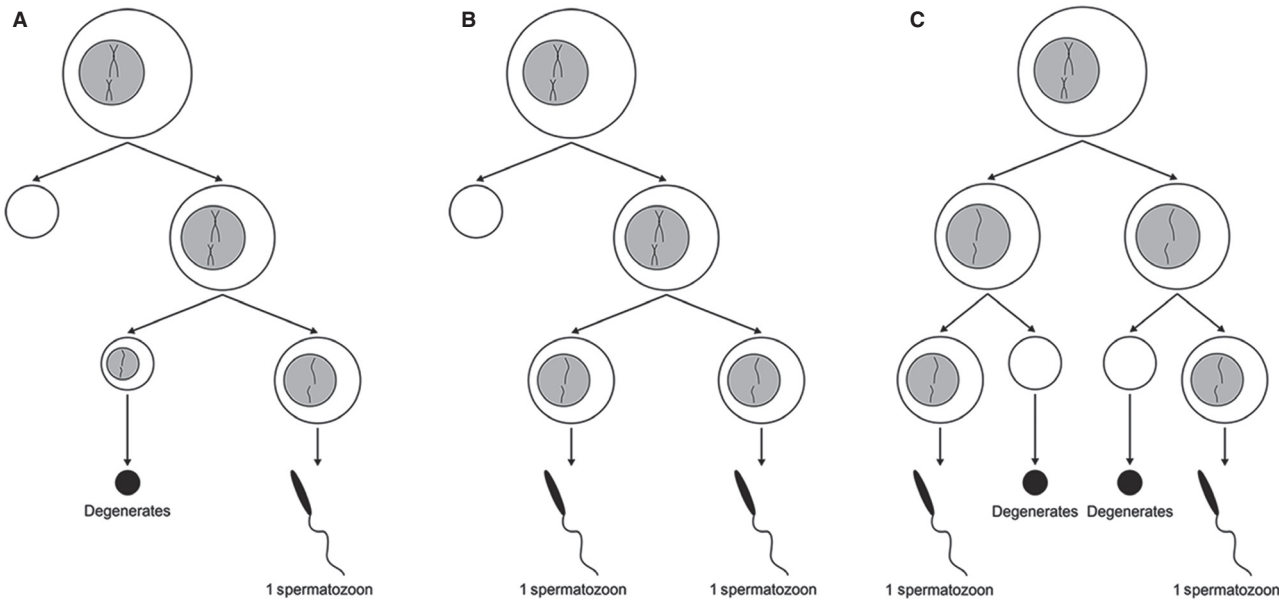

Fig. 1—Schematic representation of the different types of spermatogenesis observed in Hymenoptera. —**A.** Abortive first meiotic division followed by an unequal division of the spermatocyte, generating only one spermatozoon. —**B.** Abortive first meiotic division followed by an equal division of the spermatocyte, generating two spermatozoa. —**C.** Equational first meiotic division followed by an abortive division of the spermatocyte, generating two spermatozoa.

1 In bees, an abortive first meiotic division without chromosome reduction, where all the chromosomes migrate towards one pole resulting in the expulsion of an anucleated cytoplasmic bud. This process is followed by a second division that involves the separation of the sister chromatids and an unequal division of the cytoplasm. The smaller cell degenerates and the remaining spermatocyte generates one spermatozoon (Crozier 1975 and references therein) (Fig. 1A).
2 In other hymenopteran species, an abortive first meiotic division and an equational second division, where the sister chromatid segregation is followed by an even distribution of the cytoplasm, resulting in two spermatozoa (Crozier 1975 and references therein) (Fig. 1B).
3 In *Telenomus fariai* (Lima) (Hymenoptera: Scelionidae), during the first meiotic division the segregation of the sister chromatids occurs generating two identical spermatocytes. The second meiotic division is abortive and does not result in the division of the nucleus. A cytoplasmic constriction occurs and evolves into an anucleated bud that stays attached to the spermatocyte along the maturation of the spermatozoon. Finally, two spermatozoa are obtained, one from each spermatocyte (Dreyfus and Breuer 1944) (Fig. 1C).

A detailed description of the spermatogenesis process in Hymenoptera is limited to a handful of taxa, mostly bees and ants (Meves 1903, 1907; Sanderson and Hall 1948; Sharma *et al.* 1961; Kumbkarni 1965; Hoage and Kessel 1968; Cruz-Landim and Beig 1980; Cruz-Landim and Silva De Moraes 1980; Conte *et al.* 2005). Regarding parasitic wasps, only some species of the families Braconidae, Cynipidae, Ichneumonidae and Pteromalidae have been studied (Torvik-Greb 1935; Koonz 1936, 1939; Dodds 1938; Hogge and King 1975; Newman and Quicke 1998).

Diachasmimorpha longicaudata (Ashmead) (Ichneumonoidea: Braconidae: Opiinae) is a hymenopteran parasitoid native to Southeast Asia that is used as a biological control agent of tephritid fruit flies, which are very important pest of fruits worldwide (Sivinski 1996). Its rearing under artificial conditions is relatively easy, which allows the constant access to material for investigation in different stages of the development with the certainty of its taxonomical identity (Carabajal Paladino *et al.* 2013). The chromosome number of this parasitoid is 2n = 40 chromosomes in females and $n = 20$ chromosomes in males (Kitthawee *et al.* 1999; Carabajal Paladino *et al.* 2013). The aim of this work was to characterize the progress of spermatogenesis in *D. longicaudata* and to determine to which system it belongs.

Material and methods

Experimental insects

The parasitoid *Diachasmimorpha longicaudata* and its host, the fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), were obtained from the experimental rearing facility established at the Instituto de Genética, INTA, Hurlingham,

Buenos Aires, Argentina (Viscarret *et al.* 2006). Adult *D. longicaudata* were originally imported from México to Tucumán province (Argentina) in 1998 (SENASA, exp. N° 14054/98) and introduced to the Instituto de Genética in 2001.

Male individuals for analysis were obtained from an exposition of *C. capitata* larvae to virgin *D. longicaudata* females. Larvae of *C. capitata* were reared on artificial larval diet (Terán 1977) and offered to adult *D. longicaudata* females in plastic Petri dishes using the method described by Carabajal Paladino *et al.* (2010). The individuals of both species were maintained in an incubator under controlled conditions (25 °C, RH 85%, 18:6 h light:dark cycle) throughout all the experiments.

Squash preparations

Immature parasitoids were recovered from parasitized fly pupae by dissection under stereoscopic binocular microscope Leica EZ4 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Preparations from testes were performed daily, from the third larval instar of the parasitoid until the adult stage of *D. longicaudata*. The testes were dissected in physiological solution for *Ephestia* (Glaser 1917 cited in Lockwood 1961), fixed for 20 min in freshly prepared fixative (ethanol:acetic acid, 3:1) and then transferred into a drop of 2% iron acetic haematoxylin using ferric citrate as mordant (Carabajal Paladino *et al.* 2013). After squash under a cover slip, the preparations were sealed, and inspected and photographed using a Leica DMLB microscope equipped with a

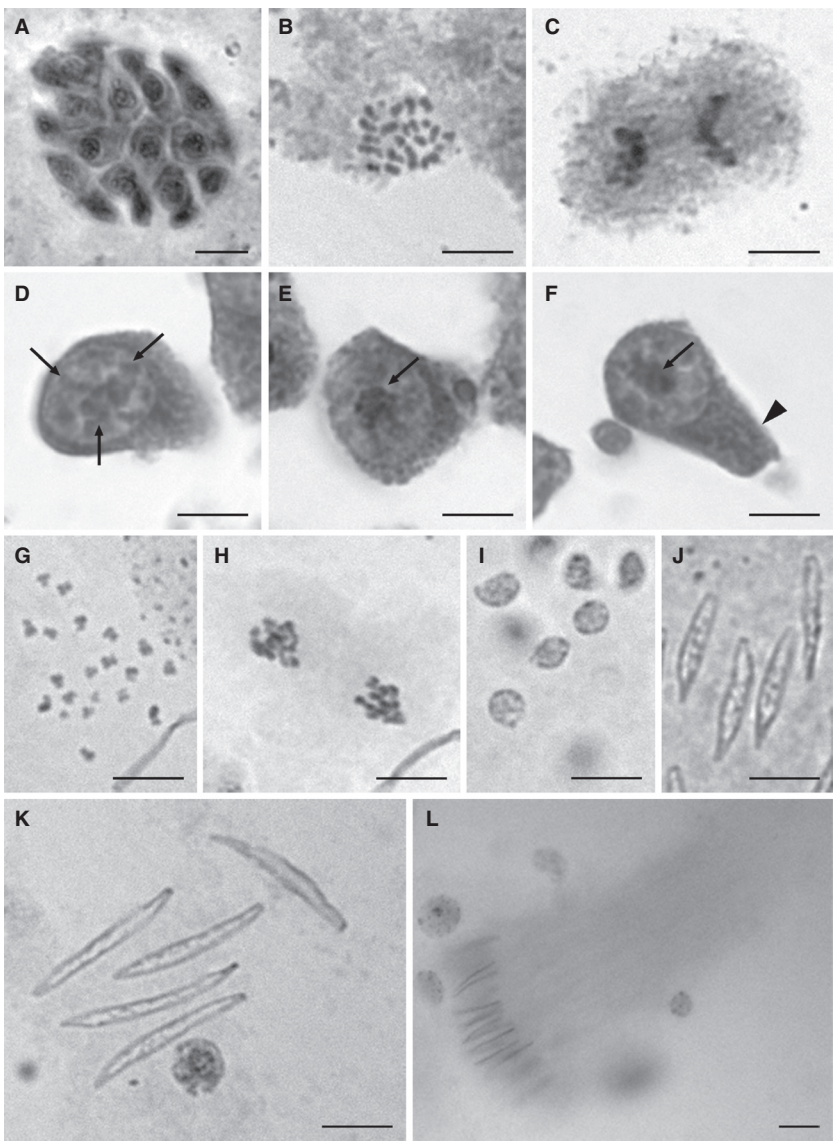

Fig. 2—Stages of the spermatogenesis observed in squash preparations of testes from *Diachasmimorpha longicaudata* haploid males (n = 20). —**A.** Rosette structure. —**B.** Spermatogonial metaphase. —**C.** Mitotic anaphase. —**D.** Spermatocyte at the abortive meiosis I stage with a large rounded nucleus showing positive heteropycnotic dots (arrows). —**E.** Spermatocyte I with a large rounded nucleus showing accumulation of positive heteropycnotic dots into a large spot (arrow). —**F.** Spermatocyte I with a large rounded nucleus showing a large heteropycnotic spot (arrow) and an anucleated cytoplasmic bud (arrowhead). —**G.** Metaphase II. —**H.** Anaphase II. —**I.** Early spermatids. —**J.** Intermediate spermatids. —**K.** Late spermatids. —**L.** Bundles of spermatozoa. Scale bar in A–K 5 μm. Scale bar in L 10 μm.

Leica DFC350FX CCD camera and Leica IM50 Software, version 4.0 (Leica Microsystems Imaging Solutions Ltd).

Semi-thin sections

Testes from 11-day-old males were fixed for 12 h at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After postfixation in 1% Osmium Tetroxide in the same buffer, blocks were contrasted in 5% aqueous uranyl acetate for 2 h, dehydrated in acetone and embedded in Araldite. Semi-thin sections (1 μm) were stained with Toluidine blue. The slides were analysed and photographed using the same microscope, camera and software mentioned above.

Results and Discussion

The analysis of the squash preparations showed rosettes of small cells at the third larval instar of the parasitoid (Fig. 2A). Later on, spermatogonial metaphases characterized by the presence of 20 chromosomes (Fig. 2B) and mitotic anaphases (Fig. 2C) are observed. Afterwards, an increase in cell size is detected, the nuclei are round and the chromatin acquires a dotted appearance (Fig. 2D). Following this meiotic stage, positive heteropycnotic dots associate forming one or two spots arranged in one side of the nucleus (Fig. 2E). Along the process, the cell losses its rounded appearance and acquires a pear-like one, due to the development of a cytoplasmic bud opposite to the side where the nucleus is located (Fig. 2F); this cytological characteristic could correspond to the early abortive meiosis I. Without a clear prophase II, 20 chromosomes with two evident chromatids each are observed in metaphase II (Fig. 2G). This stage is followed by a normal anaphase II (Fig. 2H). Then, the maturation of the spermatids, all of the same size, occurs changing from a rounded form to an elongated one (Figs. 2I–K), until the generation of mature spermatozoa of filiform morphology arranged in packages, which are predominant in newly emerged adults (Fig. 2L).

The semi-thin sections of the testes of 11-day-old males allowed a more accurate analysis of the structure of the testis in *D. longicaudata*. The gonad is organized in cysts of dividing cells, surrounded by a thin membrane. Each cyst contains cells in the same stage of division. Different cysts with cells in the different stages previously described with the squash preparations co-exist (Fig. 3A). It is also possible to observe spermatogonia among the cysts (Fig. 3A). The pear-shaped cells are organized within the cyst with their nuclei in the periphery and the cytoplasmic buds arranged in the centre (Fig. 3B). In these cells, striations are visible extending from the nucleus to the bud (Fig. 3B), and when the bud is more elongated a cone-shaped dark structure with the apex pointing towards the bud is identified (Fig. 3C). This structure may correspond to a unipolar spindle, like the one described during the first abortive meiotic division of the parasitoid *Mastrus smithii* (Packard) (Hymenoptera: Ichneumonidae) (Koonz 1936), as well as in some other wasps (reviewed in White 1973; John 2005).

The course of spermatogenesis observed in *D. longicaudata* corresponds to the second system described above (Fig. 1B), with the first abortive division and formation of an anucleated cytoplasmic bud and a nucleated cell, followed by an equational division where sister chromatids segregate and form two mature spermatozoa. It is worth mentioning that

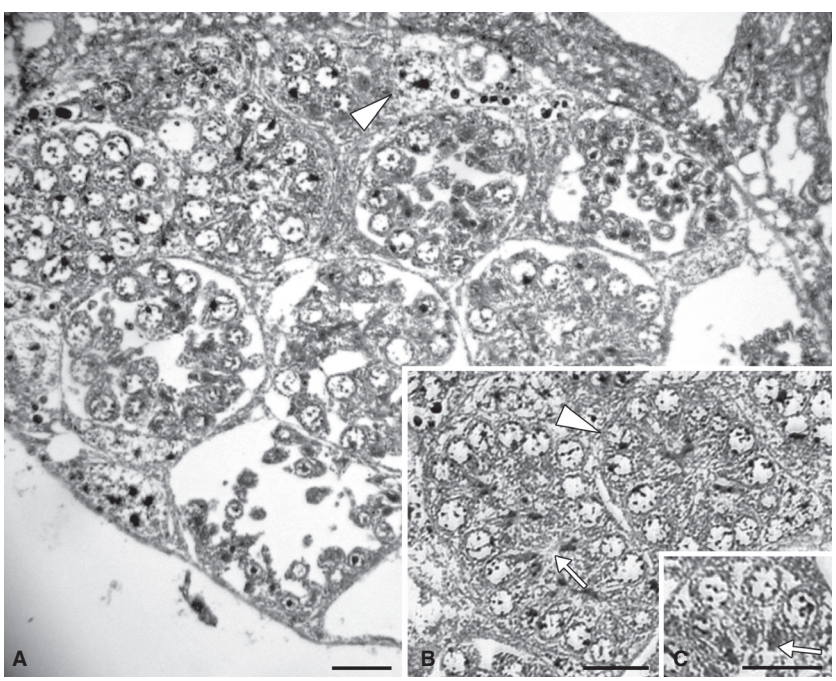

Fig. 3—Semi-thin section of the testes of an 11-day-old *Diachasmimorpha longicaudata*. —**A.** Organization in cyst of the different meiotic stages, including a spermatogonium (arrowhead). —**B.** Cyst of pear-shaped cells with the nuclei in the periphery (arrowhead) and the cytoplasmic buds in the centre (arrow). —**C.** Detail of a pear-shaped cell with a monopole spindle (arrow). Scale bar 10 μm.

while the development of *D. longicaudata* males proceeds, different stages of the cell division may co-exist, to the point that spermatogonia can be observed at the same moment as pear-shaped cells (Fig. 2A), and spermatogonial metaphases can be observed simultaneously with mature spermatozoa (nonshown). This suggests the capability of *D. longicaudata* males of generating spermatozoa along their lifespan and corresponds to the moderately sinspermatogenic characteristic of this species, previously described by Ramadan *et al.* (1991). This trait contrasts to the prospermatogenic males which emerge with the complete load of spermatozoa, being later incapable of producing more (Boivin *et al.* 2005).

It was recently demonstrated that diploid *D. longicaudata* males can occur under extreme inbreeding conditions. The size of the nuclei of their spermatocytes is significantly larger compared to that of haploid males, and they are also capable of producing mature spermatozoa (Carabajal Paladino *et al.* 2015). Diploid males have been described in at least 83 hymenopteran species (Harpur *et al.* 2012). Most of these males sire triploid daughters, implying that the gametogenesis process includes an abortive division without chromosome reduction (Inaba 1939; references in Harpur *et al.* 2012). However, the spermatogenesis of diploid males may differ from the one of haploid males, as it is the case of *Euodynerus foraminatus* (Saussure) (Hymenoptera: Vespidae), where diploid males sire diploid daughters, instead of the expected triploid daughters. In this species, it was suggested that diploid males produce haploid gametes by means of a normal reductional meiosis or alternatively, by the loss of one chromosome set at some point in the fertilization process (Cowan and Stahlhut 2004).

The spermatogenesis of haploid and diploid males was described in detail in *Habrobracon juglandis* (Ashmead) (Hymenoptera: Braconidae) (Torvik-Greb 1935). In this species, it was proved that the spermatogenesis process in haploid and diploid individuals is similar and includes a first abortive meiotic division (Fig. 1B). Hence, haploid males produce haploid gametes and diploid males produce diploid gametes (Torvik-Greb 1935). The similarities observed between *D. longicaudata* and *H. juglandis*, together with the observations made by Carabajal Paladino *et al.* (2015) permit to infer that *D. longicaudata* diploid males may produce diploid gametes as well. This should certainly be investigated in the future.

The techniques applied herein proved to be suitable for the identification and characterization of the different cytological stages and cell structures observed during spermatogenesis. The use of squash preparations allowed an accurate identification of the presence of one or two sister chromatids in the chromosomes. However, due to the fragility and the size of the sampling material, and the technique itself, the spatial organization of the testis is lost. This inconvenience was partially overcome using the semi-thin sections. With this tool, it was possible to better analyse and describe the organization of the pear-shaped cells in the cysts and to observe the presence of a unipolar spindle, which constitutes an evidence for the existence of a first abortive meiotic division in *D. longicaudata*.

The results obtained regarding the steps involved in the spermatogenesis process of *D. longicaudata* provide more information about a mechanism, which has not been studied in depth in parasitic species. This is partly due to the limited access to material from the field in the right time to perform informative preparations. The use of *D. longicaudata* as model organism overcomes this problem, and the information obtained from this species might be extrapolated to other parasitic Hymenoptera whose artificial rearing is difficult to establish.

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