Aberrant Spermatogenesis and the Peculiar Mechanism of Sex Determination in Symphypleonan Collembola (Insecta)

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Light and electron microscopy evidence have been obtained to describe the peculiar spermatogenesis in the collembolan species Sminthurus viridis and Allacma fusca (Sminthuridae). In these two species, the two sexes differ for the lack of two chromosomes (the sex chromosomes) in males (males, $2n = 10$; females, $2n = 12$). While oogenesis seems to proceed normally, spermatogenesis is peculiar because the two daughter cells of the first meiotic division have different chromosome numbers (six and four). The cell receiving four chromosomes degenerates, while the cell receiving six chromosomes completes meiosis and produces identical spermatzoa ($n = 6$). At fertilization, pronuclei with six chromosomes fuse together to form zygotes with $2n = 12$. Male embryos must lose two sex chromosomes during the first zygotic mitosis, as all male cells have $2n = 10$ chromosomes. The sex chromosome system of these species can be identified as $X_1X_1X_2X_2:X_1X_20$. Electron microscopy observations show that the same peculiar spermatogenesis occurs also in two others species of the same family, Caprainea marginata and Lipothrix lubbocki. The peculiar sex determination system described is similar but not identical to what is observed in other insect orders, and it may represent an evolutionary step toward parthenogenesis. It is suggested that this peculiar spermatogenesis is common to all Symphypleona.

Sexual dimorphism in the majority of animals is due to a difference in chromosome sets between males and females. This is usually achieved at fertilization through production of heterogametic males or females, and both XXXY and XX:XY systems have been described. In a few insect species, sex-determining chromosomal differences can be generated postzygotically by chromosome elimination during embryonic development (McCarrey and Abbott 1979; White 1973).

Springtails (Collembola) are one of the first insect orders that appeared on the Earth (Walley and Jarzembowski 1981). In spite of their importance as a major component of the edaphic fauna, collembolan species have received little attention regarding their karyology. Mitotic chromosomes are particularly difficult to study and most of the work has been concentrated on the polytene chromosomes found in the salivary glands (Cassagnau 1968; Dallai and Fanciulli 1982; Deharveng 1982; Fanciulli et al. 1991). Unfortunately these polytene chromosomes are not observed in all collembolan species, but only in members of the family Neanuridae (suborder Arthropleona), and their presence seems to be related to their piercing mouth apparatus.

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ing spermatogenesis and embryogenesis in Symphypleonan Collembola appeared recently (Dallai et al. 1999), and it was shown that males and females in two species of the family Dicyrtomidae differ in their chromosomal number, suggesting that sex is determined by the elimination of two sex chromosomes in the male embryos. As a consequence, the sex-determining system in these species could be identified as XXXX:XX0. Of interest, this system is similar to that proposed for other insect species, including Cecidomyiid (Stuart and Hatchett 1988, 1991) and Sciarid (White 1973) dipterans.

In this article, four species of Symphypleona from different genera of the family Sminthuridae have been studied in order to test whether this peculiar mechanism of sex determination, which involves modifications to normal spermatogenesis, is a common feature of all members of the suborder. Evolutionary implications of these findings are also discussed.

Materials and Methods

Material
The following species, all belonging to the family Sminthuridae, were studied: Sminthurus viridis L.; Allacma fusca (L.); Caprai-nea marginata (Schött) (= C. echinata (Stach)); and Lipothrix lubbocki (Tullberg). They were collected in the neighborhoods of Siena, Italy.

Chromosomal Preparations
Testes and young ovaries of S. viridis and A. fusca were dissected in 0.1 M phosphate buffer (PB) pH 7.2, to which 1.8% sucrose was added. The material was transferred to a small drop of 2% lactic acetic orcein. After 15–30 min the excess stain was removed and the material was squashed and observed with the light microscope. Some testes of the two species, after dissection in 0.9% NaCl, were incubated for 10 min in 1% sodium citrate, fixed in 3:1 methanol/acetic acid mixture, and then squashed in a drop of 50% acetic acid. The coverslip was removed after freezing in liquid nitrogen and stained for 10 min with 8% Giemsa solution in 0.1 M phosphate-buffered saline (PBS), incubated for 10 min with DAPI, rinsed in PBS, and mounted in 90% glycerol in PBS. Observations were performed with a Leica DMRB equipped with UV filters. Micrographs were taken with Kodak Tri-X 400 Pan negative film.

Transmission Electron Microscopy (TEM)
Testes of S. viridis, A. fusca, C. marginata, and L. lubbocki, after dissection, were dissociated in PB in min fragments by using thin needles and spread on small coverslips previously treated with 1% poly-L-lysine (SIGMA). The preparations were briefly fixed (15 min) in 2.5% glutaraldehyde in PB and dehydrated in an ethanol series and critical point dried in a Balzers CPD 010 apparatus. Samples were coated with gold by means of a Balzers Med 010 sputtering device and observed under a Philips SEM XL 20 at 10 or 20 kV.

Scanning Electron Microscopy (SEM)
Testes of A. fusca, after dissection, were dissociated in PB in min fragments by using thin needles and spread on small coverslips previously treated with 1% poly-L-lysine (SIGMA). The preparations were briefly fixed (15 min) in 2.5% glutaraldehyde in PB and dehydrated in an ethanol series and critical point dried in a Balzers CPD 010 apparatus. Samples were coated with gold by means of a Balzers Med 010 sputtering device and observed under a Philips SEM XL 20 at 10 or 20 kV.

Results

Chromosomal Observations by Light Microscopy
Spermatogonial cells of A. fusca as well as primary spermatocytes have 2n = 10 metacentric or submetacentric chromosomes. In this species it is somewhat difficult to establish which chromosomes lack their homologues, since all the smaller chromosomes have almost the same size and shape (Figure 1A).

Spermatogonial cells of S. viridis are also provided with 2n = 10 metacentric or submetacentric chromosomes, eight of which can be easily paired. The remaining two are small unpaired heteromorphic sex chromosomes (Figure 1B). These chromosomes can be identified as X,XY by comparison with the female karyotype and for their behavior during first meiotic division.

In both species, at the beginning of the first meiotic prophase of spermatogenesis, homologous chromosomes do not pair so that, at metaphase, 10 chromosomes are visible (Figures 2A and 3A). At first anaphase, a set of six chromosomes moves toward a pole and the remaining four chromosomes toward the opposite pole. The set of six chromosomes contains the two unpaired sex chromosomes (Figures 2B and 3B). The two unequal sets of chromosomes further migrate and condense. The set of four chromosomes progressively becomes a compact dense mass of chromatin material where the single chromosomes are no longer distinguishable; on the other hand, the set of six chromosomes is distinctly visible for a longer time (Figures 2B–D and 3B–D). Only the nucleus that receives the set of six chromosomes will divide again, proceeding to the second meiotic division and giving

Figure 1. (A) Allacma fusca. Spermatocyte metaphase from the first meiotic division showing 2n = 10 chromosomes and the karyotype. Lactic orcein staining. Bar = 5 µm. (B) Sminthurus viridis. Spermatogonial metaphase showing 2n = 10 chromosomes and the karyotype. Giemsa staining. Bar = 5 µm.

rise to two spermatid nuclei, each one retaining six chromosomes (Figure 3E). Spermatids will transform into spermatozoa after a long and complex spermiogenesis (article in preparation); at the end of this process, rolled-up spermatozoa provided with a long peduncle will be formed (Dallai 1970). Secondary spermatocytes with only four chromosomes, which correspond to the longer chromosomes in the full haploid set, do not divide again and eventually degenerate.

Female germ cells taken from immature gonads of both *S. viridis* and *A. fusca* show six paired chromosomes at the zygotene meiotic prophase (Figure 4). Embryos of both species show mitotic metaphases with either $2n = 10$ or $2n = 12$ chromosomes (Figure 5); embryos showing nuclei with the two different chromosome numbers in the same individual have never been observed.

**SEM Observations**

Testes of *A. fusca* show germ cells at different stages of maturation. In most cases they appear to be joined by protoplasmic bridges (Figure 6A) having an irregular shape and being about 4 μm long. These cells are likely to be spermatocytes. Some of these cells are provided with a flagellum, thus indicating that they are early spermatids (Figure 6B). Among these cells, smaller spheroidal cells, only 1.3 μm in diameter, are visible. These small cells are often in close vicinity or even adhere to the interconnected cells, but they are clearly separated from them (Figure 6A,B). They presumably correspond to the aberrant secondary spermatocytes, which do not proceed with the second meiotic division.

**TEM Observations**

In all the species examined, testes consist of somatic cells with large polyploid nuclei and clusters of germ cells. Each cluster of spermatogonia is formed by interconnected cells (Figure 7A); in cross section germ cells are arranged with their long axis radiating from a common central region. In the ribosome-rich cytoplasm of each cell, two orthogonally arranged centrioles surrounded by several mitochondria are visible near to the central region (Figure 7A). Centrioles consist of microtubular doublets rather than triplets (Figure 7C). The nucleus is roundish and contains patches of chromatin material, many of which are distributed along the inner face of the nuclear membrane. Spermatogonial mitoses are occasionally visible in the cell cysts. When spermatogenesis starts, the two centrioles elongate and then replicate to form two couples of centrioles (Figure 7A,B). The nuclear material does not seem to change its appearance compared to the previous stage and no synaptonemal complex was seen within the dense chromatin masses. The meiotic spindle has a normal shape and at metaphase I chromosomes are arranged at the equatorial level (Figure 7D). At anaphase I, chromosomes move toward the poles, which contain two centrioles each (Figure 7E). At the telophase, one secondary spermatocyte has a normal nucleus and a cytoplasm rich in organelles. At the opposite pole, a sister cell is formed; it consists of a homogeneous, dense spheroidal nucleus surrounded by a thin layer of cytoplasm, which contains only two centrioles (Figures 6C and 8A–C). These centrioles are sometimes hosted in a cytoplasmic protuberance, and in a few cases one of them elongates to form a rudiment of axoneme that remains confined within the scanty cytoplasm (Figures 6C and 8A). These aberrant secondary spermatocytes, 1.3 μm in diameter, are free cells, having lost their connections with neighboring cells, and are intermingled with normal secondary spermatocytes (Figures 6C and 8A–C). These aberrant cells do not divide again and eventually degenerate. On the contrary, normal secondary spermatocytes, after the second meiotic division, give rise to spermatids (Figure 8D). These cells are distinguishable for the loose aspect of their chromatin material and for the presence of a large and active Golgi apparatus (Figures 6C and 8B,C). In *Lipothrix*, however, the general aspect of chromatin condensation in the functional and aberrant secondary spermatocytes does not differ much; the functional spermatocytes, however, have a greater size and are interconnected to each other, while the aberrant ones are free cells (Figures 8C). Early spermatids show a short flagellum surrounded by three mitochondria (Figures 6C and 8C).

**Discussion and Conclusions**

In the different collembolan species examined, the structural organization of male gonads before meiosis is comparable to what is known in other insects (Jamieson et al. 1999; Phillips 1974). Spermatogonia are clustered in cysts and interconnected by cytoplasmic bridges; they have two orthogonally arranged centrioles, close to the nucleus. However, the centrioles consist of microtubular doublets rather than triplets, but this finding seems to be a general feature occurring in most insect orders. *Drosophila* included (Callaini et al. 1999; González et al. 1998).

When meiosis starts, the two centrioles replicate and give rise to elongated microtubular organelles; no vesicle and primary cillum are found, as it has been described in several insects (Daub and Hauser 1988;
The peculiarity of spermatogenesis in the Collembola examined becomes evident at the end of the first meiotic division. At this point, in fact, two different types of secondary spermatocytes are formed and only one of them will proceed to the second meiotic division to give origin to two spermatids, each one receiving six chromosomes. The other secondary spermatocyte is a smaller cell with very reduced cytoplasm, two centrioles, and a condensed nucleus; this cell is unable to continue the second meiotic division and eventually degenerates. This might explain the finding by Tuzet and Manier (1956), who observed only one mitotic division in the secondary spermatocytes of *S. viridis*.

The reason for such an anomalous spermatogenesis is clear when it is considered that the nuclei of the smaller spermatocytes contain an incomplete set of haploid chromosomes consisting of only four chromosomes, the sex chromosomes being lacking. The small size of the aberrant secondary spermatocytes could be the consequence of an asymmetrical cytokinesis, although a direct observation is still lacking. Cytologically such cells, which can be unequivocally defined as secondary spermatocytes for the presence of two centrioles (Friedländer and Warman 1971; Gonzales et al. 1998), do not have enough cytoplasm to organize a mitotic spindle; they can sometimes elongate only one of the two centrioles. Moreover, the loss of cytoplasmic connections between these secondary spermatocytes, as well as with the neighboring cells, would impair the synchronism of their development as it occurs in normal secondary spermatocytes. The fact that these particular secondary spermatocytes are free cells makes the first meiotic division of Collembola strongly different from what has been described in the similar aberrant spermatogenesis occurring in the gall midge (*Monarthropalpus buxi*) (Jazdowska Zagrodzinska and Dallai 1988). In this dipteran, two unequal cells are formed at the end of the first meiotic division, a normal spermatocyte and a large residual cell. This latter cell does not divide again, but it retains the cytoplasmic bridge with the sister cell and also with the two spermatids that will be formed after the second division.
meiotic division. As a consequence of the aberrant spermatogenesis described here, the efficiency of the meiotic process in these species is reduced by 50% and all spermatocytes carry the same chromosomal set.

Looking at the number of chromosomes observed in the primary spermatocytes of *S. viridis* and *A. fusca*, one could reasonably argue that the males have heteromorphic sex chromosomes and in both species the diploid number is $2n = 8 + XY$. However, this is a wrong interpretation and the observation that the haploid chromosome number in normal secondary spermatocytes is $n = 6$, while the degenerating secondary spermatocytes receive only four chromosomes, lets us conclude that these species have two nonhomologous sex chromosomes ($X_1X_2$).

Female germinal cells of either *S. viridis* and *A. fusca* have $2n = 12$ chromosomes, which undergo synopsis at the zygotene stage to form six bivalents as already described by Kiauta (1970). In two other species of Symphypleona from a different family having the same aberrant spermatogenesis, there is a clear evidence of chiasmata during diakinesis in all bivalents of primary oocytes (Dallai et al. 1999). Although chiasma were not observed in *A. fusca* and *S. viridis*, the presence of six bivalents in the oocytes of these species reinforces the idea that oogenesis proceeds in a similar way in all Symphypleona. We cannot exclude that chromosomes can be eliminated in the later stages of oogenesis, which in insects is completed only after egg laying, but it seems to be more reasonable that oogenesis is normal and eggs have $n = 6$ chromosomes. At fertilization, male and female pronuclei, both with six chromosomes, form the zygote that starts its development with $2n = 12$ chromosomes. The analysis of the chromosome number of embryos revealed that some had $2n = 10$ and others $2n = 12$, but individual embryos with both chromosome numbers in their cells have never been found. Since somatic and primordial male germ cells have the same chromosome number, the chromosome elimination will likely occur at the first zygotic division. The evidence of such chromosome elimination has not yet been observed. Thus two chromosomes are eliminated from the cells of those embryos that will become males, while chromosomes will be retained in those embryos that will become females. The two chromosomes eliminated are evidently two sex chromosomes.

The fact that male germinal cells begin spermatogenesis with an unbalanced number of chromosomes may place some constraints, inducing the loss of one set of autosomes and the correct maturation of only those spermatocytes with the complete haploid set. The sex-determining system in the collemblan species examined can be identified as $XXXX:XX0$, although $X_1X_1X_2X_2:XX0$ is probably the case here. Hence the sex of the embryo is not determined at syngamy by fertilization with sperm having different chromosomal sets. On the contrary, it is determined by the elimination of two sex chromosomes during early cleavage in male embryos, resulting in a reduced number of chromosomes in both somatic and germ cells (Figure 9). So far the same phenomenon has been described in members of two families of the suborder Symphypleona (Dallai et al. 1999; this work). On the contrary, karyological and ultrastructural data available on members of Arthropleona (Dallai et al. 1999; Hemmer 1990; Nunez...
1962) suggest that the aberrant spermatogenesis described here does not occur in this suborder. Therefore it can be argued that the peculiar sex-determining system has appeared very early in Collembolan evolution and it is now peculiar of the Symphypleona.

A similar aberrant spermatogenesis and a postzygotic sex determination has been described in few other insect species. In Sciarids and Cecidomyiids, which also have homogametic males and a postzygotic sex determination, the sex-determining chromosome elimination occurs after germ-line segregation, so that somatic and germ cells of males have different chromosome numbers (Metz 1938; Sanchez and Perondini 1999; Stuart and Hatchett 1991; White 1973). Also aphids (Homoptera), which are characterized by cyclical parthenogenesis, have a similar selective segregation of chromosomes at the end of the first meiotic division of spermatogenesis and produce identical spermatozoa. The sex-determining mechanism in aphids is characterized by the loss of one sex chromosome during oogenesis in the sex-uparae female destined to give rise to males (Cognetti 1962). Finally, in some scale insects (Homoptera, Diaspidoidea), males are produced by the elimination of the complete chromosomal set of paternal origin during the late cleavage division, thus giving haploid males that do not undergo meiosis at spermatogenesis (White 1973).

The set of chromosomes lost during the first meiotic division in the three examples above is always the paternal one (Metz 1938). If this also occurs in Collembola, females could produce two different types of oocytes: those predetermined to eliminate two X chromosomes and those predetermined not to lose any X chromosome. In this way, females could control the sex ratio. This may explain why collembolan population sex ratios are quite variable and often are far from 1:1 (Christiansen 1964; Hopkin 1997; Huther 1961). In populations sampled for the present study, females always exceeded males. Moreover, in a screening of 30 embryos from a single female of A. fusca, only 7 were males ($\chi^2 = 8.5, df = 1, P = .0036$), suggesting that the distorted sex ratio is an intrinsic property of the sex-determining mechanism.

From an evolutionary point of view, sex determination during embryogenesis may offer some selective advantages. In sed-
Figure 8. (A) Sminthurus viridis: cross section through secondary spermatocytes; small cells almost devoid of cytoplasm (arrows) correspond to degenerating secondary spermatocytes; large cells (asterisks) are normal secondary spermatocyte; the arrowhead indicates a second meiotic division (bar = 2 μm). (B) Caprainea marginata: a very early spermatid (asterisk) and degenerating secondary spermatocytes (arrows) are visible (bar = 2 μm). (C) Lipothrix lubbocki: free degenerating secondary spermatocytes (arrows) are intermingled with normal secondary spermatocytes and early spermatids which are connected by cytoplasmic bridges (bar = 2 μm). (D) Sminthurus viridis. Telophase of the second meiotic division (asterisk).

entary species, the inbreeding rate is likely to be high, and one should expect some deleterious consequences. The possibility of producing monogenic progenies increases the amount of outbreeding, therefore counterbalancing the negative effect of a sedentary lifestyle. Of interest, Collembola have low mobility and are usually strictly dependent on the soil environment; allozyme electrophoresis screenings have shown that populations often have reduced levels of genetic variability (Fanciulli et al. 1994; Frati et al. 1992). A second advantage may be that this chromosome loss mechanism of sex determination allows these species to avoid the 1:1 sex ratio intrinsic to normal meiosis. Collembola perform an indirect sperm transfer through spermatophores laid on the substrate by the males which are then picked up by females (Betsch-Pinot 1977). Each male produces a large number of spermatophores and is therefore potentially able to fertilize many females. The fecundity of these species is proportional to the number of females and the number of males is of lesser importance; the distorted sex ratio probably more than outweighs the loss of 50% of the spermatocytes in males.

We can also speculate that the aberrant meiosis and the large number of females in the species examined here may be considered a step toward parthenogenesis, a mechanism of reproduction occurring in other springtails (Cassagnau 1972; Goto 1960; Petersen 1971; Pomorski 1989). The strong similarities observed between the chromosome cycle of S. viridis and A. fusca...
and that of the cyclically parthenogenetic aphids reinforce this interpretation.

References


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