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Spermatogenesis in the Testes of Diapause and Non-Diapause Pupae of the Sweet Potato Hornworm, *Agrius convolvuli* (L.) (Lepidoptera: Sphingidae)

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Dichotomous spermatogenesis was examined in relation to diapause in the sweet potato hornworm, *Agrius convolvuli*. In non-diapause individuals, eupyrene metaphase began during the fifth larval instar and eupyrene spermatids appeared in wandering larvae. Bundles of mature sperm were found after pupation. Apyrene spermatocytes also appeared during the fifth larval instar, but meiotic divisions occurred irregularly and their nuclei were discarded from the cells during spermiogenesis. Morphometric analyses of flagellar axonemes showed a variable sperm number in apyrene bundles. The variation ranging from 125 to 256 sperm per bundle indicated abnormal divisions or the elimination of apyrene spermatocytes. In diapause-induced hornworms, spermatogenesis progressed similarly during the larval stages. The cessation of spermatogenesis during diapause is characterized by 1) secondary spermatocytes and sperm bundles degenerating gradually as the diapause period lengthens, and 2) spermatogonia or primary spermatocytes appearing throughout diapause. A TUNEL (TdT-mediated dUTP-biotin nick end-labeling) assay revealed that DNA fragmentation occurred in the nuclei of secondary spermatocytes and early spermatids. Aggregates of heterochromatin along the nuclear membrane indicated the onset of apoptosis, and condensed chromatin was confirmed by electron microscopy to be the apoptotic body. These results show that the degenerative changes in spermatogenic cells during pupal diapause were controlled by apoptosis.

Key words: *Agrius convolvuli*, hornworm, diapause, spermatogenesis, apoptosis, reproduction

INTRODUCTION

The dimorphic spermatozoa (eupyrene and apyrene) in lepidopteran species have been studied for a long time (Sugai, 1965), although a question about the role of apyrene spermatozoa remained unsettled (Friedländer, 1997; Jamieson *et al.*, 1999). Ultrastructural studies have demonstrated the difference between the two types of spermatozoa in the cabbage white butterfly, *Pieris brassicae* (Zylberberg, 1969), the silkworm, *Bombyx mori* (Friedländer and Gitay, 1972; Katsuno, 1987; Yamashiki and Kawamura, 1997), and the swallowtail *Atrophaneura alcinous* (Kubo-Irie *et al.*, 1998). Especially in *A. alcinous*, eupyrene spermatozoa differ from apyrene spermatozoa not only in the possession of nuclei and appendages but also in the presence of a

sheathed acrosome and large mitochondrial derivatives. However, primary spermatocytes destined to become either eupyrene or apyrene are difficult to recognize in the testes of fifth-instar larvae or pupae. The difference in spermiogenesis resulting in the two types of spermatozoa in *B. mori* is due to the special relationship between their nuclei and centrioles (Friedländer and Wahrman, 1971; Yamashiki and Kawamura, 1998). The mechanism of eupyrene and apyrene spermiogenesis was recently studied by *in vitro* cultivation of the spermatocytes in *B. mori* (Kawamura and Sahara, 2002).

In early studies of pupal diapause in the swallowtail, *Papilio xuthus*, the difference in spermatogenesis between diapausing and non-diapausing types was described (Numata and Hidaka, 1980). In diapause-destined male larvae, spermatogenesis stopped completely and the existing spherical spermatids began to degenerate during the fifth larval instar. This degeneration occurred in all spermatid cysts and sperm bundles during pupal diapause. In the testes of another swallowtail, *Atrophaneura alcinous*, diapause occurred in the pupal stage and spermiogenesis was

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arrested during pupal diapause. The degenerating secondary spermatocytes were shown to be apoptotic by supravital staining, a TUNEL assay, and ultrastructural observations (Kubo-Irie *et al.*, 1999).

In the North American tobacco hornworm, *Manduca sexta*, fluctuations of ecdysteroid titer in wandering laevae may affect the initiation and maintenance of spermatogenesis (Friedländer and Reynolds, 1988), and no fluctuations of ecdysteroid titer during pupal diapause may control eupyrene and apyrene sperm formation (Friedländer and Reynolds, 1992).

As compared with *M. sexta*, the sweet potato hornworm, *Agrius convolvuli*, is a cosmopolitan species widely distributed in tropical, subtropical and temperate zones from Europe to Asia. Its developmental, physiological, and behavioral characteristics have been studied for over a decade (Kiguchi and Shimoda, 1994; Shimoda and Kiuchi, 1997, 1998). The seasonal occurrence of *A. convolvuli* is regulated by pupal diapause, which is strictly controlled by temperature and photoperiod (Kamiwada *et al.*, 1990; Shimoda *et al.*, 1995). However, little is known about how dichotomous spermatogenesis relates to pupal diapause.

We examined *A. convolvuli* using supravital staining with a fluorescent dye to define the normal timetable of spermatogenesis in the larval and pupal stages, with special attention to eupyrene and apyrene spermatogenesis in non-diapause and diapause-induced individuals. Furthermore, the TUNEL assay and electron microscopy confirmed that the nuclei of secondary spermatocytes or early spermatids undergo apoptosis in diapausing individuals.

MATERIALS AND METHODS

Insects

Larvae and pupae of the sweet potato hornworm, *Agrius convolvuli*, were obtained from a laboratory colony established from pupae collected at Tsukuba, Ibaraki, Japan, in 1989 and maintained on artificial diets containing sweet potato leaf powder (Kiguchi and Shimoda, 1994). Diapause was induced by rearing at 23°C under a 12L–12D photoperiod throughout the larval stage, and non-diapause pupae were produced by rearing larvae at 27°C under a 16L–8D photoperiod (Shimoda *et al.*, 1995). The diapausing and non-diapausing pupae were maintained on a wet paper towel in a plastic box to prevent drying during the experiment (Shimoda and Kiuchi, 1997).

Supravital staining by fluorescent dye

Supravital staining with Hoechst 33342 (H342) fluorescent dye was used to examine the nuclear number and shape of spermatogenic cells, as described by Locke *et al.* (1990). Testes were dissected out from fifth instar larvae, pupae and adults, and their contents were spilled into a modified Kiev solution (Peng *et al.*, 1990) in which the contents were stained by incubation with H342 (5 µg/ml; Sigma B2261) for 10 min. The stained cells were mounted on poly-L-lysine-coated glass slides and were observed under a Nikon Optiphot microscope equipped with fluorescence optics.

TUNEL assay

We examined apoptotic cell degradation in testes of diapausing pupae using the TUNEL method to detect DNA fragmentation in spermatogenic cells, as described by Gavrieli *et al.* (1992). The testes of diapausing pupae were removed and their contents were mounted on poly-L-lysine-coated glass slides and fixed in 4% buffered formaldehyde. These samples were nick end labeled in situ

with biotinylated poly-dU introduced by TdT, and then stained using avidin-conjugated peroxidase (Apoptosis Detection Kit, Promega Corp., Madison, WI). The stained cells were observed under the microscope.

Electron microscopy

Testes were dissected out of fifth instar larvae, pupae, and adults, and directly placed into a primary fixative (2.5% glutaraldehyde and 1% tannic acid in 0.2 M sodium cacodylate buffer, pH 7.4). After post-fixation with 1% OsO₄ in the same buffer, samples were dehydrated through a graded ethanol series and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were made with a Sorvall ultramicrotome, double stained with uranylacetate and lead citrate, and then observed using a JEOL 1200 EX II electron microscope. For morphometric analysis on electron micrographs, we developed a computer code using C++ compiler language with Microsoft Visual Studio Ver. 6.00 (Microsoft, Tokyo) to count the number of nuclei (eupyrene) or flagellar axonemes (apyrene) in a transverse section of each cyst in randomly selected areas from five adult testes.

RESULTS

Spermatogenesis in non-diapause and diapause individuals under the optical microscope

After whole mount testes of *Agrius convolvuli* were stained with fluorescent dye, we observed the nuclear number and shape of spermatogenic cells in each cyst. Primary spermatocytes destined to become either eupyrene or apyrene were not distinguishable in the testes of fifth instar larvae or pupae. Both kinds of spermatozoa could be clearly distinguished one from the other after the meiosis to secondary spermatocytes.

In the testes of larvae destined to become non-diapause pupae, primary spermatocytes with 64 cells and secondary spermatocytes with 128 cells were seen in each cyst. The meiotic metaphase of eupyrene spermatocytes, characterized by the regular arrangement of bivalents at the equator, was observed in the fifth instar larvae and lasted until pupae were about 7 days old (Fig. 1a). After meiosis, early spermatids dramatically decreased in cell volume, the nuclei became small and spherical, and their flagellum was organized. The nucleus subsequently became lance shaped at the anterior end of the cell in day-1 wandering larvae. The nuclei of spermatids started to elongate before pupation. Bundles of nearly mature sperm were found after the pupal molt (Fig. 1b).

Apyrene spermatogenesis occurred later than eupyrene spermatogenesis. Secondary spermatocytes were seen in day-2 fifth-instar larvae, and early apyrene spermatids appeared only after pupation. The meiotic divisions of apyrene spermatocytes were irregular, and the chromosomes clumped together and moved asynchronously during anaphase (Fig. 1c). The nuclei of apyrene spermatids were eventually discarded, whereas their flagella became elongated. Apyrene sperm bundles, which were smaller than eupyrene bundles, were observed in day-5 pupae. In day-10 pupae, testes were filled with both mature eupyrene bundles and mature apyrene bundles.

In diapause-induced individuals, eupyrene spermatids with both spherical and elongated nuclei existed in the early diapause period. Eupyrene sperm bundles decreased gradually and were nearly absent within about 60 days after

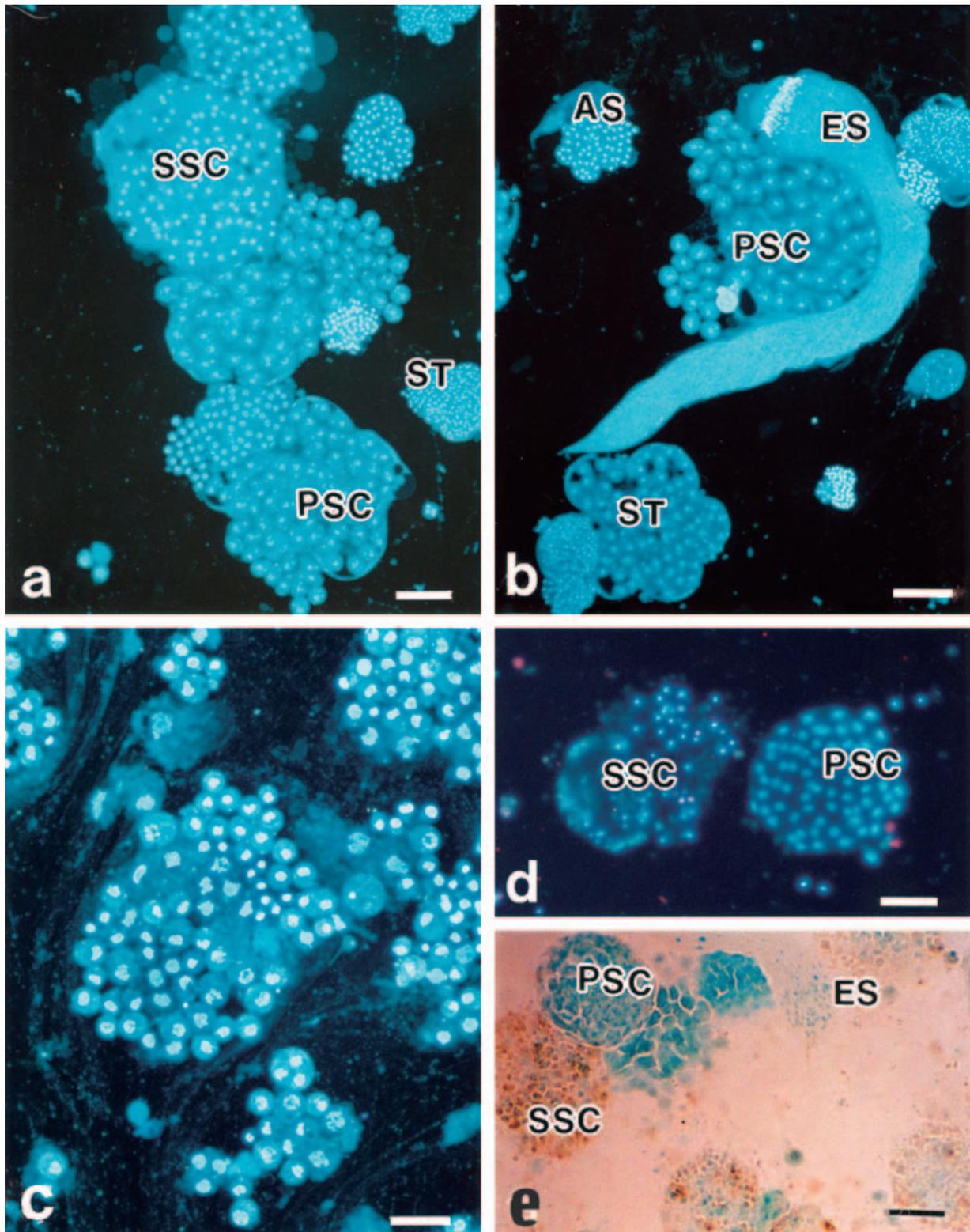


Fig. 1. Optical micrographs of whole-mounted, spread testes of non-diapause (a–c) and diapause-induced (d–e) *A. convolvuli* after staining with the fluorescent dye Hoechst 342 (a–d) or after staining by the TUNEL method (e). (a) Various stages of spermatogenic cells in a day-3 wandering larva. The nuclei appear uniformly in each cyst. (b) Both eupyrene and apyrene sperm bundles are seen in a day-3 pupa. (c) Abnormal nuclei of apyrene spermatocytes in a day-3 wandering larva. Note deformation of condensed nuclei. (d) Two adjacent cysts of spermatocytes in the testis of a day-5 diapausing pupa. In one cyst are normal-appearing primary spermatocytes; in the other are degenerated secondary spermatocytes. (e) The testis of a day-5 diapausing pupa assayed by the TUNEL method. TUNEL-positive nuclei were detected within cysts of secondary spermatocytes, whereas nuclei of primary spermatocytes and eupyrene sperm bundles were TUNEL-negative. AS, apyrene sperm bundle; ES, eupyrene sperm bundle; PSC, primary spermatocyte; ST, spermatid; SSC, secondary spermatocytes. Bars: a, b, d, e, 100 μ m; c, 50 μ m.

pupation. Apyrene spermatocytes lysed before the meiotic division, so no apyrene spermatids remained during diapause. In the testis of day-5 diapausing pupae, the cyst of primary spermatocytes with 64 cells seem to be normal, but the degradation of secondary spermatocytes resulted in the loss of their unity (Fig. 1d). Therefore the testes of day-5 pupae were examined using the TUNEL assay for apoptosis. TUNEL-positive nuclei were found in cysts containing 128 secondary spermatocytes, whereas the 64 nuclei of primary spermatocytes and eupyrene sperm bundles were TUNEL-negative (Fig. 1e).

Fig. 2 shows a summarized timetable of spermatogenesis in both non-diapause and diapause individuals from the last larval instar to pupae, based on both optical and electron microscopical observations.

Spermiongenesis in non-diapause insects

Early eupyrene spermatids have dispersed chromatin in the nuclei. The formation of an acrosome and a flagellum occurred simultaneously in the cytoplasm. An acrosomal vesicle derived from a Golgi complex was located at the anterior end of the spherical nucleus. Flagellar formation started from the centriole in a small depression of the nucleus at the posterior end (Fig. 3a). As spermiongenesis advanced, the nucleus gradually elongated and was covered by a flattened acrosome on the anterior end. The dispersed chromatin transformed to a fibrous appearance (Fig.

3b). In nearly mature spermatozoa, the nuclei had completely condensed chromatin, and the acrosome covered the anterior end of the tapered nucleus (Fig. 3c).

Apyrene spermatids could be recognized by the positions of nuclei and centriole. Two nuclei were occasionally seen in the cytoplasm, due to irregular nuclear divisions. Such abnormal nuclei were evident under the optical microscope (Fig. 1c). Intercellular bridges were found near these nuclei. The flagellum was organized from the centriole separately from the nuclear depression (Fig. 3d). The nucleus was transferred to the posterior end of the cell along the axoneme during flagellar elongation (Fig. 3e). In longitudinal sections of apyrene spermatozoa, a cap-shaped structure was seen at the tip, followed by the centriole and the axoneme. Neither the nucleus nor the acrosome was found in the anterior region of apyrene spermatozoa (Fig. 3f).

We counted 256 sperm nuclei surrounded by an acrosome in a eupyrene sperm bundle in transverse section (Fig. 4a). The sperm flagellum was organized with two central singlets, nine peripheral doublets, and nine accessory singlets, as well as two fused large mitochondria. Reticular and laciniate appendages attached to the outside of the cell membrane (Fig. 4b). In an apyrene bundle, no acrosome was found in the sperm heads, and 128 flagellar axonemes were counted (Fig. 4c). Two smaller, equal-sized mitochondria were positioned at the side of the axoneme, and no appendage was seen outside the cell membrane

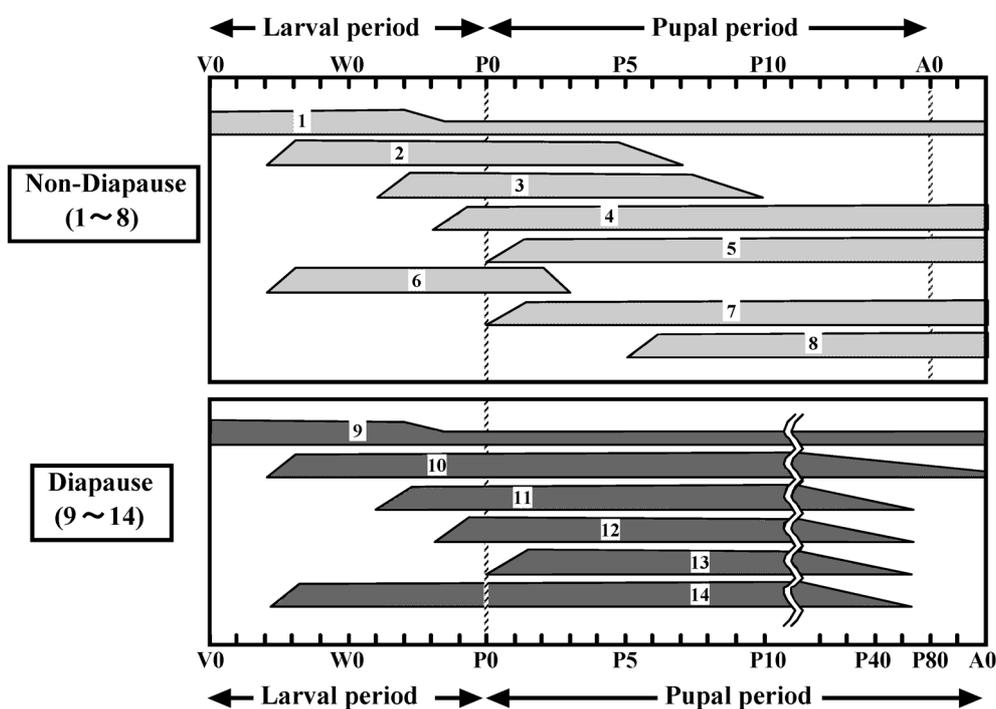


Fig. 2. Schematic time course of spermatogenesis in non-diapause (lanes 1–8) and diapause-induced (lanes 9–14) *A. convolvuli* individuals. The normal timetables of the dichotomous spermatogenesis were defined on the basis of morphological observation after supravital staining with Hoechst 33342, and electron micrographs. (1) Primary spermatocyte. (2) Spermatocyte meiosis. (3) Eupyrene spermatid spherical nuclei. (4) Eupyrene spermatid elongated nuclei. (5) Eupyrene sperm bundle. (6) Apyrene secondary spermatocyte. (7) Apyrene spermatid. (8) Apyrene sperm bundle. (9) Primary spermatocyte. (10) Spermatocyte meiosis. (11) Eupyrene spermatid spherical nuclei. (12) Eupyrene spermatid elongated nuclei. (13) Eupyrene sperm bundle. (14) Apyrene degenerating spermatocyte. The tapered ends of each trapezoidal bar indicate variation among individuals. Lanes 10–13 exclude the degenerating forms in diapausing pupae in the supravital staining. V0 represents the day of ecdysis to the fifth larval instar; W0, the first day of wandering; P0, the day of ecdysis to the pupal stage; A0, the day of eclosion.

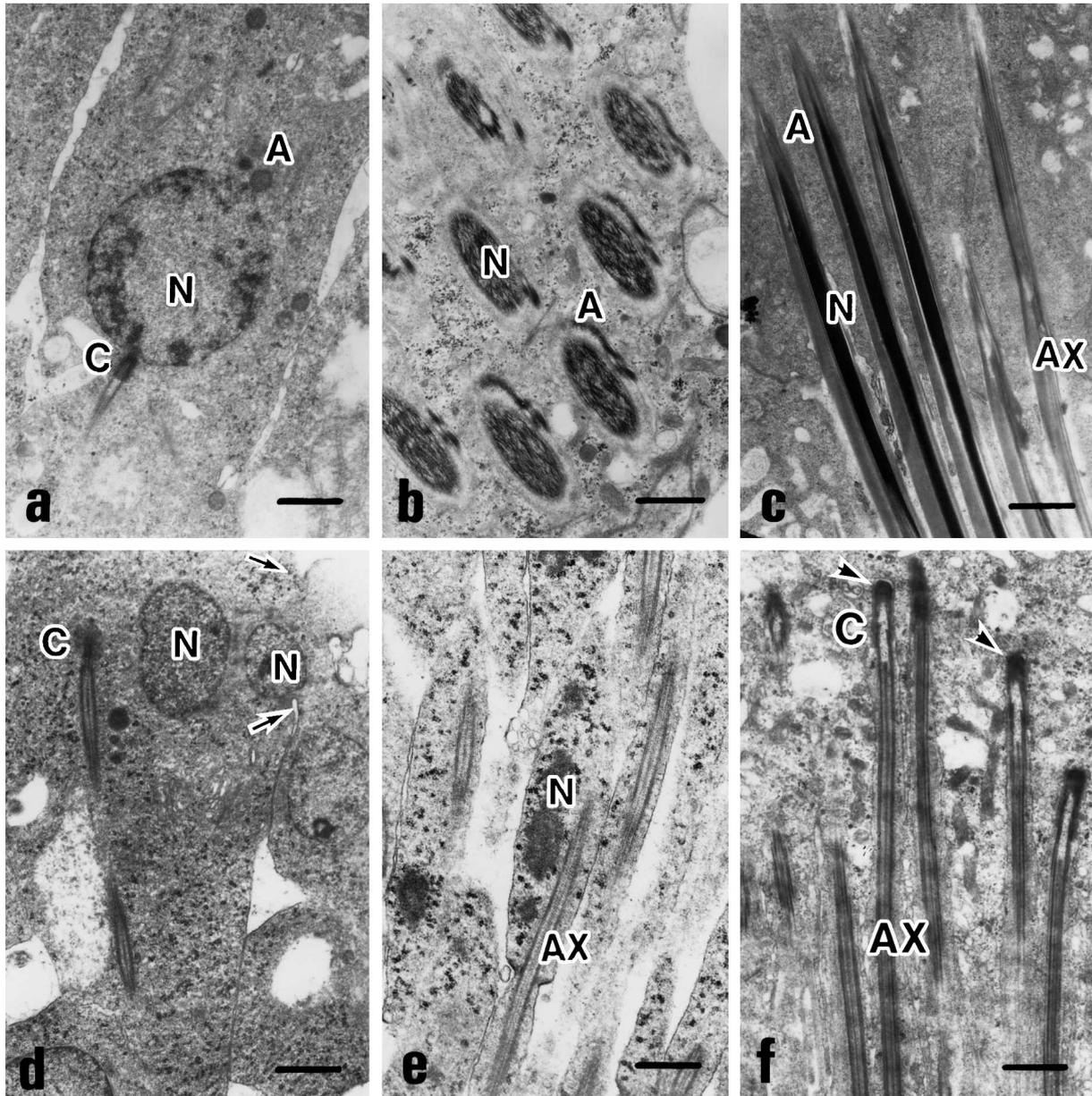


Fig. 3. Electron micrographs of spermatozoa in non-diapausing *A. convolvuli* pupae. Note the transformation of nuclei in both eupyrene (a–c) and apyrene (d–f) spermiogenesis. A, acrosome; AX, axoneme; C, centriole; N, nucleus. Arrows indicate intercellular bridges (d); arrowheads indicate the cap-shaped structure (f). Bar, 1 μ m.

(Fig. 4d).

The number of nuclei surrounded by the acrosome in eupyrene sperm bundles and the number of flagellar axonemes in apyrene bundles were counted in transverse sections on electron micrographs. The sperm number in the eupyrene bundles was around 256, whereas the sperm number in apyrene bundles ranged from 125 to 256, with a peak of about 128 (Fig. 5).

Spermatogenesis in diapause-induced insects

In diapause-induced individuals, the morphological changes of spermatogenic cells were observed by electron microscopy (Fig. 6). Fig. 6a shows a few cysts of sper-

matogonia with ovoidal and somewhat irregularly shaped nuclei with randomly distributed chromatin in the testis of day-4 diapausing pupae. Spermatogonia can be distinguished from primary spermatocytes by the number of nuclei in each cyst. One or two nucleoli were visible in each nucleus. Some mitochondria were found in the cytoplasm. In day-10 diapausing pupae, the cysts of primary and secondary spermatocytes were next to each other. The nuclei of primary spermatocytes (Fig. 6b, above) had dispersed chromatin, with a nucleolus and some mitochondria gathered in the cytoplasm. Some primary spermatocytes had synaptonemal complexes in the nucleus. These complexes were composed of a central core and lateral

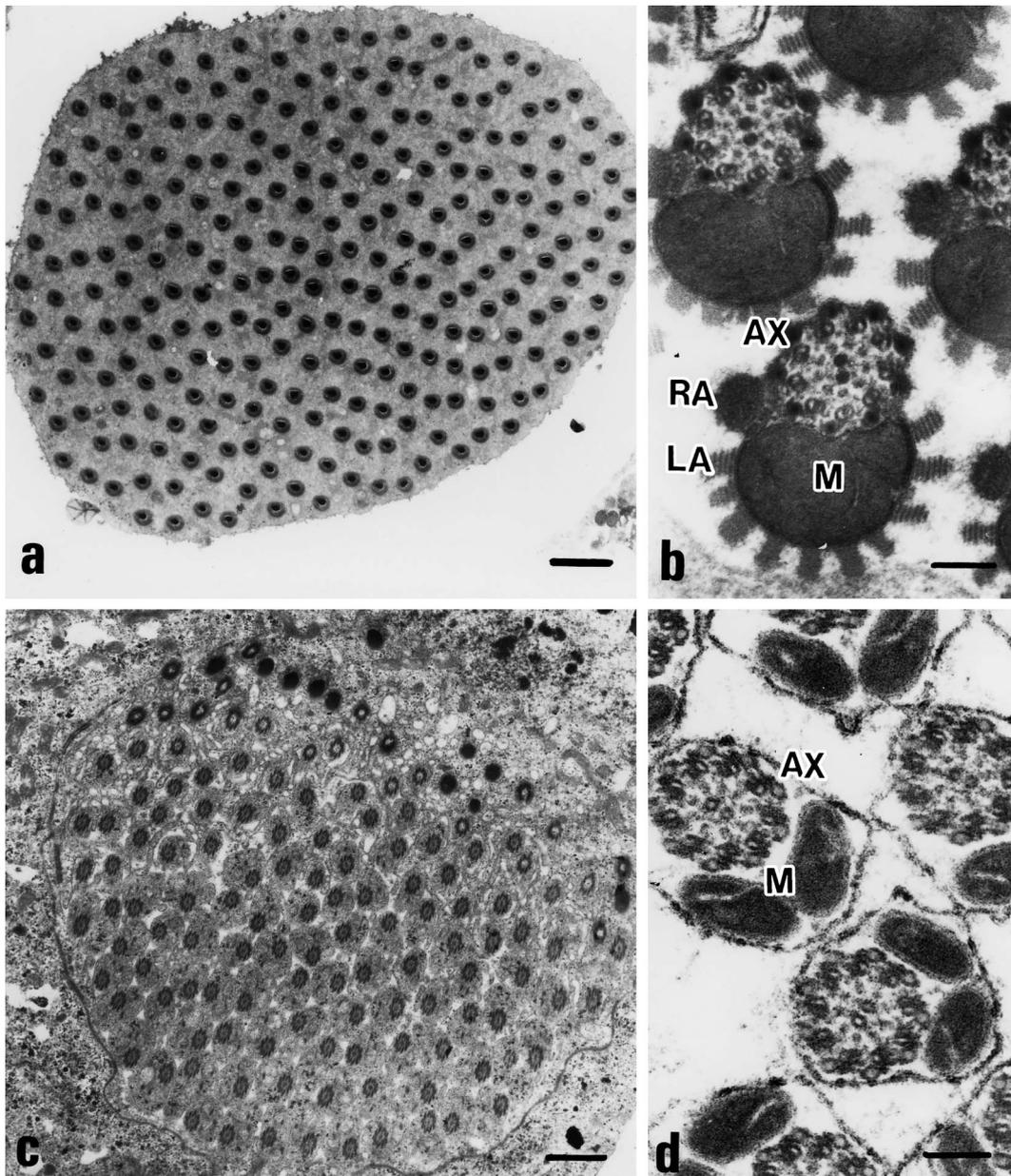


Fig. 4. Electron micrographs of eupyrene (a, b) and apyrene (c, d) sperm in bundles of *A. convolvuli*. **(a)** In this transverse section of a eupyrene sperm bundle, the number of nuclei surrounded by the acrosome is 256. The sperm flagellum is organized with 9+9+2 microtubules and two large, fused mitochondria. **(b)** Reticular and laciniate appendages attached to the outside of the cell membrane. **(c)** In this apyrene bundle, no acrosome is evident in the sperm head, and the number of flagellar axonemes is 128. **(d)** Two equal-sized mitochondria are positioned at the side of the axoneme, and no appendage is seen outside the cell membrane. AX, axoneme; LA, laciniate appendage; M, mitochondria; RA, reticular appendage. Scale Bars: a, c, 1 μ m; b, d, 100 nm.

elements and ends that terminated in the nuclear envelope, suggesting that they were in the pachytene stage. The shape of nuclei in cysts of secondary spermatocytes (Fig. 6b, below) developed asynchronously; some had completely condensed chromatin, whereas others had partly aggregated chromatin. Mitochondria gathered in the cytoplasm to form Nebenkern.

Fig. 6c shows two neighboring cysts of degenerating spermatids. One cyst consisted of elongated spermatids with aggregated chromatin along the nuclear membrane,

and the other had nuclei with completely condensed chromatin. In the former, the onset of apoptosis was indicated by the aggregation of heterochromatin along the nuclear membrane. Nebenkern had begun to elongate, as well as the long flagella. In the latter, spermatids were irregularly shaped, smaller in diameter, and separated from each other. The condensed nuclei seemed to be apoptotic bodies. Therefore, mature spermatozoa were never produced in the testes of diapausing pupae.

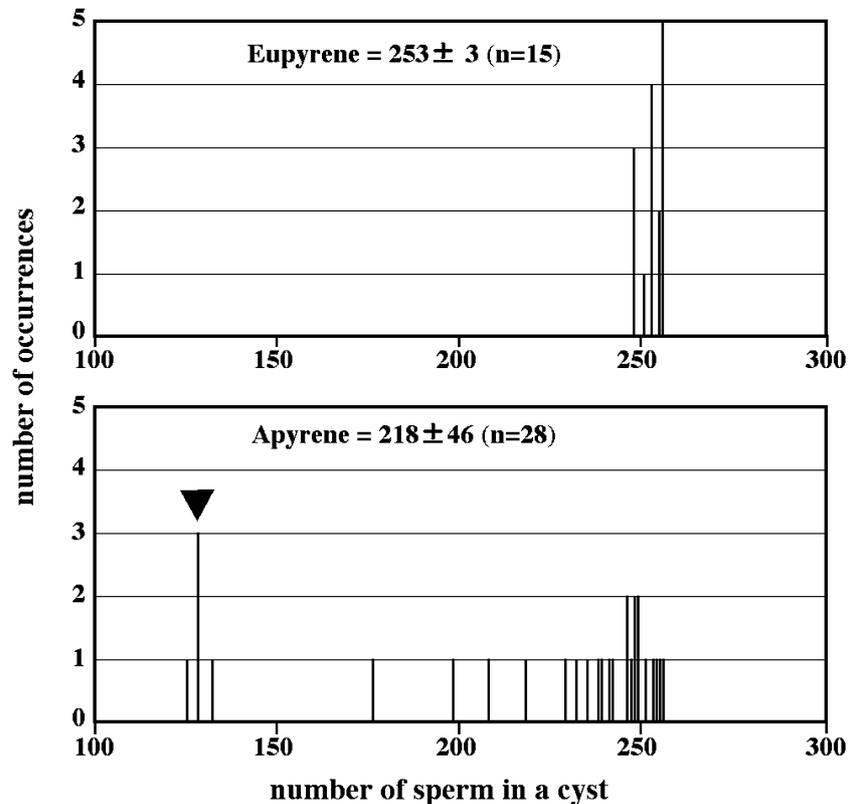


Fig. 5. Morphometric analysis of sperm bundles in *A. convolvuli*. Either the number of nuclei surrounded by an acrosome (eupyrene) or the number of flagellar axonemes (apyrene) within each cyst was counted from micrographs of transverse sections. Note that there are two peaks in sperm number in apyrene bundles, with one around 128 (arrowhead), whereas there is a single peak around 256 in eupyrene sperm bundles. Mean number of sperm \pm SD is shown in each panel.

DISCUSSION

The male sweet potato hornworm, *A. convolvuli*, concomitantly produces eupyrene and apyrene spermatozoa, like other lepidopteran species. The number and shape of nuclei in a cyst was determined using supravital staining with a fluorescent dye. Primary spermatocytes consisting of 64 cells and secondary spermatocytes consisting of 128 cells were easily recognized in each cyst (Figs. 1a, b)

By electron microscopy, we demonstrated ultrastructural differences between eupyrene and apyrene development from the secondary spermatocyte stage. We established a timetable of spermatogenesis in the larval and the pupal stages to distinguish between non-diapause and diapause phases (summarized in Fig. 2). Apyrene spermiogenesis began a few days after eupyrene spermiogenesis. A similar time lag has been reported in the silkworm, *B. mori* (Sugai, 1965); in the swallowtail *P. xuthus* (Numata and Hidaka, 1980); in the codling moth, *Laspeyresia pomonella* (Friedländer and Benz, 1981); and in the tobacco hornworm, *M. sexta* (Friedländer and Reynolds, 1988). Furthermore, we clearly distinguished by electron microscopy the difference in nuclear transformation during eupyrene and apyrene spermiogenesis. The divisions of eupyrene spermiogenesis proceeded as usual, but those of apyrene were irregular and asynchronous, forming abnormally shaped nuclei (Figs. 1c and 3d). In apyrene spermatids, the nuclei were separated from the centriole, where the flagellum was built, and were

subsequently discarded from the cells (Figs. 3d to 3f). Our observations indicate the importance of nuclear shape in the positioning of nuclei and centrioles during spermiogenesis. A similar relationship was reported in the silkworm, *B. mori* (Friedländer and Wahrman, 1971; Yamashiki and Kawamura, 1998).

The morphometric analysis of electron micrographs revealed a difference in sperm number between eupyrene and apyrene bundles (Fig. 5). The primary spermatocytes with 64 cells per cyst (shown in Figs. 1a, b, d) resulted in the eupyrene bundles with 256 spermatozoa, because the meiotic divisions advanced smoothly. The sperm number in apyrene bundles ranged from 125 to 256, indicating abnormal divisions and the elimination of apyrene spermatocytes, as observed by electron microscopy. Interestingly, apyrene bundles peaked at about 128 sperm, a value never found in eupyrene bundles. Variation in the cell number of primary spermatocytes (7, 8, 12, 16, 18, 24, 32, or 64 per cyst) has also been found in the Drosophilidae by means of phase contrast microscopy (Kurokawa and Hihara, 1976). Cell number may depend on the number of multiplication divisions of spermatogonia, which may be strictly genetically regulated. The peak of 128 apyrene sperm suggests two hypotheses: 1) the nuclei of spermatogonia or spermatocytes degenerate before the second meiosis, and 2) spermatocytes skip either the heterotypic or homotypic division with relation to centrioles during spermatogenesis. Further studies are needed to determine which hypothesis is correct.

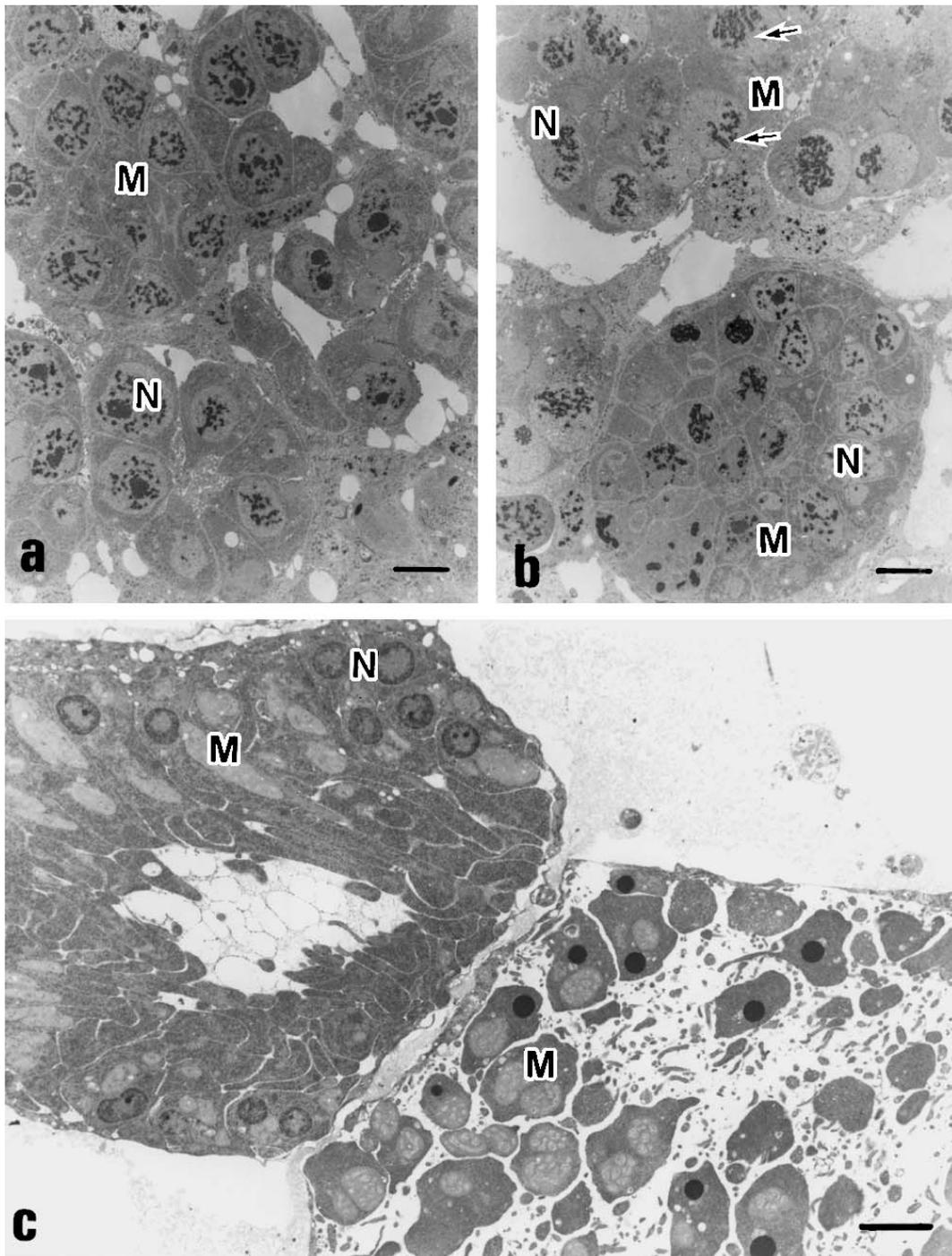


Fig. 6. Electron micrographs of spermatogenic cells in diapausing *A. convolvuli* pupae. **(a)** Cyst of spermatogonia. **(b)** Two neighboring cysts of primary and secondary spermatocytes. Arrows indicate the synaptonemal complex in primary spermatocytes. **(c)** Two neighboring cysts of degenerating spermatids. M, mitochondria; N, nucleus. Bar, 4 μ m

Until pupation, the timetable of spermatogenesis was nearly parallel in diapause-induced and non-diapause individuals. In diapausing pupae, primary and secondary spermatocytes and sperm bundles were observed up to 60 days after pupation, but these cysts degenerated as the diapause period lengthened. Apyrene spermatids and sperm bundles did not develop in the testis, and the decrease in spermatogenic cells was related closely to diminished testis size. The pupal diapause was sustained for 120–180 days at a con-

stant temperature of 25°C in *A. convolvuli*, and most spermatocytes and sperm bundles had disintegrated halfway through the diapause period. The seasonal occurrence of *A. convolvuli* in sweet potato fields in Kagoshima, Japan, has been extensively studied. The duration of diapause in the natural environment is estimated to be more than 200 days (Kamiwada *et al.*, 1990). Mature spermatozoa do not survive such a long diapause and do not participate in the next reproductive season.

When larval diapause was induced by low temperature and a short-day photoperiod in the codling moth, *Laspeyresia pomonella*, the testes of the last-instar larvae showed degeneration of spermatocytes at the diffuse stage of the meiotic prophase (Friedländer and Benz, 1982). Larvae of the wax moth, *Galleria mellonella*, were induced to diapause under similar rearing conditions (Cymborowski, 2000). Analysis of spermatogenesis at their larval diapause suggested that meiotic divisions do not occur or are altered because of the absence of secondary eupyrene spermatocytes (Friedländer and Benz, 1982). A TUNEL assay demonstrated that the degenerative changes in eupyrene cells were apoptotic (Polanska *et al.*, 2005). Here, the TUNEL assay clearly revealed distinct staining in secondary spermatocytes and early spermatids, indicating an apoptotic process in *A. convolvuli*. However, the cysts of primary spermatocytes with 64 nuclei and the sperm bundles were negatively staining by TUNEL assay (Fig.1e). In the testes of the swallowtail *Atrophaneura alcinous*, supravital staining, TUNEL assay, and ultrastructural observations showed that degenerating secondary spermatocytes were apoptotic (Kubo-Irie *et al.*, 1999). Spermatogenic cells, excluding primary spermatocytes, were eliminated by apoptotic degradation in these diapausing insects. The discontinuity of spermatogenesis is controlled by programmed cell death in the pupal diapause, as well as in the larval diapause.

We analyzed the degeneration of spermatogenic cells and arrest of spermatogenesis during diapause, with special attention to dichotomous spermatogenesis in the testes of diapausing pupae. In a subsequent study, we will examine the starting point of spermatogenesis in post-diapause development.

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