Aspects of the reproductive biology of Pieris brassicae (Lepidoptera, Pieridae) with special reference to eupyrene and apyrene spermatozoa

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ASPECTS OF THE REPRODUCTIVE BIOLOGY OF
Pieris brassicae (Lepidoptera, Pieridae)
WITH SPECIAL REFERENCE TO
Eupyrene and Apyrene Spermatozoa

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INTRODUCTION

The presence of two types of spermatozoa, nucleated eupyrene and anucleated apyrene, in Lepidoptera was first discovered by Meves (1903). Since then numerous microscopic and ultrastructural studies, as well as in situ observations, in the male and female of different species have been carried out. Both types of spermatozoa are derived from bipotential primary spermatocytes (Leviatan and Friedländer 1979). In the late larva or early pupa a shift in commitment from eupyrene to apyrene meiosis occurs. An apyrene spermatogenesis inducing factor responsible for the switch-over from eupyrene to apyrene spermatogenesis was postulated by Friedländer and Benz (1981).

Free apyrene and bundled eupyrene spermatozoa are stored in the duplex of adult males. During copulation both types of spermatozoa are transferred to the female. The apyrene spermatozoa are activated by a secretion of the male ejaculatory duct (Omura 1938b; Shepherd 1974a), but the eupyrene spermatozoa remain immotile until they reach the spermatheca (Holt and North 1970; Ferro and Akre 1975; Katsuno 1977d; Nabi and Harrison 1983). The possible role of the apyrene spermatozoa, in view of their seemingly costly production and transport to the female spermatheca, is a matter of great discussion (Goldschmidt 1916, 1920; Thibout 1981; Silberglied et al. 1984; Osanai et al. 1987).

Ultrastructural studies of spermatozoa in the testes of Pieris brassicae were carried out by Zylberberg (1963, 1965, 1969). However, a detailed account of the timing of the different stages of eupyrene and apyrene spermatogenesis, allowing for comparison with other species, was not made. The fate of both types of spermatozoa outside the testes has not been analyzed so far. Benz (1970, 1977) described some of the mechanisms involved in copulation and insemination in this species, but did not differentiate between eupyrene and apyrene spermatozoa. A detailed analysis of the spermatozoa in the male and female with regard to their fate during reproduction was thus necessary. In an attempt to gain a better understanding of the mechanisms involved in reproduction in this species and of the function of the different parts of the male and female reproductive organs, experiments were carried out and different parts of the reproductive system extirpated.
MATERIALS AND METHODS

1 The Insects

Stocks of the white cabbage butterfly *Pieris brassicae* are being kept at the Entomological Institute since more than ten years. The last addition of eggs occurred in January 1987 from a stock colony in Sussex, GB. The rearing methods used in maintaining the culture are similar to those described by David and Gardiner (1952). The larvae are reared on a standard diet of fresh cabbage leaves. Adults have access to sugar-water and a cabbage leaf for ovipositing. The culture is kept in 30 x 55 x 45 cm mesh cages in a glasshouse under variable conditions of light and humidity. The temperature ranges between 20-25°C.

2 Saline Solutions

The following saline solutions were used:

Belar (1929) *Chortippus lineatus*: spermatocytes
9 g NaCl, 0.2 g KCl, 0.2 g CaCl₂, 0.2 g NaHCO₃, 2.5 g glucose, and dest. water to 1 liter.

2.05 g NaCl, 2.68 g KCl, 1.33 g CaCl₂, 3.25 g MgCl₂·H₂O, 49.36 g glucose, to 1 liter dest. water.

Ephrussi and B(eadle) (1936) *Drosophila melanogaster*: transplantation
7.5 g NaCl, 0.35 g KCl, 0.21 g CaCl₂, to 1 liter dest. water.

Pieck and Mantel (1970) *Philosamia cynthia*: muscle
0.5 g NaCl, 2.4 g KCl, 0.6 g CaCl₂, 10.2 g MgCl₂·6H₂O, 0.08 g NaHCO₃, 10.8 g glucose, to 1 liter dest. water.

Ruttner (1975) *Apis mellifera*: semen
2.43 g trisodium-citrate 2-hydrate, 0.21 g NaHCO₃, 0.04 g KCl, 0.30 g sulfanilamide, 0.30 g glucose, and dest. water to 1 liter.

Stevenson (1969) *Antheraea polyphemus* (adult): muscle
2.98 g KCl, 0.71 g MgCl₂, 1.10 g NaH₂PO₄·H₂O, in 1 liter dest. water.

Vago (cited by Lender and Duveau-Hagege, 1963) *Galleria mellonella* (larva): gonads
5.5 g KCl, 0.6 g CaCl₂, 1 g MgCl₂·6H₂O, 5.5 g MgSO₄·7H₂O, 1.1 g NaH₂PO₄·H₂O, 1.6 g trehalose, in 1 liter dest. water.

Weevers (1966) *Antheraea pernyi* (pupa) and *A. mylitta* (pupa): hemolymph analyses
0.2 g NaCl, 3.0 g KCl, 0.3 g CaCl₂, 3.6 g MgCl₂·6H₂O, 8 mg NaHPO₄·H₂O, 5 mg NaHCO₃, 43.8 g glucose, to 1 liter dest. water.

Wyatt (1956) *Bombyx mori* (larva), *Samia walkeri* (pupa) and *Malacosoma disstria* (larva): ovary
2.98 g KCl, 0.81 g CaCl₂, 3.04 g MgCl₂·6H₂O, 3.70 g MgSO₄·7H₂O, 1.30 g NaH₂PO₄·H₂O, 0.70 g glucose, 0.40 g fructose, 0.40 g sucrose, 0.67 g malic acid, 0.37 g α-ketoglutaric acid, 0.06 g succinic acid, 0.06 g fumaric acid, to 1 liter dest. water.

All solutions were sterilized for 30 min in an autoclave. Osmolality was measured with a Knauer osmometer and pH with a Metrohm precision-pH-meter before the addition of sperm.

### 3 Morphological Studies

The butterflies were dissected in saline solution. The entire reproductive system was separated from the abdominal cavity and freed of attached tracheae and fat body tissue. The parts were measured and either drawn to scale with the help of a drawing tube attached to the stereo-microscope or a picture was taken and a drawing made of the enlargement. For detailed studies, certain parts were transferred to a glass slide and observed under higher magnification with the help of a light microscope equipped with Nomarski optics.

### 4 Analysis of Spermatogenesis

Squash preparations were made of the testes of accurately aged larvae, pupae and adults. The testes were either fixed and stained in lactic acid-orcein as described by Camenzind (1966) or left unfixed in a drop of saline.
The latter method proved more effective for analyzing elongating spermatids. The preparations were then observed with phase contrast under a light microscope.

For analyzing the influence of the juvenile hormone titer on apyrene spermatogenesis, one-day-old fifth instar larvae were neck-ligated. A thin thread was tied tightly around the larvae between the head and thorax. After a few hours the head was cut off, thus avoiding any possible hormone release. Testes squash preparations were made 12 days later as described above.

5 Observations of Copulation and Sperm Transmission

Sperm transmission was studied by the techniques of serial morphology as described by Callahan (1958). Adult butterflies were anesthesized with CO₂ and dissected in Belar's or Weevers' saline solution before, during and at various intervals after mating. The parts were transferred in a drop of saline to a glass slide and observed under a light microscope with Nomarski optics. The following criteria were recorded: (1) activity and progress of the two types of spermatozoa, (2) muscular contractions of the different parts of the male and female reproductive system, (3) production and presence of secretions, and (4) general state of the different parts.

Copulating pairs were frozen (-20°C) at different time-intervals after copulation began. The genitalia could then be dissected locked in the mating position and examined to determine the mechanics of mating and spermatophore formation.

6 Muscular Paralysis

To study the influence of muscular contractions of different parts of the female reproductive system on sperm transport to the receptaculum seminis, females were paralysed after copulation. Nitrogen or CO₂ was passed slowly over the insects for different periods of time. Paralysis ensued within a few minutes. After the treatment the females were dissected as described above. Other females were injected with Rotenone (0.01 μg per insect; blocks the respiratory chain), or Parathion (0.01-0.04 μg per insect; inhibits acetylcholinesterase) as described by Thibout (1977).
7 Extirpation of Different Parts of the Male and Female Reproductive System

Freshly emerged butterflies were anaesthesized with CO₂ so that they remained sedated for 10-15 min. The anaesthesized insects were secured by their wings in a self-constructed wooden mounting-block under a stereomicroscope. The wings were folded ventrally and clamped between the two wooden squares leaving the dorsal side free for the operation. A small V-shaped incision was made in the cuticle on the left side of the 6th abdominal segment of the female (for extirpating the caput bursae, accessory glands, or glandula receptaculi) or on the right side of the 5th abdominal segment of the male (for extirpating the accessory glands or the testes). Since the parts lie well below the dorsal cuticle, they had to be flushed into view before they could be removed. The physiological solution of Ruttner (1975) was used. This solution was gently flushed into the cavity with the help of a glass pipette until the part to be extirpated became visible. Great care had to be taken to avoid internal injuries in the process. The flap of skin was then folded back in place and the wound sealed with low-melting paraffin wax.

The testes of male pupae were removed in much the same way. The anaesthesiszed pupa was secured in a petri-dish with the dorsal side facing up. The petri-dish had been filled with wax and a mound formed in the wax in the shape of the pupa. The pupa was pinned down with a rubber band. A V-shaped incision was made in the 4th or 5th abdominal segment and the flap folded back. By slightly pressing on the thorax the testes usually popped into view and could easily be removed. The cuticle flap was pulled forward, the protruding hemolymph dabbed away and the wound sealed with paraffin.

8 pH Measurements of Different Parts of the Reproductive System

Measurement of the pH in the different parts of the male and female reproductive system was difficult, due to the small amount of secretion available (0.5-1.5 µl/insect). This necessitated a two- to three-fold dilution with dest. water (pH 6.5) prior to measurements with "Neutralit Indikatorstäbchen" (Merck).
9 Preparation of Sperm Suspensions

Unmated adult males were dissected in Belar's or Weevers' saline solution and the duplex removed, dried shortly on filter paper, and placed in 5 μl of the saline solution to be tested on a glass slide. The membrane was ruptured with fine needles. The semen and saline solution was collected in a glass capillary and transferred to a fresh glass slide with a small vaseline ring over which a cover slip could be fitted. The vaseline ring prevented the preparation from drying out.

Sperm from a fresh spermatophore or from the spermatheca of a female 1-2 days after copulation were collected in much the same way.

10 Analysis of Influence of Oxygen Deprivation

The influence of oxygen deprivation due to CO₂ or N₂ treatment on sperm motility was studied in vitro. A drop of sperm suspension containing active sperm from a fresh spermatophore or from the spermatheca was placed on a cover slip, which was then inverted onto a glass slide with a vaseline ring (hanging-drop method). A fine glass capillary was inserted and the humidified gas directed through the enclosure. Sperm motility was observed under a microscope equipped with a red filter for more than an hour.

11 Preparation of Secretions

The different secretions of the male ejaculatory duct and accessory glands were prepared as described by Shepherd (1974). The duct was placed in 10-50 μl 0.03 M ammonium bicarbonate-acetic acid buffer (a volatile buffer) pH 7.0. Cuts were made and the secretion was collected with forceps or with the help of a glass capillary. The secretion was either homogenized and then centrifuged for 10-15 min at 5600 g or assayed directly as described below.

12 Assay of Activating Capacity

The activating capacity of a secretion was tested by mixing an equal volume of sperm and secretion (usually 1-2 μl). The mixture was placed on a glass slide and the cover slip sealed with vaseline. Motility was analyzed
at intervals, starting not less than 15 min after mixing, under a microscope equipped with Nomarski optics.

13 Assay of Possible Motility-Blockers

The following solutions (5 µl of each) were mixed with active spermatozoa from a fresh spermatophore and the motility of the spermatozoa was then analyzed as described above:

- **saponin**: 0.2 g in 20 ml saline of E and B.
- **extraction medium of Mohri and Yanagimachi (1980)**: 0.1% Triton X-100 (Sigma), 0.2 M sucrose, 0.025 M potassium glutamate, 1 mM MgSO₄, 1 mM dithiothreitol, 0.02 M Tris-HCl buffer (pH 7.9). These two solutions demembranate salmonid spermatozoa (Morisawa et al. 1982). Triton X-100 is also used to demembranate hamster (Mohri and Yanagimachi 1980) and bull (Lindemann 1978) spermatozoa.
- **CTAB (cetyltrimethylammonium bromide)**: 10⁻² M in saline of E and B. This cationic detergent was used by Davey (1958) as an effective spermicide in *Rhodnius prolixus*. It is a surface-active substance.
- **colchicine**: 10⁻² M in saline of E and B. Colchicine is known to inhibit the formation and depolarization of microtubules, but colchicine insensitive microtubules have also been described (Dustin 1984).

14 High Temperature Sterilization of Males

Fresh fifth instar larvae were transferred to a 30°C chamber and kept there until they pupated. They were then returned to the standard culture conditions. Some larvae were kept from the first instar up to pupation in a 30°C chamber. The pupae were sexed and kept separately in different cages. After ecdysis the adult males were either dissected and the sperm in the testes and duplex analyzed or the males were allowed to copulate with normal females and the sperm transferred to the female analyzed.

15 Attempts at Artificial Insemination

Sperm suspensions were collected as described above under paragraph 9 (those from the duplex were activated with the ductus ejaculatorius simplex secretion no. 2 in Fig. 1) or undiluted from a fresh spermatophore or from the spermatheca. The sperm mass (usually 2-5 µl, according to dilution)
was sucked into a fine glass capillary with attached rubber tube and mouth-piece. The mouth-piece enabled strong pressure to be exerted when needed to press the sperm mass into the bursa and left both hands free for other manipulations.

Females were anaesthetized with CO₂ and secured by their wings in the wooden mounting-block described above (paragraph 7), leaving the abdomen with the ventral side up free for the manipulations. Under a stereo-microscope the ostium bursae was laid free with fine forceps. This was best achieved by gently pulling the triangular clitoris located between the ostium bursae and the oviporus. The ostium bursae is opened and the ductus bursae straightens (see bending of ductus bursae in Figs. 2 and 3), allowing insertion of the fine glass capillary and the pressing of its contents into the bursa without injuring the duct.

In some females the glass capillary was inserted into the oviporus and the sperm mass injected directly into the common oviduct (see Fig.2).

In other females the tip of a fresh spermatophore was inserted into the ostium bursae and the sperm mass and male secretory secretions pressed into the ductus bursae. The corpus of the spermatophore was held firmly between fine forceps so that the collum was free and could be inserted into the ostium bursae (see Fig. 4). By applying pressure to the forceps the contents of the spermatophore collum where easily pressed into the ductus bursae.
RESULTS

1 Gross Morphology of the Reproductive Systems of Male and Female *P. brassicae*

In describing the different parts of the male and female reproductive systems the terminology of Callahan (1958), Omura (1938b) and Petersen (1907) was used.

1.1 Male Reproductive System

The complete male reproductive system is shown in Fig. 1.

**Testes**

The crimson colored testes lie dorsally between the fourth and fifth abdominal segments. In the adult they are paired and so closely united that they appear as a single round organ enclosed by a common scrotum.

**Vas Deferens**

The vasa deferentia are paired tubes extending from the testes to the ductus ejaculatorius duplex. Each vas deferens consists of an upper wider tubular portion proximal to the testes, a swollen enlargement, called the seminal vesicle, and a long narrow tubular portion that empties into the duplex. The seminal vesicles are covered by a tightly packed mass of fat bodies. The whole length of the vasa deferentia of freshly emerged males contains free, immobile apyrene spermatozoa and eupyrene sperm bundles, but the main sperm reservoir is the ductus ejaculatorius duplex.

**Ductus Ejaculatorius Duplex**

The duplex is a paired V-shaped structure connected to the accessory glands at one end and joining to form the ductus ejaculatorius simplex at the other end. The outer surface of the duplex is covered by fat bodies. The duplex is the actual reservoir for sperm. Here the eupyrene spermatozoa are tightly compacted in bundles, while the apyrene spermatozoa appear as non-motile single sperm. During copulation the contents of the duplex are emptied into the simplex. Within 60 to 120 min. after the termination of
copulation the sperm reservoir is again filled with eupyrene sperm bundles and free apyrene spermatozoa that travelled down the vasa deferentia.

**Accessory Glands**

The male accessory glands are about 5 cm long, blind-ending extensions of the ductus ejaculatorius duplex. They can be divided into two distinct secretory sections: a proximal yellowish, granular secretory section, the glandula accessoria proximalis, and a much longer distal portion with a viscous, transparent secretion, the glandula accessoria distalis. During copulation the secretions of both sections are depleted and can be found in the collum of the freshly produced spermatophore. The yellowish secretion is caudad of the sperm mass filling most of the lumen of the spermatophore collum and the transparent secretion is found around the spermatophore orifice forming a cap (see Fig. 3).

**Ductus Ejaculatorius Simplex**

The ductus ejaculatorius simplex is a long tube extending from the duplex to the aedeagus. Five regions containing different secretions are visible in fresh unstained preparations. These regions are numbered one through five in Fig. 1 in the same manner as was done by Shepherd (1974) for *Antheraea pernyi* and *Hyalophora cecropia*. The most cephalad region (no. 5) is about 5 cm long and contains a whitish, highly elastic secretion. The fourth region is 3 cm long and much wider than all the others. It contains a clear, homogeneous secretion that turns white and hard when the ductus is ruptured. The next section (no. 3) is about 1 cm long and contains a transparent, granular secretion. It is separated from the former by a tiny secretory plug. Section no. 2 is only 0.3 cm long and filled with a viscous, transparent, gelatinous secretion. The most caudad section (no. 1) is 0.8 cm long and contains a sticky, white, granular secretion. All five sections are separated by distinct constrictions. During copulation the secretions are depleted, and most of them can be retraced to the female copulatory pouch. Secretion no. 1 fills the caput bursae and the uppermost portion of the corpus bursae. Secretion no. 4 builds the spermatophore wall and secretion no. 5 is found filling most of the spermatophore corpus (see Fig. 4). Secretions nos. 2 and 3, being transparent and small in quantity, are hard to localize.
1.2 Female Reproductive System

The internal reproductive system of the female is illustrated in Figs. 2, 3, 4 and 5.

**Bursa Copulatrix**

The bursa copulatrix (Figs. 2 and 3) is a large (8 mm long and 2 mm wide), conspicuous, white organ lying on the ventral side of the abdominal cavity. It is divided into three different parts: a tubular portion adjacent to the copulatory orifice or ostium bursae, called the ductus bursae, and two membranous sac-like pouches connected in series, namely, the large corpus bursae and the much smaller caput bursae. The basal portion of the ductus bursae is highly sclerotized and holds the aedeagus during copulation. The rest of the ductus membrane is covered by tiny tooth-like structures and striated muscle fibers. Similar tooth-like structures also cover most of the inner surface of the corpus bursae. Two highly sclerotized toothed plates (signa or lamina dentata) are found adjacent to each other in the dorsal wall. The corpus bursae is enclosed in a muscular sheath that is attached laterally to the signa.

The caput bursae, also called appendix bursae by Rutowski (1984), is a tiny cuticular sac that lies cephalad of the corpus bursae and farthest away from the copulatory orifice (Fig. 3). During copulation the caput bursae is filled with white secretion of section 1 of the male ductus ejaculatorius simplex.

**Ductus Seminalis**

The seminal duct connects the bursa copulatrix with the vestibulum of the unpaired oviduct. It is a highly contractile tube emerging dorsally at the base of the ductus bursae and joining the vestibulum opposite the spermathecal duct. Older, mostly virgin, females were often found with from one to eight eggs in the elastic, dorsal portion of the ductus seminalis. Only the egg closest to the vestibulum was intact, while the others had all been crushed. Traces of egg yolk were sometimes found in the bursa. The ventral portion of the seminal duct is covered with tiny spines pointing away from the bursa copulatrix.
Vestibulum

The vestibulum is an enlargement of the unpaired oviduct and the site of insemination of the eggs with eupyrene spermatozoa descending the canaliculus fecundans. The egg is positioned with the micropyle region closely pressed against the opening of the ductus receptaculi (see Fig. 5). While in the vestibulum the egg also receives the yellow secretion of the accessory glands.

Ductus Receptaculi or Ductus Tortuosus

The ductus receptaculi is a convoluted tube surrounded by well-developed musculature and leading from the vestibulum to the utriculus of the receptaculum seminis. Within this tube is a much thinner, highly sclerotized, spiral tube, called the canaliculus fecundans (Fig. 5). The canaliculus starts about half-way down from the utriculus in a wide mouth and narrows gradually till it reaches the vestibulum. Sperm travelling to the utriculus are found in the outer wide lumen of the ductus receptaculi, while eupyrene spermatozoa, on their way to fertilize an egg in the vestibulum, pass through the narrow canaliculus fecundans.

Receptaculum Seminis or Spermatheca

The receptaculum seminis of *P. brassicae* is a bilobed organ consisting of an elongated utriculus and a rounded lagena (Fig. 5). Both lobes communicate near the base. The utriculus is surrounded by muscle fibers allowing for peristaltic contractions. It is the main storage place for eupyrene and apyrene spermatozoa after copulation. The lagena is a thin-walled, elastic sac that is highly inflated in virgin and freshly copulated females. Only very few sperm were ever found in the lagena.

Glandula Receptaculi

The utriculus continues posteriorly into a blind-ending tubular gland, the glandula receptaculi. It consists of a thick layer of secretory cells around a narrow inner lumen. Active eupyrene spermatozoa are found in the lumen of the glandula of copulated females (see 3.4 for means of differentiating the spermatozoa).
Ovaries

The largest organs in the female abdomen are the paired ovaries, each of which consists of four ovarioles that empty into a short oviduct. The paired oviducts join together to form the common or unpaired oviduct. The ovaries of freshly emerged females are filled with developing eggs. Mature eggs are found in copulated and older virgin females.

Accessory Glands

In *P. brassicae* the accessory glands are two long narrow tubes that unite in a large common reservoir. They then join the common oviduct caudad of the ductus receptaculi at about mid-height of the vestibulum (Fig. 2). In mature females (about 24 hours after emergence) the paired glands are very conspicuous due to the yellow secretion they produce.

2 Testicular Development and Spermatogenesis

2.1 Testicular Development

In the larva two red bean-shaped testes are found just below the dorsal cuticle of the fifth abdominal segment. They lie with their concave sides facing each other on either side of the dorsal vessel. Each testis is composed of four compartments or follicles covered by a common envelope. The follicles are broadest laterally and narrow down medially to the vasa efferentiata that converge and continue into the vas deferens.

Towards the end of the fifth larval instar the testes move towards the dorsal mid-line, where they fuse a few hours before pupation. After pupation the testes become so tightly packed in the common scrotum that the two parts are no longer discernable. The spherical organ sinks away from the dorsal surface and comes to lie between the fourth and fifth abdominal segments.

2.2 Spermatogenesis

In the testes of the white cabbage butterfly, as in other Lepidoptera, two kinds of spermatozoa are produced in different cysts: nucleate (eupyrene) and anucleate (apyrene) spermatozoa. Cysts containing eupyrene or
apyrene spermatocytes, spermatids, and developing spermatozoa are easily distinguished on the basis of shape, size, and order of the nuclei. The earliest stage at which the two kinds of cysts could be differentiated was the primary spermatocyte undergoing meiotic division.

**Eupyrene Spermatogenesis**

The meiotic divisions of the spermatocytes are regular with conspicuous metaphase plates, distinct anaphase chromosomes moving towards the poles of the spindle, and well separated chromosomal masses at telophase. The spermatocytes elongate forming pear-shaped spermatids with spherical nuclei. Further elongation of the spermatids and of the nuclei follows. The mature eupyrene spermatozoa remain bundled in the membranous cyst envelope even after they have emerged from the testes.

**Apyrene Spermatogenesis**

Apyrene metaphases display an unusual distribution of chromosomes that never form a real equatorial plate. Some of the chromosomes remain unpaired while others clump together. During anaphase the chromosomal masses split irregularly forming bridges. These bridges are especially conspicuous in late anaphase and telophase and were used as a sure indication of apyrene meiosis. Later on, the bridges break-up leaving chromosomal fragments lying outside the newly forming cells. In the apyrene spermatids only the flagella elongate. The nuclei remain as irregular clumps that travel down the length of the tails and are finally eliminated from the cells. The apyrene sperm bundles disrupt before the spermatozoa leave the testes. Only free apyrene spermatozoa are found in the vasa deferentia and duplex.

**Normal Timetable of the Spermatogenesis**

The normal timetable of the eupyrene and apyrene spermatogenesis of *P. brassicae* and its relationship to the different stages of development is shown in Fig. 6. The testes of one-day-old fifth instar larvae contain first signs of meiotic divisions. The primary spermatocytes enter the first metaphase of eupyrene meiosis. Early spermatids with spherical nuclei appear the next day, the third day from the moult to the fifth instar. Elongation of the spermatid nucleus begins early on the sixth day before
the two testes fuse. The earliest signs of apyrene meta-, ana-, and telophases are also found at about this time, four days later than the first eupyrene metaphases. The resulting apyrene spermatids with micronuclei appear the next day in the fresh pupa. Elongating apyrene spermatids with the nuclei distributed along the flagella are first found in one-day-old pupae. All stages of eupyrene and apyrene spermatogenesis continue in the adult. The oldest males examined (eight days old) contained cysts with all the different stages of spermatogenesis listed above. Only very few spermatocysts were found, though, containing either eupyrene or apyrene meiotic divisions. Thus, it can be said that the testes of *P. brassicae* males continue to produce both types of sperm side by side for as long as they live.

**Spatial Separation of Spermatocysts**

No spatial separation in the testes of the two types of cysts could be found, but immature cysts (with earlier stages of spermatogenesis) are arranged laterally around the wide edge of the follicles, while the more mature cysts with spermatids and developing spermatozoa are found towards the narrow edge leading to the vasa deferentia.

**Influence of Juvenile Hormone Titer**

According to Varjas et al. (1976), the juvenile hormone titer of the hemolymph of *P. brassicae* is at its lowest (only traces) in one and two-day-old fifth instar larvae. Following this period, on the last day before pupation, a peak in the hormone titer was measured (70-110 GU/ml). First signs of eupyrene meiotic divisions are found in fifth instar larvae when the juvenile hormone titer is at its lowest, whereas the normal start of apyrene meiosis coincides with the peak in the juvenile hormone titer. In order to find out if spermatogenesis is influenced by the juvenile hormone titer, one-day-old fifth instar male larvae were neck-ligated. Since the juvenile hormone is produced in the corpora allata, located in the head, the hormone titer in the hemolymph of these neck-ligated larvae remains very low. Twelve days later squash preparations made from the testes of these neck-ligated males contained cysts undergoing eupyrene as well as apyrene spermatogenesis. Apyrene spermatogenesis was not blocked, although the juvenile hormone titer did not increase as it normally does towards the beginning of apyrene spermatogenesis. Thus, it can be said that in this
species the juvenile hormone titer has no influence on the onset of apyrene spermatogenesis.

3 Mating, Sperm Transport, and Remating

3.1 Copulation and Sperm Transfer to the Female

Successful copulations last from one to two hours. No relationship could be found between the onset of copulation and the actual start of ejaculation. A certain duration of copulation seems necessary to position the aedeagus properly in the ductus bursae before ejaculation can start. During ejaculation the secretions of the male reproductive glands are transferred serially to the bursa copulatrix of the female by peristaltic contractions. A certain degree of mixing does occur in the simplex of the male, though, due to the different viscosities of the secretions. No spermatophore precursor is produced in the male ejaculatory duct. The first material transferred to the corpus bursae is the white, granular secretion (no. 1) of the caudal portion of the male simplex. This is followed by the secretions nos. 2 and 3. Secretion no. 1 is pushed into the caput bursae and the uppermost portion of the corpus bursae when the secretion no. 4, forming the wall of the spermatophore, is pressed into the bursa.

The mechanism by which the spermatophore wall is formed is not completely understood. Although the form of the spermatophore with its oval corpus and tubular collum corresponds to the shape of the bursa, it is not molded by the walls of the bursa. The following observations show that the shape is genetically determined: if the corpus bursae of a female in copula is cut away before the secretion no. 4 has been ejaculated by the male, a normal-shaped spermatophore is still produced. Furthermore, males injected with physostigmine salicylate (10 μl of a 0.01 M aqueous solution) and kept in a chamber lined with moist filter paper, evert their aedeagus and continue to produce a normal spermatophore even without mating. These spermatophores have the same typical form as those produced during copulation.

The collum of the empty spermatophore is held firmly in the everted endophallus while it is being filled. The male first secretes the highly elastic, whitish secretion no. 5 into the lumen of the spermatophore. This
secretion largely fills the corpus. The discrete sperm package (see Fig. 4) consisting of motile apyrene and bundled eupryrene spermatozoa comes to lie at the base of the spermatophore corpus where it narrows down towards the collum. The collum of the spermatophore is filled with the yellow granular secretion of the proximal part of the male accessory glands, while the orifice at the tip of the collum is sealed with a transparent cap (Fig. 3) produced by the secretion of the distal part of the accessory glands. Before copulation is terminated, the collum of the spermatophore is properly positioned in the ductus bursae with the orifice close to the opening of the ductus seminalis.

Ejaculation lasts for about 30 to 60 min. After this period of time the lumen of the bursa is completely filled by the spermatophore.

The male ejaculatory ducts and accessory glands are emptied during copulation. No sperm are left in the duplex, but the vasa deferentia are still filled with immobile apyrene and bundled eupryrene spermatozoa. Within two hours of copulation the duplex is refilled and secretion of the glands of the ejaculatory duct has started. Males were observed to recopulate successfully the following day.

3.2 Emptying of the Spermatophore

The stretch stimuli exerted on the walls of the bursa by the spermatophore trigger muscular contractions that lead to the grating open of the spermatophore by the signa located in the walls of the corpus bursae. Experiments in which either saline solution or glycerol was used to inflate the copulatory pouch led to similar strong contractions of the bursal wall. These contractions start soon after copulation and within 30 to 60 min the imprint of the signa is visible in the form of a hole in the spermatophore wall (see Fig. 4).

One hour after copulation the cap around the orifice of the spermatophore (Fig. 3) is no longer visible. At about the same time the bundles of eupryrene spermatozoa in the spermatophore start to fall apart beginning at the tail end. As seen in Table 1, a group of apyrene spermatozoa leaves the spermatophore 5.5-8 hours after copulation, but the main sperm mass only leaves about three hours later.
The white secretion (no. 5) in the spermatophore is still elastic 24 h after copulation, but becomes liquefied later on, probably due to some chemical reaction, and continuously disappears. Two days after copulation the spermatophore is completely deflated. The colllum is transparent showing no signs of remaining secretions and the corpus is collapsed and concave where the signa dug into it.

Deflation of the old spermatophore leaves space in the bursa for additional spermatophores. Up to three spermatophores were found in the bursa of older *P. brassicae* females. The fresh spermatophore was usually lodged between the deflated old ones and the signa. In some cases, though, the new spermatophore was placed ventrad of the old spermatophore and away from the signa or it was smaller than normal and did not reach up to these teeth. In both cases the spermatophores were nevertheless emptied and the sperm transferred to the spermatheca.

Spermatophores removed from the bursa immediately after copulation (with the cap still around the orifice and no imprint of the signa), as well as those removed at a later time (when the cap has disintegrated and a hole is clearly visible in the corpus wall), and stored in saline solution in a petri-dish are emptied of the yellow accessory gland secretion and the sperm mass within less than five hours. In both cases the white secretion (no. 5) filling the corpus expanded and pushed the contents of the colllum out of the spermatophore. These emptied spermatophores remained inflated, even after a week, although the white secretion within liquefied. The different salines listed in Table 2 were used to store the spermatophores. Only in the saline of Wyatt (pH 3) were other observations made. In this saline the spermatophores remained intact. Neither the accessory gland secretions nor the spermatozoa were pressed out.

### 3.3 Sperm Transport to the Spermatheca of the Female

About five hours after copulation strong rhythmic contractions of the seminal duct, vestibulum and spermathecal duct can be observed. Soon after this, active apyrene spermatozoa are found in the receptaculum (see Table 1). Eight to ten hours after copulation the main sperm mass, made up of immobile eupyrene spermatozoa (some still partially bundled) surrounded by highly motile apyrene spermatozoa, begins to enter the seminal duct. Spermatozoa are then also found throughout the reproductive tract leading
to the spermatheca. The sperm pass through the seminal duct and cross the vestibulum to the spermathecal duct. No special duct or groove could be detected in the inner surface of the vestibulum leading from the ductus seminalis to the ductus receptaculi, and yet the sperm seem to follow a straight path. They were never found scattered about within the unpaired oviduct, but always in a compact group cutting across it. The spermatozoa ascend the outer lumen of the ductus receptaculi and enter the utriculus. Many can be seen swimming up the narrow lumen of the glandula. The lagena remains more or less free of sperm. As seen in Table 1, the transport from the spermatophore to the receptaculum seminis lasts only a few minutes. In most females dissected the spermatozoa were either still in the spermatophore or already in the receptaculum seminis.

3.4 Sperm Activity

Apyrene and eupyrene spermatozoa can easily be distinguished by their different size and activity patterns. Eupyrene sperm motility is in the form of a simple wave of low frequency with a wave length almost as long as the flagellum. Apyrene motility is of a much shorter wave length, a higher frequency and also consists of a spiral almost coiling motion. Apyrene spermatozoa are much thinner than eupyrene spermatozoa. The two types of spermatozoa are also activated at very different times.

The activity pattern of the spermatozoa in male and female *P. brassicae* is given in Fig. 7. Males emerge with their testes containing bundles of eu- and apyrene spermatozoa covered with a sheath, while eupyrene sperm bundles and free apyrene spermatozoa are already stored in the vasa deferentia and the ductus ejaculatorius duplex. During copulation the spermatozoa in the duplex are transported through the simplex and the aedeagus to the spermatophore in the bursa copulatrix. Apyrene spermatozoa are activated early in their descent down the simplex of the male and remain highly motile in the spermatophore in the female bursa. Eupyrene sperm bundles became loose in the spermatophore, but other than occasional and very weak gyrating they remain immobile. Vigorous eupyrene sperm motility is not observed until the spermatozoa enter the spermatheca. In the spermatheca both types of spermatozoa are active, but the apyrene soon clump together and become immobile. Continuous filling of the utriculus results in excessive clumping of spermatozoa hindering further motility. One day after copulation the utriculus is packed with
spermatozoa. Active eupyrene spermatozoa are only found towards the
glandula receptaculi where the sperm mass is less compact, in the
glandula itself, and in the wide portion of the ductus receptaculi leading to
the canaliculus fecundans. Only clumps of inactive apyrene spermatozoa
can be found.

In the spermatheca the eupyrene spermatozoa are stored until they are
used for fertilization. Before travelling down the canaliculus fecundans to
the vestibulum to inseminate the egg, the eupyrene spermatozoa hatch
from their enveloping sleeves along a predefined slit. The empty sleeves or
extracellular sheaths (Riemann and Gassner 1973) are barely visible in the
lower portion of the utriculus and in the wide mouth of the canaliculus
fecundans when observed under a light microscope. Electron microscopic
observations of spermatozoa in the receptaculum seminis of female
*P. brassicae* were made by Junquera and Benz (in preparation).

### 3.5 Insemination of Eggs and Oviposition

After an egg has been properly positioned in the vestibulum, the utricular
muscles start contracting rhythmically forcing hatched eupyrene
spermatozoa back down the ductus receptaculi. Its muscular walls contract
restricting sperm flow to the narrow sclerotized canaliculus fecundans.
Since the lumen of the canaliculus fecundans is very narrow, sperm motility
is not possible and the observed contractions alone must be responsible
for sperm transport to the site of fertilization. The egg in the vestibulum is so
positioned that the spermathecal duct leads directly to the micropyle region
ensuring entry of the eupyrene spermatozoon (see Fig. 5).

Females start ovipositing around 24 hours after copulation and continue
doing so for at least four days without copulating a second time. Fertilized
eggs were even collected eight days after copulation. Ten days after
copulation only inactive spermatozoa were found in the spermatheca.
Since females in our culture live for two to three weeks, the sperm mass
received during one mating alone is not sufficient to inseminate all the eggs
produced by the female.

### 3.6 Rematingings

As stated above under 3.2. females were found with up to three
spermatophores in their bursa. The older the females the greater the probability that they remate. Whether or not a female remates is not dependant upon the presence of active spermatozoa in the spermatheca. Some females dissected shortly after remating still had large amounts of active spermatozoa and these were still active when the new batch of spermatozoa started entering the spermatheca.

The rate of remating of females copulating with normal and with castrated males (castrated as pupae) was compared. As seen in Table 3 females remate after a shorter period of time after having copulated with a castrated male. Castrated males produce a normal-sized spermatophore in the bursa copulatrix. The male glandular secretions, but no spermatozoa, are transferred to the female (see also below, 4.2. testes extirpation).

4 Experiments concerning the Transport of Spermatozoa in the Female

4.1 The Effect of Muscular Contractions

At about the time spermatozoa exit from the spermatophore, rhythmic contractions of the ductus seminalis and the unpaired oviduct can be observed. These contractions take the form of peristaltic waves travelling towards the bursa. Spermatozoa within the ductus seminalis were observed to be sucked towards the vestibulum due to these contractions. Similar contractions are also observed in virgin females, but they are weaker and irregular in frequency.

To test the importance of these muscular contractions for sperm transport, females were exposed to nitrogen or carbon dioxide for certain periods of time after copulation. Muscular paralysis results under such conditions, but commences again some time after the treatment. The motility of apyrene spermatozoa is unaffected by oxygen deprivation resulting from exposure to either nitrogen or carbon dioxide as tested in vitro (see 5.3.).

As can be seen in Tables 4 and 5, such treatment delays the transport of spermatozoa from the bursa to the receptaculum seminis. Females paralysed before spermatozoa normally leave the bursa, and for a period of time exceeding that needed for all the spermatozoa to reach the
receptaculum, contained no spermatozoa outside of the bursa if they were dissected immediately after the treatment. It is interesting to note that the spermatozoa were not always trapped in the spermatophore. A big mass of spermatozoa was sometimes found in the ductus bursae just outside of the spermatophore. Controls, consisting of females treated in the same manner, but allowed to recover for some hours before dissection, had large quantities of spermatozoa in the spermatheca. Females exposed to nitrogen or carbon dioxide for 15 hours or more remained paralysed until they died. The spermatozoa were never transferred to the spermatheca.

Sperm transport from the bursa to the receptaculum seminis is also blocked in females stored in 2°C shortly after copulation. Females survive such treatment and after a period of recovery the spermatozoa are transported to the receptaculum seminis.

Rotenone (0.01 μg per insect) and Parathion (0.01-0.04 μg per insect) were also tested, but were unsatisfactory in as much as the insects injected never recovered from the treatment. Sperm transport out of the bursa remained blocked in the paralysed individuals.

4.2 Influence of Extirpation of Different Parts of the Male and Female Reproductive Tracts on Sperm Transfer

In an attempt to acquire additional information on the function of the different parts of the male and female reproductive organs and on their influence on sperm transport, the different parts were extirpated. Due to the fragility of the internal organs, only few animals survived the operations and copulated normally. The following results are usually from only a few individuals in which the surgical manipulations were successful.

Testes Extirpation

Extirpation of the testes of a male pupa has no influence on copulation or on the formation of a spermatophore in the female, but since all the spermatozoa were removed with the testes no spermatozoa are transferred to the female. If the testes are removed immediately after eclosion a normal amount of spermatozoa is transferred during the first copulation and a highly reduced amount of spermatozoa during the second copulation. This corresponds to the sperm masses stored in the duplex and vasa deferentia,
respectively, of the adult male prior to the extirpation. None of these males copulated a third time. The testes of more than 100 males were successfully extirpated.

**Extirpation of the Male Accessory Glands**

Extirpation of the accessory glands of freshly eclosed male butterflies led to the production of a much smaller spermatophore during copulation. Not only were the accessory gland secretions missing, but also the spermatozoa. Six operated males mated and produced such a spermatophore. Dissection of the males showed that the duplex was full of spermatozoa, but that these were not pressed out due to the lack of the adjacent secretions. Extirpation of only the distal portion of the accessory glands of two males led to the same result.

**Extirpation of the Caput Bursae**

The caput bursae of one virgin female was successfully extirpated. The wound healed and left the corpus bursae intact. During copulation a normal spermatophore was produced. The spermatophore was emptied and the spermatozoa transferred to the spermatheca.

**Extirpation of the Glandula Receptaculi**

Extirpation of the glandula less than 24 hours after eclosion (before secretion has started) did not influence copulation, or the transport of spermatozoa to the receptaculum seminis, but sperm motility and survival was reduced. Only a few fertilized eggs were oviposited. Surgical manipulations were carried out on 53 females, but only eight females copulated. The females were dissected after they started ovipositing or when they died. Of a total length of about 16.5 mm of glandula less than 0.5 mm was ever left attached to the utriculus.

Similar manipulations were also carried out on isolated adult female abdomina soon after copulation. The abdomina were dissected dorsally and the glandula receptaculi alone or together with the whole receptaculum seminis removed. The isolated abdomina were stored in saline solution in a petri-dish. Provided that the nerve endings to the different organs were left intact, the spermatozoa were transported normally
as far as possible. Active spermatozoa were found near the stumps of the extirpated parts. Controls consisted of isolated abdomina that were cut open dorsally, but left otherwise intact. In the controls sperm transport was slightly delayed compared to that in the intact female.

**Extirpation of the Female Accessory Glands**

Four females were successfully operated shortly after eclosion. The accessory glands of these females were still immature. The reservoirs were completely deflated and transparent with no signs of secretion. The females copulated during the proceeding days. One day after copulation eggs were found scattered on the floor of the cage around the cabbage leaf. These eggs dried out, but some eggs held back by folds in the cabbage leaf hatched. All of the eggs had been fertilized, but due to the lack of the sticky, yellow secretion of the accessory glands, the eggs could not be attached to the cabbage leaf as they normally are.

5  **Sperm Activity in Vitro**

5.1  **General**

The foregoing description (3.4. and Fig. 7) indicates that apyrene spermatozoa are activated at some point during their transfer from the duplex to the spermatophore. To determine the secretion of the male simplex responsible for the activation, each of the five secretions (see Fig. 1 and section 1.1. ductus ejaculatorius simplex) was combined with inactive spermatozoa from the duplex. Eupyrene spermatozoa are activated on their way to the receptaculum seminis and a search was made for the activator by mixing immobile spermatozoa from the spermatophore with different secretions. Since these techniques necessitate prior dilution of the sperm mass, it was essential first of all to analyse the conditions under which sperm motility is maintained.

5.2  **Osmolality and pH**

The conditions necessary for the maintenance of sperm activity *in vitro* were first analysed by testing different saline solutions of known osmolality and pH. Active apyrene spermatozoa collected from fresh spermatophores
remain active in very different salines with osmolalities ranging from 110-530 mOsmols/kg and pH from 4.6-7.6. As seen in Table 6, apyrene spermatozoa remain active in vitro for up to two days. Bacterial contamination usually ended motility.

Table 7 shows that active eupyrene spermatozoa collected from the receptaculum seminis only remain active for 4-5 hours. Motility was not normal and consisted more of a slow dying-off. Eupyrene sperm motility seems to be hindered by dilution alone. Spermatozoa in an intact receptaculum seminis observed in vitro were still active 24 hours after the organ had been transferred in 5 μl saline (Ephrussi and Beadle) to a slide and sealed with vaseline.

To further test the influence of different pH on sperm activity, the pH of a single saline (Belar) was varied by adding a few drops of either buffer solution pH 9.2 or buffer solution pH 4. This did not affect the osmolality which remained 330 mOsmols/kg. Active apyrene spermatozoa remained motile for at least 10 hours in a pH of 4.5 to 8.8.

The pH of the different parts of the male and female reproductive tracts is listed in Table 8. The values were the same in virgin and mated adults. Spermatozoa are usually stored in a neutral to slightly alkaline environment (pH 6.5-8), while the bursa itself seems to be slightly acidic (pH 5.5-6).

5.3 Oxygen

The importance of oxygen for sperm motility was analysed by observing the activity in vitro under CO₂ and N₂ influence. A steady flow of humidified gas was directed through a glass capillary into the slide enclosure while observing the whole process under a light microscope. No noticeable drop in apyrene motility was observed even after an hour. Proof of the flow of gas was seen in the form of tiny bubbles forming in the saline solution used to dilute the sperm mass extracted from a fresh spermatophore.

Since some of the solutions used to dilute the spermatozoa included no possible exogenous energy source such as a carbohydrate, a lipid or an amino acid, apyrene spermatozoa must have an endogenous energy
supply. Furthermore, motility must be sustained by anaerobic glycolysis alone.

5.4 Apyrene Sperm Activator

The standard medium used in the apyrene spermatozoa activator assay consisted of 5 µl Weevers' or Belar's saline. This corresponds to a 5-fold dilution of the sperm mass. Immobile spermatozoa from the male duplex were mixed with each of the five different simplex secretions (see 1.1. ductus ejaculatorius simplex and Fig. 1). Only secretion no. 2 contained the activating substance capable of inducing normal apyrene motility.

5.5 Eupyrene Sperm Activator

Different female secretions were tested as to their ability to activate immobile eupyrene spermatozoa taken from a fresh spermatophore. Neither the addition of accessory gland secretion, a ruptured glandula, a receptaculum seminis, nor an egg succeeded in inducing motility. Certain findings indicate that a "ripening" process, possibly coinciding with morphological changes in the spermatozoon as described by Riemann and Thorson (1971), leads to motility. Some in vitro preparations of spermatozoa from spermatophores mixed with either Weevers' or E + B saline solution (see Table 6) had active eupyrene spermatozoa 24-26 h after copulation. Furthermore, in three isolated female abdomina (see 4.2. under: Extirpation of Glandula) active eupyrene spermatozoa were found in the ductus bursae 20-25 hours after copulation. Nerve endings to the bursa and ductus seminalis had been severed, inhibiting contractions of the ducts. The sperm mass consisting of active apyrene and eupyrene spermatozoa (a few active) had been partially pressed out of the spermatophore, but remained stuck in the ductus bursae.

5.6 Sperm Activating Capacity of Glycerol

Immobile sperm dissected from the male duplex were placed in 5 µl of each of the following solutions: 5, 10, 20 and 50% glycerol-E + B saline. After an hour the sperm suspensions were checked for motility. Active apyrene spermatozoa were found in the 5, 10 and 20% glycerol-saline solutions, but motility persisted only in the solution containing 10% glycerol.
Addition of 10% glycerol to a sperm diluent enhances apyrene sperm motility. Fig. 8 illustrates the activity of apyrene spermatozoa from a fresh spermatophore in 5 μl of a 10% glycerol-E + B saline solution. Two different activity patterns of apyrene spermatozoa are observed. Some spermatozoa show directional movement to the periphery of the drop, while others stay clumped in the middle together with the eupyrene bundles and their motility consists of a circular or coiling motion. With time the eupyrene bundles break apart and the freed spermatozoa are transported to the periphery of the drop by the apyrene spermatozoa. This differential behavior of apyrene spermatozoa might explain the fact that a group of apyrene spermatozoa leaves the spermatophore in the bursa so much earlier than the main sperm mass (see Table 1).

5.7 Factors Hindering Sperm Activity

Different substances were tested to find out if they are capable of inhibiting apyrene sperm motility: (1) saponin (0.2 g in 20 ml E + B saline), (2) the extraction medium of Mohri and Yanagimachi (1980) containing 0.1% Triton X-100, (3) a 10⁻² M CTAB-E + B saline solution. These substances are known to demembranize spermatozoa. In all three substances apyrene sperm motility ceased. Neither the addition of 1.6 mMol cAMP nor of 3.4 mMol Mg²⁺ ATP succeeded in completely reactivating the spermatozoa. Only in very few preparations were signs of activity observed, and for a maximal period of 5-10 min.

A 10⁻² M colchicine-E + B saline solution had no effect on sperm motility. The apyrene spermatozoa remained motile even after 4-5 hours. Colchicine, a microtubuli-blocker, binds to singlet but not doublet tubulin at a site usually binding GTP.

6 Attempts to Influence the Ratio of Apyrene and Eupyrene Spermatozoa in the Male

Attempts were made to influence the ratio of apyrene and eupyrene spermatozoa produced in the male and transferred to the female during copulation. The optimal temperature for development in P. brassicae is between 20 and 25°C. Constant temperatures of 30°C resulted in highly reduced numbers of eupyrene spermatozoa in the duplex of males.
exposed as fifth instar larvae to this treatment. Only very few eupyrene spermatozoa were found in the receptaculum seminis, while the number and motility of apyrene spermatozoa remained normal. Males exposed during their whole larval period to 30°C had no eupyrene spermatozoa in the duplex. Only active apyrene spermatozoa were ever found in the receptaculum of females mated to these males. High temperature sterilization in this species is not the result of reduced apyrene sperm motility or even sperm death, but is due to reduced eupyrene sperm production or the complete lack of these functional spermatozoa.

7 Attempts at Artificial Insemination

Numerous attempts were made to artificially inseminate females. Although very different methods were used, no spermatozoa were ever transported to the spermatheca. No normal egg batches were laid by these females and only unfertilized eggs were oviposited and these were scattered about as is done by virgin females. Clumps of inactive sperm were only found in the bursa or the common oviduct, depending on whether the females were injected through the ostium bursae or the oviporus.

To ensure the presence in the bursa copulatrix of all the male secretions normally transferred to a female during copulation, females were allowed to copulate with castrated males prior to artificial insemination. The males that were castrated as pupae produced a normal spermatophore in the bursa void of spermatozoa. Insemination was then carried out, either immediately after copulation or at variable time intervals corresponding to the normal time-lapse between the termination of copulation and the emergence of spermatozoa from the spermatophore (5-11 hours, see Table 1). The results remained negative as described above. The spermatozoa remained stuck in the ductus bursae and were inactivated.
DISCUSSION

1 Eupyrene-Apyrene Spermatogenesis

The eupyrene-apyrene dichotomous spermatogenesis of Lepidoptera is a synapomorphy of this order and emphasizes the phylogenetic branching between the two sister orders of Trichoptera and Lepidoptera. Whereas in Trichoptera only eupyrene spermatozoa were found, eupyrene and apyrene spermatozoa were found in such primitive Lepidoptera as the Zeugloptera and the Exoporia (Friedländer 1983), as well as in the higher Lepidoptera or Ditrysia (Table 9).

Information on the spermatogenesis of lower Lepidoptera is scarce, but in all the higher Lepidoptera studied in detail spermatogenesis in the testes follows the same pattern. At the beginning all meiotic divisions are eupyrene, while spermatocytes undergoing apyrene spermatogenesis are only found a few days later.

In *P. brassicae* eupyrene spermatogenesis was first detected in one-day-old fifth instar larvae, four days before apyrene spermatogenesis. Zylberberg (1963) reports for the same species that first signs of apyrene meiotic divisions were made in one-week-old pupae, although no detailed time-table for the different stages of spermatogenesis is otherwise given. This discrepancy cannot be explained, although it seems important to emphasize that the observations reported here were made in a laboratory strain cultured at the Entomological Institute of the Swiss Federal Institute of Technology, as well as in individuals from a culture from the Glasshouse Crops Research Institute in Sussex, England.

Unlike reports from most other species (Table 10), the dichotomous spermatogenesis of *P. brassicae* (and *Calpodes ethlius*, Lai-Fook 1982a) continues in the pupa and throughout adult life. Not only are cysts found with signs of eupyrene and apyrene spermiogenesis, but both types of meiotic divisions as well. No actual switch-over from eupyrene to apyrene spermatogenesis occurs. The start of apyrene spermatogenesis is simply delayed by a few days.

Friedländer and Benz (1981) postulate the presence of an apyrene
spermatogenesis inducing factor (ASIF) that becomes active towards pupation and is responsible for the change in commitment of the "bipotential" primary spermatocytes from eupyrene to apyrene spermatogenesis. Conclusions are based on the fact that in the codling moth, *Cydia pomonella*, as well as in the carob moth, *Ectomyelois ceratoniae*, eupyrene spermatogenesis preceeds apyrene, and an obvious switch-over from one form of spermatogenesis to the other occurs. The short period in which both types of spermatogenesis take place can be explained by the fact that the prophase of the apyrene spermatocyte is much shorter than that of the eupyrene spermatocyte (Friedländer and Hauschteck-Jungen 1986). Thus, in the late last larval instar and young pupae of *C. pomonella* both types of metaphases can be found. Testes cultures of young fifth instar larvae of *C. pomonella* (explanted before the fourth day) in a medium containing mammalian serum, but neither hemolymph nor insect hormones showed no signs of apyrene spermatogenesis. Only eupyrene spermatids developed, even after 12 days of culture (Friedländer and Benz 1981). It is concluded that the ASIF is not yet active at this early stage. Testes cultures from a later stage produced eupyrene and apyrene spermatocytes and spermatids.

Contradictions to this hypothesis do exist, though. In *P. brassicae* eupyrene and apyrene spermatogenesis continues in the adult. Thus, although eupyrene spermatogenesis preceeds apyrene by four days, both types of meiotic divisions continue side-by-side for at least 20 days until the male dies. Since the two types of spermatocytes are not separated in the testes, but are found intermingled in the same follicle, these observations can hardly be the result of exposure to different environmental conditions. Within a single cyst all stages are either eupyrene or apyrene.

An alternative explanation for the production of two different types of spermatozoa could be that the seemingly bipotential, morphologically undifferentiated primary spermatocytes are in fact two different types of cells. Whether or not they are genetically differentiated is unknown. Zylberberg (1969) states that no difference was found in the base composition of the DNA.

2 The Activity of Sperm in the Male and Female

In *P. brassicae*, as in most other species studied, sperm are released from
the testes during, or a few hours after emergence. Only in *Bombyx mori* are sperm released already in the late pupa (Omura 1938b; Katsuno 1977c), and in *Heliothis zea* and *Trichoplusia ni* three days after emergence (Callahan and Cascio 1963; Holt and North 1970 resp.). They are stored in the adult male duplex as immobile apyrene and bundled eupyrene spermatozoa, but also in the vasa deferentia and/or seminal vesicles. During copulation only the spermatozoa stored in the duplex are incorporated into the spermatophore, while the spermatozoa found in the seminal vesicles are used to refill the duplex after mating (Holt and North 1970; Riemann and Thorson 1971; Etman and Hooper 1979b).

During copulation both types of spermatozoa are transferred to the female bursa copulatrix. In *P. brassicae*, as in *Cydia pomonella* (Tschudi, unpublished data), *Bombyx mori* (Omura 1938b), and various saturniid moths (Shepherd 1974a, b, 1975), the apyrene spermatozoa are activated during copulation by a secretion of the caudal portion of the male simplex. In *P. brassicae* and *B. mori* (Omura 1938b) apyrene spermatozoa are activated while passing through the male simplex, whereas in *C. pomonella* (Ferro and Akre 1975), *Trichoplusia ni* (Holt and North 1970), *Acrolepiopsis assectella* (Thibout 1977), and *Spodoptera litura* (Etman and Hooper 1979b), apyrene spermatozoa are only activated upon, or a few minutes after entering the spermatophore. An exceptional case is reported by Nabi and Harrison (1983) in *Phthorimaea operculella* where apyrene spermatozoa apparently become mobile already in the duplex during copulation.

In the monarch butterfly, *Danaus plexippus*, the production of a sperm activator, also located in the male ejaculatory duct, was shown to be stimulated by juvenile hormone (Hermann and Peng 1976). Unfortunately the authors failed to differentiate between eupyrene and apyrene spermatozoa.

In *P. brassicae* apyrene spermatozoa are activated by the secretion no. 2 of the distal portion of the male ejaculatory duct (see Fig. 1). They are also activated in a 10% glycerol solution. Similar observations were also made by Shepherd (1974b) with *Antheraea pernyi*. Addition of glycerol to a sperm diluent greatly increases the extracellular osmotic pressure. Thibout (1981), on the other hand, showed that apyrene spermatozoa of *Acrolepiopsis assectella* are activated by a decrease in osmotic pressure. Apyrene
spermatozoa from the duplex or seminal vesicles were only activated in solutions with an osmotic pressure of around 100 mOsm regardless of the chemical composition of the solutions. It is possible that different systems exist for the activation of apyrene spermatozoa in Lepidoptera.

The apyrene sperm activator of different saturniid moths was analysed in vitro by Shepherd (1974a, b, 1975). His findings suggest that the substance is a polypeptide with a molecular weight between 1600 and 4500. Aigaki et al. (1987) demonstrated that a specific endopeptidase (arginine ester-hydrolyzing enzyme) is present in the glandula prostatica, the lower region of the male ejaculatory duct of Bombyx mori that produces the apyrene sperm activator. This endopeptidase, with a molecular weight of 30,000, seems to be responsible for apyrene sperm activation in this species (Aigaki, personal communication).

In the spermatophore the apyrene spermatozoa are highly active and the immobile eupyrene sperm bundles soon start to break apart. Only very weak gyrating is observed in eupyrene spermatozoa in the spermatophore of P. brassicae, as observed in other species by Holt and North (1970), Ferro and Akre (1975), Katsuno (1977d), and Nabi and Harrison (1983). Normal eupyrene activity is only observed when the spermatozoa reach the receptaculum seminis. According to Thibout (1977), though, both apyrene and eupyrene spermatozoa of Acrolepiopsis assectella are active upon emerging from the spermatophore. The eupyrene spermatozoa are activated in the spermatophore soon after the bundles disrupt, but activity remains slight until the spermatozoa enter the spermatheca.

In Diatraea saccharalis (Miskimen et al. 1983) immobile eupyrene sperm bundles and motile apyrene spermatozoa leave the spermatophore. The same seems to be true in the Tortiricid, Zeiraphera diniana. In this species the eupyrene sperm bundles are transported to the bulla seminalis, an enlargement of the ductus seminalis, where they break apart (Benz, 1988). No mention is made in the former article as to where the eupyrene sperm bundles disrupt, but a bulla seminalis is absent in D. saccharalis.

No eupyrene sperm activator could be found in P. brassicae, neither could the start in activity be traced to a change in pH, osmotic pressure, nor salt concentration. Thibout (1981) reports the same negative results for his search for the eupyrene sperm activator of Acrolepiopsis assectella. So far,
eupyrene sperm activation cannot be explained, but a "ripening process" might be necessary before the spermatozoa become motile. Ultrastructural changes as described by Riemann and Thorson (1971), Friedländer and Gitay (1972), and Riemann and Gassner (1973) could be evidence of such a process. Eupyrene spermatozoa in the testes are surrounded by a series of appendages. These are replaced by a complex extracellular sheath and an electron dense matrix when the spermatozoa leave the testes. In the spermatheca the sheath splits along a clearly defined slit and the spermatozoon "hatches" before descending the canaliculus fecundans to fertilize the egg. The comparison of light and electron microscopic observations by Junquera and Benz (in preparation) shows that in *P. brassicae* hatched, as well as unhatched, eupyrene spermatozoa in the spermatheca can be highly motile. The "ripening process" mentioned above can, therefore, not simply be a matter of hatching from an extracellular sheath.

In *P. brassicae* most of the sperm remain in the utriculus and only very few are found in the lagena. Etman and Hooper (1979a) report that in *Spodoptera litura* sperm are only found in the utriculus, none in the lagena, whereas in most other species with bilobed spermatheca the spermatozoa enter the utriculus and the lagena (Holt and North 1970; Katsuno 1977d; Miskimen et al. 1983). Eupyrene spermatozoa are found in the utriculus, while apyrene pass via the utriculus into the lagena. In *Phthorimae operculella* (Nabi and Harrison 1983) eupyrene spermatozoa are found in the utriculus and lagena, but they are only active in the former. Benz (1970, 1977) reports that the spermatozoa of *P. brassicae* are immobilized in the utriculus by a secretion of the glandula receptaculi, but that portions of the sperm are later transported to the lagena, where they become active. These observations could, however, not be confirmed in the present study (neither with the same laboratory strain, nor with *P. brassicae* from a stock colony in England).

In *Bucculatrix thruberiella* (Lingren et al. 1987), *Acrolepiopsis assectella* (Thibout 1977), and *Cydia pomonella* (Ferro and Akre 1975) both types of spermatozoa are transferred to the spermatheca that consists of a single vesicle. In *A. assectella* the apyrene spermatozoa disappear one to two days after copulation.

Sperm survival in the spermatheca of the female varies widely in the
different species. In *P. brassicae* fertile eggs were collected up to eight days after copulation. Ten days after mating only inactive spermatozoa were found in the spermatheca. In *Phthorimaea operculella* eupyrene spermatozoa remain active for 1-12 days (Nabi and Harrison 1983) after mating as determined by egg-hatch. In both these species one mating alone (under laboratory conditions) is insufficient to ensure complete or near complete fertilization of all the eggs. In *Spodoptera litura* both sperm types persist throughout the life of the female (Etman and Hooper 1979a). Motile eupyrene and apyrene spermatozoa are found in the utriculus up to nine days after mating. In *Ephestia kühniella* spermatozoa remain active for 7-9 days, while females usually deposit all their eggs within 4-5 days (Riemann and Thorson 1971).

Riemann and Gassner (1973) report that in *Heliothis zea*, *H. virescens*, *Spodoptera frugiperda*, *Manduca sexta*, and *Pectinophora gossypiella* most apyrene sperm disappeared shortly after arriving in the spermatheca, while in *Trichoplusia ni* apyrene spermatozoa persisted, although they became immotile. In *P. brassicae* apyrene spermatozoa clump together in the spermatheca and are immobilized, but no signs of degeneration were observed in ultrastructural analyses (Junquera and Benz, in preparation).

3 The Function of the Signa and the Mechanism by which the Spermatophore is Emptied

Different hypotheses exist regarding the function of the sclerotized teeth (signa or lamina dentata) in the lepidopteran bursa and the method by which the spermatozoa exit from the spermatophore (summaries are given by Mann 1984; Rogers and Wells 1984).

(1) According to the simplest hypothesis the sclerotized teeth hold the spermatophore in place while the male retracts the aedeagus at the end of copulation.

In *Heliothis zea* (Callahan and Cascio 1963) and *Cydia pomonella* (Ferro and Akre 1975) the signa are reported to aid in retaining the spermatophore in the corpus bursae while the male aedeagus is removed at the end of copulation. The tiny spines found distributed along the inner surface of the bursa of *P. brassicae* could also have such a function, as they point away from the ostium bursae. Yet, the inflated spermatophore
itself fits snugly into the bursa with the corpus filling the corpus bursae limiting its retraction at least to a certain degree. The large signa, on the other hand, could hardly serve such a purpose.

(2) According to a second hypothesis the teeth hold the spermatophore in place while chemical reactions, such as swelling or tumefaction, force the sperm out through the orifice.

In *Heliothis virescens* and *H. subflexa* (Proshold et al. 1975) an increase in pressure within the spermatophore leads to the ejection of sperm and seminal fluid. A sudden release of the sperm from the spermatophore is observed *in vivo* as well as *in vitro* in saline. The reason for this sudden increase in pressure is unknown, but two events occurring prior to the ejection of the spermatozoa seem to be of importance: the milky secretion within the spermatophore corpus becomes transparent; and the spermatophore collum undergoes additional coiling. The bursa copulatrix appears to contract just prior and during ejection, and this apparently holds the spermatophore in place.

According to Benz (1977) secretions located in the bursa of *P. brassicae* enter the spermatophore through the hole produced by the lamina dentata. These secretions react with the secretions located within the spermatophore corpus, leading to the swelling of this secretion, forcing the accessory gland secretions and spermatozoa out through the spermatophore orifice. Personal observations contradict such a mechanism being responsible for the emptying of the spermatophore. First of all, emptied spermatophores without a hole produced by the signa are sometimes found. Either the spermatophore was smaller than normal and didn't come into contact with the signa, or the spermatophore was placed behind an old one away from the signa instead of between the signa and the emptied spermatophore. In both cases the spermatozoa were transported to the spermatheca. Secondly, in females paralysed with CO$_2$ or N$_2$ the spermatozoa remain stuck in the spermatophore, although the signa had already produced a hole in the wall of the spermatophore before the females were treated (see Tables 4 and 5). *In vitro* experiments did show, though, that the white secretion filling the spermatophore corpus does expand when isolated spermatophores are kept in different saline solutions, whether or not a hole had already been produced in the spermatophore wall.
According to a third hypothesis the teeth hold the spermatophore in place, while the spermatozoa and male secretions are squeezed out through the orifice at the tip of the spermatophore by muscles located in the walls of the bursa.

Petersen (1907) after analyzing several hundred different lepidopteran species came to the general conclusion that the spermatozoa are squeezed out of the spermatophores by pressure exerted by the muscles of the bursa. The spermatophores are positioned within the bursa with the orifice lying close to the opening of the ductus seminalis. The lamina dentata or signa located in the wall of the bursa of most species simply hold the spermatophore in place while these contractions are taking place.

In *Ephestia kühniella* and *Plodia interpunctella* (Norris 1932) no holes are made in the walls of the spermatophores by the lamina dentata. The muscles of the bursal sac are so arranged that when they contract pressure is exerted on the body of the spermatophore and the sperm squeezed into the ductus seminalis. In *Acrolepiopsis assectella* (Thibout 1977) sperm seem to exit actively from the spermatophore, as inactive sperm remained stuck in the spermatophore, but muscular activity of the bursa aids in the emptying of the spermatophore.

In *Diatraea saccharalis* (Miskimen et al. 1983) the spermatophore corpus is held in place by spines in the cuticular wall of the corpus bursae, while the corpus bursae muscles control the springlike action of the spermatophore collum. Both ensure precise positioning of the orifice close to the opening of the ductus seminalis. The sperm are believed to leave the spermatophore under their own power. Displacement of the spermatophore results in the spilling of the spermatozoa into the corpus bursae. This prevents them from reaching the ductus seminalis, as was revealed by the histological study of laboratory multiple matings resulting in the production of sterile eggs.

In *P. brassicae* contractions of the muscles of the corpus bursae cause the signa to bore a hole into the wall of the spermatophore while muscular contractions of the bursa and the ductus seminalis seem to be important for the emptying of the spermatophore. In paralysed females the spermatozoa remained mostly stuck in the spermatophore for the duration of the
treatment. However, apyrene sperm activity also seems to aid in the emptying of the spermatophore.

(4) A further hypothesis states that the sclerotized teeth open the spermatophore allowing the sperm and male secretions to escape through the hole(s).

In *P. brassicae* the hole produced by the signa (Fig. 3) is surrounded by the secretion of the proximal portion of the ejaculatory duct and is well away from the sperm mass (see Fig. 4). Furthermore, no seminal fluid was ever found to escape through this hole. Male secretions and the seminal fluid leave the spermatophore by way of the orifice at the tip of the collum and pass directly on to the ductus seminalis. Although this hypothesis is not relevant in *P. brassicae*, it is discussed here in view of certain interesting findings made in other species in which such a mechanism seems to exist.

Already Hagen (1882) reported that the spermatophores of the Yucca-moths, *Pronuba yucassella* and *Prodoxus* spec. are possibly torn open by the star-shaped, sclerotized teeth located in the wall of the bursa allowing the spermatozoa to pass via a special groove into the ductus seminalis.

The bursa copulatrix of the Monarch butterfly, *Danaus plexippus*, was studied in detail by light and electron microscopy by Rogers and Wells (1984). Other than the usual sclerotized teeth, they also found sclerotized plates with bristles on the inner surface of the corpus bursae. They conclude from their observations that the spermatophores deposited into the bursa expand the bristle areas. This triggers muscular contractions, pulling the teeth into the wall of the spermatophore and producing the holes through which seminal fluid can escape. From the bursal lumen the sperm then make their way to the spermatheca.

In *P. brassicae* light microscopic analysis of the bursal wall did not reveal any bristle structures as observed in *Danaus plexippus*, but expansion of the bursa also triggers muscular contractions, grating the signa through the spermatophore wall. Experiments in which either saline solution or glycerol was used to expand the bursa of a virgin female led to similar strong contractions of the bursal wall around the signa.

It is obvious that different species employ different mechanisms, and that a
few of the hypotheses listed above are true for a single species. In _P. brassicae_ muscular contractions seem to be paramount for the release of the secretions and the spermatozoa from the spermatophore. In most females paralysed with CO₂ or N₂ the sperm mass remained in the spermatophore, although the signa had already dug a hole in the corpus wall, and the apyrene spermatozoa within remained active. In a few cases, though, spermatozoa were found in the ductus bursae just outside of the spermatophore orifice. This observation, as well as the fact that in untreated insects a group of apyrene spermatozoa leaves the spermatophore a few hours before the main sperm mass and male accessory gland secretions, implies that sperm motility also plays an important role. Since spermatophores without a hole made by the signa are also emptied and, on the other hand, spermatophores with a hole in paralysed females are not, tumefaction as stated by Benz (1977) can hardly be responsible for the ejection of spermatozoa from the spermatophore.

The signa are believed to hold the spermatophore in place while muscular contraction primarily of the ductus bursae and ductus seminalis assisted by the activity of apyrene spermatozoa transports the sperm mass and accessory gland secretion to the ductus seminalis. The hole made by the signa allows chemical processes (reaction with secretions located in the bursa) to take place that lead to the liquefaction of the secretion in the corpus bursae and to the collapse of the spermatophore, thus leaving space in the bursa for a fresh spermatophore.

4 **The Function of the Male Accessory Glands**

The accessory glands of _P. brassicae_ are divided into two secretory sections. The proximal portion produces a yellowish, granulär secretion, and the distal portion a viscous, transparent secretion. The former secretion fills the spermatophore Collum, while the latter forms a cap around the orifice.

The accessory glands of _Bombyx mori_ (Omura 1938b) can also be divided into two secretory parts: a proximal part with a milky white secretion and a distal part with a transparent viscous secretion. After copulation the secretion of the proximal part can be found in the spermatophore caudad of the sperm mass close to the orifice. The secretion of the distal part forms a plug just outside of the orifice. By means of artificial insemination Omura
(1936) showed that both secretions are not essential for the insemination and fertilisation of the eggs. In fact only the sperm mass from the duplex and the apyrene sperm activator from the caudal portion of the ductus ejaculatorius of the male are essential for the successful insemination of females in this species. This is not the case in *P. brassicae*. The accessory glands play an important role first of all in the transport of spermatozoa from the duplex to the bursa: extirpation of the glands prior to copulation blocked spermatozoa from emerging from the duplex. Secondly, it is believed that the secretion of the proximal part induces muscular contractions of the female genital ducts responsible for the transport of the spermatozoa from the bursa to the spermatheca. However, injection of this secretion into the bursa of a virgin female did not succeed in inducing similar strong contractions. Perhaps the secretion has to undergo some chemical change before it becomes active, or it might need to be transported to a distant receptor.

In *Trichoplusia ni* sperm migration within the female is inhibited when mated with males deprived of their paired accessory glands (Leopold and Degrugillier in Leopold 1976). Unfortunately no details are given as to the possible cause for these observations.

In the heteropteran *Rhodnius prolixus* (Davey 1958) removal of the opaque lobe of the male accessory glands, considered homologous to the accessory glands of male Lepidoptera, prevents the normal migration of spermatozoa in the female. The opaque secretion induces rhythmic contractions of the oviducts. Davey (1958) suggests that the mode of action of the secretion is through the nervous system rather than directly on the muscle. In *Heliothis zea* (Callahan and Cascio 1963) the accessory glands produce an opaque secretion that is believed to have the same function. Contraction of the female seminal duct start when the secretion and sperm mass reach the orifice of the spermatophore collum and start to enter the bursa.

5 **The Function of the Female Accessory Glands**

Peterson (1907) states that the secretion of the female accessory glands attaches the eggs to the surface on which they are oviposited. Species that do not attach or stick their eggs to a surface lack these glands. Numerous other authors also state the same function, but to my knowledge no
experiments have been undertaken to test this hypothesis. Extirpation of the glands in *P. brassicaceae* does not influence copulation, the transport of sperm to the spermatheca, the fertilization of eggs, or oviposition, but the eggs that are normally attached in clusters to a cabbage leaf are found scattered around the leaf. This proves the lack of the agglutinant in the extirpated females. Some of the eggs that were held back by folds in the leaf hatched, but most dried out. The importance of attaching eggs to the food plant seems to be two-fold: to prevent them from drying out; and to ensure a direct source of food for the larvae.

6 The Function of the Glandula Receptaculi

Numerous functions are attributed to the secretion of the glandula receptaculi (for a complete summary see Drummond 1984):

Norris (1932) and Callahan and Cascio (1963) report that in *Ephestia* and *Plodia*, and *Heliothis zea* resp., spermatozoa are drawn up the spermathecal duct and into the spermatheca by the pumping action of the spermathecal gland and the spermatheca itself.

Weidner (1934) believes that the spermatozoa travel to the spermatheca due to chemical attraction of the secretion of the glandula, and experiments carried out with *Bombyx mori* clearly show an attraction. Benz (1977) reports that the spermatozoa of *P. brassicaceae* are transported to the vestibulum by muscular contraction of the female reproductive tracts, but that they actively ascend the ductus receptaculi chemically attracted by the secretion of the glandula. However, removal of the spermatheca and the glandula, or only of the glandula, did not influence the transport of spermatozoa to the ductus receptaculi or spermatheca resp., but sperm motility and survival was reduced. Only few eggs (fertilized) were oviposited. *In vitro* experiments with active spermatozoa from a spermatophore or from the receptaculum, to which a piece of glandula or a drop of secretion was added, did not show any attraction of the spermatozoa. Similar observations were made by Thibout (1977) who showed that removal of the spermatheca (including the glandula) did not hinder the spermatozoa of *Acrolepiopsis assectella* from entering the vestibulum. Sperm survival was reduced, morphological changes in the spermatozoa could be seen, and motility outside of the spermatheca did not last long. According to Norris (1932) the secretion of the glandula
supplies or provides nutrients to the sperm stored in the spermatheca. This may be a general function in higher insects, since even in the honey bee queen (Ruttner and Koeniger 1971) extirpation of the spermathecal gland results in the rapid reduction in sperm motility and the loss in ability of sperm to inseminate the egg. The number of sperm in the spermatheca was reduced and only unfertilized eggs were oviposited.

Stockel (1973) reports that secretions of the spermathecal gland of *Sitotroga cerealella* fill and lubricate the spermathecal duct, facilitating the migration of spermatozoa to the spermatheca.

Ultrastructural analysis of the spermathecal gland of *Plodia interpunctella* (Lum and Arbogast 1980) led to the discovery of mechanoreceptor setae, suggesting that the gland may have a role in determining the presence of motile sperm in a mated female.

7 The Role of Apyrene Spermatozoa

A number of hypotheses have been set up regarding the seemingly costly production of apyrene spermatozoa in Lepidoptera. For complete summaries see Goldschmidt (1916, 1920), Thibout (1981), and Silberglied et al. (1984). The following hypotheses shall be discussed here in detail:

1. The activity of apyrene spermatozoa in the spermatophore aids in the dissociation of eupyrene sperm bundles (Katsuno 1977e; Osanai et al. 1987).

In *P. brassicae*, as in many other lepidopteran species, apyrene spermatozoa are activated during ejaculation into the spermatophore in the female bursa copulatrix, while eupyrene spermatozoa are still bundled and completely immobile (*Spodoptera litura*, Etmans and Hooper 1979a; *Acrolepiopsis assectella*, Thibout 1977) or only show very slight gyratory motion (*Phthorimea operculella*, Nabi and Harrison 1983; *Bombyx mori*, Katsuno 1978; *Cydia pomonella*, Ferro and Akre 1975). It could well be that the vigorous flagellar motion of the apyrene spermatozoa is necessary for the breaking-apart of eupyrene bundles and the separation of the individual spermatozoa. *In vitro* observations show that active apyrene spermatozoa adhere to a clump of eupyrene bundles and that with time the latter are dissociated (see Fig. 8).
By mixing different batches of eupyrene sperm bundles of *Bombyx mori in vitro* with active, inactive or no apyrene sperm, Katsuno (1977e) showed that eupyrene sperm bundles only began to separate when active apyrene sperm were present. Osanai et al. (1987) demonstrated in the same species that the dissociation of eupyrene bundles is promoted by the vigorous flagellating motion of apyrene spermatozoa. The active apyrene spermatozoa stir the heterogenous, highly viscous, male secretions in the spermatophore and thus promote dissociation of eupyrene bundles and separation of each individual eupyrene spermatozoon both mechanically and by biochemical reactions brought about in the secretions, i.e. activation of peptidases, etc..

(2) The activity of apyrene spermatozoa assists in the digestion of secretions blocking the spermatophore orifice, allowing the sperm mass to escape (Osanai et al. 1987).

In *P. brassicae* a group of apyrene spermatozoa first leaves the spermatophore around five to eight hours after copulation, at a time when the cap around the spermatophore orifice (see Fig. 3) produced by secretions of the distal portion of the male accessory glands has already disintegrated since a few hours, but the secretions of the proximal portion of the accessory glands still fill the spermatophore collum. It seems likely that the activity of these apyrene spermatozoa is directly or indirectly responsible for eliminating the secretory mass blocking the passage and allowing the remaining spermatozoa to leave the spermatophore. As discussed above (under no. 6.), it is believed that the secretion of the proximal part of the accessory glands induces contractions of the ductus seminalis, but that a biochemical reaction is necessary to activate the secretion. The active apyrene spermatozoon could do just this, as well as transport some of the active substance to its receptor.

In *Bombyx mori* Osanai et al. (1987) found that the flagellating apyrene spermatozoa in the spermatophore are responsible for the digestion of the soft plug around the spermatophore orifice. In this species only this soft plug, produced by secretions of the distal portion of the male accessory glands (glandula pellucida), separates the sperm mass from the ductus seminalis. The mass of granular substance from the proximal portion of the accessory glands (glandula lacteola) lying within the orifice immediately
after copulation disappears and is probably mixed with the semen (Omura 1938b). Digestion of the plug opens the passage for the spermatozoa and allows them to enter the ductus seminalis.

(3) Apyrene spermatozoa assist the eupyrene spermatozoa in their migration from the bursa copulatrix to the spermatheca (Iriki 1941; Holt and North 1970).

Iriki (1941), working with *Bombyx mori*, separated the two types of spermatozoa from the spermatophore by centrifugation. He then injected the eupyrene spermatozoa by means of artificial insemination back into the bursa copulatrix of the female. The sperm were not transported to the receptaculum seminis, but remained in the bursa. The same results were obtained when inactive apyrene and eupyrene spermatozoa from the male seminal vesicles (corresponds to the duplex) or ampulla ductus deferentis (enlargement of the vas deferens close to the duplex) were injected. Unfortunately, no mention was made as to the influence of centrifugation on the spermatozoa and whether or not any controls were made. Thus, it remains unclear whether the absence of active apyrene spermatozoa alone is responsible for his results. Artificial insemination in *B. mori*, unlike in *P. brassicae*, is a successful technique developed by Omura (1936). Omura showed that only sperm from the duplex mixed with apyrene sperm activator, but neither the male accessory gland secretions, nor the ejaculatory duct secretions, were needed to successfully inseminate females in this species.

Gamma-irradiated male cabbage loopers, *Trichoplusia ni*, fail to transfer sperm to the spermatheca, due to their inability to properly incorporate the sperm into the spermatophore bulb (Holt and North 1970). Instead, they ejaculate directly into the bursa. Only a small portion of the apyrene spermatozoa manage to reach the spermatheca. The authors interpret the results as follows: due to the lack of sperm concentration and the highly reduced numbers of active apyrene spermatozoa (the biochemical environment of the bursa is obviously unsuitable for maintaining their activity) the immobile eupyrene spermatozoa had no means of reaching the spermatheca.

In *P. brassicae*, as in *Acrolepiopsis assectella* (Thibout 1977), muscular contractions of the female genital tract were shown to be responsible for the
transfer of sperm to the spermatheca. Paralysis of the female after copulation delays the transport of both types of spermatozoa for the duration of the treatment, although the sperm remain active. Peristaltic contractions of the ductus seminalis after copulation, when the sperm mass are first seen to enter, of the oviduct, and of the ductus receptaculi were observed in different species: *Ephesia kühniella* and *Plodia interpunctella* (Norris 1932), *Bombyx mori* (Omura 1938a), *Leucinodes orbonalis* (Srivastava and Srivastava 1957), *Pseudaletia unipuncta* and *Peridroma margaritosa* (Callahan and Chapin 1960), *Heliothis zea* (Callahan and Cascio 1963), *Spodoptera litura* (Etman and Hooper 1979a), *Diatraea saccharalis* (Miskimen et al. 1983). It is thus believed that passive transport of sperm in the female might be the general rule in Lepidoptera and that sperm motility plays only a secondary role.

Concerning the freeing of the sperm from the spermatophore, apyrene sperm motility might play an important role at least in some species. In *P. brassicae*, as in most other species studied: *Trichoplusia ni* (Holt and North 1970b), *Cydia pomonella* (Ferro and Akre 1975), *Spodoptera litura* (Etman and Hooper 1979b), *Diatraea saccharalis* (Miskimen et al. 1983), *Danaus plexippus* (Rogers and Wells 1984), eupyrene spermatozoa remain immobile until they reach the receptaculum seminis or as in the case of *Bombyx mori* only reach full motility there (Katsuno 1978). Only in *Acrolepiopsis assectella* (Thibout 1981) and *Manduca sexta* (Silberglied et al. 1984) are eupyrene and apyrene spermatozoa independently motile while leaving the spermatophore. As can be seen in Fig. 8, active apyrene spermatozoa of *P. brassicae* are quite capable of transporting loose eupyrene spermatozoa under certain conditions. It could very well be that a similar mechanism also takes place *in vivo*, at least in those species in which only apyrene spermatozoa are active when leaving the spermatophore. This could also explain the observations of Holt and North (1970) with gamma-irradiated cabbage loopers. The effectiveness of apyrene sperm transport under the confined conditions of a spermatophore is not given when the sperm are deposited directly into the large lumen of the bursa copulatrix. Therefore, in spite of the fact that a few active apyrene sperm left the bursa, no eupyrene sperm were transported along to the spermatheca.

The fact that in *P. brassicae* a group of apyrene spermatozoa leaves the spermatophore before the main mass of male accessory gland secretions
indicates that the passage of the spermatozoa is at least assisted by their own movements. Otherwise the sperm and the more caudal lying secretions would be squeezed out of the spermatophore and transferred to the receptaculum together. Similar observations are also reported by Norris (1932) in *Plodia interpunctella* and *Ephestia kühniella*.

High temperature sterilization of butterflies is due to the failure of eupyrene spermatozoa to be transferred to the spermatheca. Katsuno (1977f), and Benito-Espinal and Laugé (1980) attributed the fact that in *Bombyx mori* and *Spodoptera littoralis*, respectively, such treatment leads to the production of abnormal apyrene spermatozoa as proof that normal apyrene spermatozoa are responsible for transporting eupyrene spermatozoa to the spermatheca. Yet, it could well be that the eupyrene sperm only remained bundled and stuck in the spermatophore due to the lack of normal apyrene spermatozoa, but that they would have been transported to the spermatheca had they managed to enter the seminal duct. Zylberberg (1965) reports that treatment of *P. brassicae* to temperatures of 30°C results in sterility due to an increase in apyrene and a decrease in eupyrene sperm production during spermatogenesis. My own findings show that high temperature sterilization leads either to the complete absence of eupyrene spermatozoa, or to greatly reduced production of this type of sperm. However, if normal eupyrene spermatozoa are produced, they are then also transferred to the spermatheca.

(4) Apyrene spermatozoa represent some form of nutrient reserve for eupyrene spermatozoa, the female butterfly, or the zygote.

This hypothesis is widely discussed in connection with apyrene spermatozoa for the simple reason that in numerous molluscs such a trophic function of atypical spermatozoa seems to exist. The atypical spermatozoa contain yolk bodies composed of glycoproteins that are believed to serve as a source of nutrition for the eupyrene sperm and for the female (Melone et al. 1980; Buckland-Nicks et al. 1982). The cytoplasmatic secretions of these sperm are released in the copulatory pouch or in the seminal receptacle.

The apyrene spermatozoa of lepidopterans have a simple morphological structure. Electron micrographs show them to consist of little more than a centriole, an axial filament or axoneme, and two small mitochondrial
derivatives surrounded by two thin concentric sleeves or sheaths (Zylberberg 1969; Riemann and Thorson 1971; Friedländer and Gitay 1972; Riemann and Gassner 1973; Friedländer 1982; Lai-Fook 1982b). In some species apyrene spermatozoa disappear shortly after arriving in the spermatheca (Riemann and Gassner 1973), but in many other species both types of spermatozoa remain motile (Etman and Hooper 1979b), or the apyrene spermatozoa become immobile and clump together, but were never observed to degenerate (Friedländer and Gitay 1972; Riemann and Gassner 1973; Junquera and Benz, in preparation). Although this hypothesis cannot be completely rejected as of now, it remains highly unlikely that apyrene spermatozoa constitute any kind of nutrient resource. Furthermore, males are known to transfer large amounts of secretions to the female during copulation (4% to 8% of the male's precopulatory body weight: Boggs and Gilbert 1979; Greenfield 1982; Rutowski 1984). These secretions would constitute a far simpler and cheaper means of transferring nutrients to the female than in the form of apyrene spermatozoa.

(5) The production of apyrene spermatozoa in the Lepidoptera is the result of competition between rival spermatozoa deposited by different males in the female. Apyrene spermatozoa can either displace or inactivate eupyrene spermatozoa from previous matings, or they can delay further mating by the female (Silberglied et al. 1984).

In most species studied so far, the apyrene spermatozoa are inactivated shortly after arriving in the spermatheca (references listed above). In *P. brassicae* active apyrene spermatozoa were only found in the spermatheca within 24 hours of copulation. After this period of time only clumps of inactive apyrene spermatozoa were found.

Comparison of the number of spermatophores (after a period of nine days) in the bursa copulatrix of females that mated with castrated males with those of females allowed to copulate with normal males, shows that the presence of spermatozoa in the spermatheca delays mating for a longer period of time. It is hard, though, to imagine how such relatively small clumps of inactive apyrene spermatozoa should be responsible for these observations. The mass of active eupyrene spermatozoa themselves would be much more likely to induce female refractivity.

When females of different lepidopteran species mate more than once, it is
usually the sperm of the last male that inseminate the eggs (Pair et al. 1977; Etman and Hooper 1979a). In Spodoptera litura (Etman and Hooper 1979a) a second mating results in the expulsion from the spermatheca of the sperm received from the first male. Within 30 min of completion of copulation the spermathecae of these females are found to be void of sperm, and they remain empty until the sperm from the second male began to enter (within 60 min of copulation). Little or no mixing of sperm of different males occurs. In P. brassicae no such expulsion of spermatozoa was observed. Active eupryene spermatozoa from the first male were present in the spermatheca when the spermatozoa from the second male started entering. Since the spermatheca is blind-ending, it is to be expected that the spermatozoa from the first male are pushed back when fresh spermatozoa enter, and that only the latter then manage to leave the spermatheca and travel down the canaliculus fecundans to inseminate the eggs. How apyrene spermatozoa should contribute, under these circumstances, to the displacement or inactivation of eupryene spermatozoa already present in the spermatheca, remains unclear.
SUMMARY

The normal timetable of spermatogenesis in relation to the different stages of development of male *Pieris brassicae* is given. Unlike in most other lepidopteran species studied so far, eupyrene and apyrene spermatogenesis continues in the testes throughout adult life.

The mechanisms involved in the transfer of spermatozoa were studied by live dissection of males and females before, during and after copulation. Different parts of the male and female reproductive tracts were extirpated. The possible function of the male and female accessory glands and of the glandula receptaculi as concluded from the results of these extirpation experiments is discussed.

During copulation the apyrene spermatozoa are activated by a secretion of the distal part of the male ejaculatory duct. The eupyrene sperm bundles fall apart in the spermatophore, but active eupyrene spermatozoa are only found in the spermatheca. The spermatozoa are transported to the spermatheca by peristaltic contractions of the female reproductive tract. A portion of the apyrene spermatozoa leaves the spermatophore and is transported to the spermatheca 5.5-8 hours after copulation, but the main sperm mass consisting of the immobile eupyrene spermatozoa surrounded by the remaining active apyrene spermatozoa only leaves the spermatophore about three hours later. Differential behavior of apyrene spermatozoa as observed *in vitro* could be responsible for this. The transport of spermatozoa from the spermatophore to the spermatheca involves only a short period of time. In most females analysed the sperm mass was either still in the spermatophore or already in the spermatheca.

Two possible roles of apyrene spermatozoa in this species are discussed: (1) the activity of apyrene spermatozoa in the spermatophore aids in the dissociation of eupyrene sperm bundles, and (2) apyrene sperm motility assists in the freeing of the immobile eupyrene spermatozoa from the spermatophore.

No spermatophore precursor is formed in the male, but instead the secretions of the ductus ejaculatorius and the accessory glands are transferred serially. The spermatophore is produced in the female bursa copulatrix.
The mechanisms involved in the emptying of the spermatophore after copulation in this species are rather complicated and are discussed in connection with the peristaltic contractions of the female reproductive tract, apyrene sperm motility, and the function of the signa located in the wall of the corpus bursae.
ZUSAMMENFASSUNG

Der zeitliche Verlauf der eupyrenen und apyrenen Spermatogenese bei *Pieris brassicae* wurde analysiert. Im Unterschied zu Befunden bei anderen Lepidopterenarten findet man bei *P. brassicae* alle Stadien beider Typen von Spermatogenese noch bis zum Tode des Männchens. Es findet also kein Wechsel von eupyrener zur apyrener Spermatogenese statt, sondern nur ein zeitlich verzögerter Beginn der apyrenen Spermatogenese.


Folgende Funktionen der apyrenen Spermatozoen bei *P. brassicae* werden diskutiert: (1) apyrene Spermien unterstützen die Auflösung der eupyrenen Spermienbündel in der Spermatophore, und (2) durch die Aktivität der apyrenen Spermien werden die noch unbeweglichen eupyrenen Spermien aus der Spermatophore befreit.
Die Sekrete des Ductus ejaculatorius und der Akzessorischen Drüsen des Männchens werden seriell ins Weibchen transportiert und die Spermatophore dann in der Bursa gebildet.

Die Vorgänge, welche sich bei der Spermatophorentleerung abspielen, sind bei dieser Art recht kompliziert. Sie werden in Zusammenhang mit peristaltischen Kontraktionen des weiblichen Geschlechtsapparates, der Aktivität der apyrenen Spermatozoen und der Funktion der Signa in der Wand des Corpus bursae diskutiert.
REFERENCES


Friedländer, M., Jans, P. and Benz, G. 1981. Precocious reprogramming of eupyrene-apyrene spermatogenesis and commitment induced by
allatectomy of the penultimate larval instar of the moth *Actias selene*. *J. Insect Physiol.* 27: 267-269.


Table 1  Transmission of apyrene (ap) and eupyrene (eup) spermatozoa from the spermatophore in the bursa copulatrix to the receptaculum seminis (r.s.).

<table>
<thead>
<tr>
<th>time after copulation [h]</th>
<th>N</th>
<th>number of ap sperm under way</th>
<th>number of ap sperm in r.s.</th>
<th>number of females with eup sperm under way</th>
<th>number of eup sperm in r.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.5</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.5</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>1b</td>
<td>16</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>8.5</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>9.5</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*a* spermatozoa in ductus seminalis, vestibulum and/or ductus receptaculi

*b* eupyrene spermatozoa still in spermatophore.
Table 2  The emptying of isolated spermatophores in different saline solutions in petri-dishes.

<table>
<thead>
<tr>
<th>saline solution</th>
<th>spermatophore emptied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belar (pH 7.6; 330 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>Ephrussi + Beadle (pH 6.5; 270 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>Vago (pH 4.6; 280 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>Weevers (pH 6.2; 420 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>Wyatt (pH 3; 410 mOsm/kg)</td>
<td>no</td>
</tr>
<tr>
<td>Wyatt (pH 6; 370 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>0.05 M NaCl (pH 6.5; 279 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>0.1 M NaCl (pH 6.5; 186 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>0.15 M NaCl (pH 6.5; 94 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>dest. H2O (pH 6.5; 0 mOsm/kg)</td>
<td>yes</td>
</tr>
<tr>
<td>moist filter paper (no saline)</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 3  Comparison of number of copulations between females mating with castrated and with normal males within a period of nine days.

<table>
<thead>
<tr>
<th>treatment of males</th>
<th>N \textsuperscript{a}</th>
<th>no.of females with spermatophores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>females</td>
<td>males</td>
</tr>
<tr>
<td>none</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>none</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>none</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>none</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>castrated</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>castrated</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} total number of females and males together in a cage; insects 1-2 days old.
### Table 4  Effect of N₂ paralysis of the muscles of the female on sperm transport from the spermatophore to the receptaculum seminis.

<table>
<thead>
<tr>
<th>N</th>
<th>duration of treatment [h]</th>
<th>time after copulation analysed [h]</th>
<th>time after treatment analysed</th>
<th>females with sperm mass in spermatophore</th>
<th>receptaculum seminis</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6</td>
<td>13</td>
<td>⩽10 min</td>
<td>4ᵃ</td>
<td>1ᵇ</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>24</td>
<td>13-14 h</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>15-16 c</td>
<td>19-19.5</td>
<td>⩽10 min</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15-16 c</td>
<td>22-23</td>
<td>⩽10 min</td>
<td>3ᵃ</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ also in ductus bursae  
b only apyrene spermatozoa  
c females irreversibly paralysed

### Table 5  Effect of CO₂ paralysis of the muscles of the female on sperm transport from the spermatophore to the receptaculum seminis.

<table>
<thead>
<tr>
<th>N</th>
<th>duration of treatment [h]</th>
<th>time after copulation analysed [h]</th>
<th>time after treatment analysed [h]</th>
<th>females with sperm mass in spermatophore</th>
<th>receptaculum seminis</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8</td>
<td>12-13</td>
<td>⩽0.5</td>
<td>3ᵃ</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>17-18</td>
<td>⩽0.5</td>
<td>3</td>
<td>3ᵇ</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>20-21</td>
<td>3.5-4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>12-13</td>
<td>15-17</td>
<td>1-1.5</td>
<td>3</td>
<td>1ᵇ</td>
</tr>
<tr>
<td>4</td>
<td>12-13</td>
<td>19-20</td>
<td>⩽0.5</td>
<td>4</td>
<td>2ᵇ</td>
</tr>
<tr>
<td>3</td>
<td>12-13</td>
<td>19-20</td>
<td>4.5-5</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>16 c</td>
<td>19-20</td>
<td>⩽0.5</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>16 c</td>
<td>24</td>
<td>2.5-3</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ also in ductus bursae  
b only apyrene spermatozoa  
c females irreversibly paralysed
Table 6  Motility of apyrene (ap) and eupyrene (eup) spermatozoa from the spermatophore in various saline solutions.

<table>
<thead>
<tr>
<th>saline solution</th>
<th>N</th>
<th>pH</th>
<th>osmolality [mOsm/kg]</th>
<th>motility</th>
<th>max. time active (^a) [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belar</td>
<td>2</td>
<td>7.6</td>
<td>330</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>Belton</td>
<td>7</td>
<td>6.5</td>
<td>530</td>
<td>+</td>
<td>48</td>
</tr>
<tr>
<td>E + B</td>
<td>7</td>
<td>6.5</td>
<td>270</td>
<td>+</td>
<td>43</td>
</tr>
<tr>
<td>P + M</td>
<td>2</td>
<td>7.0</td>
<td>310</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>Stevenson</td>
<td>4</td>
<td>6.0</td>
<td>110</td>
<td>+</td>
<td>46</td>
</tr>
<tr>
<td>Vago</td>
<td>3</td>
<td>4.6</td>
<td>280</td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td>Weevers</td>
<td>9</td>
<td>6.2</td>
<td>420</td>
<td>+</td>
<td>46</td>
</tr>
<tr>
<td>Wyatt</td>
<td>2</td>
<td>3.0</td>
<td>410</td>
<td>+</td>
<td>48</td>
</tr>
<tr>
<td>dest. H(_2)O</td>
<td>1</td>
<td>6.5</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>hemolymph</td>
<td>1</td>
<td>6.5</td>
<td>-</td>
<td>+</td>
<td>19</td>
</tr>
</tbody>
</table>

+ : motility; - : no motility observed

\(^a\) activity time influenced by bacterial contamination

\(^b\) signs of activity 24-26 h after copulation over a period of 1-2 hours
Table 7  Motility of apyrene (ap) and eupyrene (eup) spermatozoa from the receptaculum seminis in various saline solutions.

<table>
<thead>
<tr>
<th>saline solution</th>
<th>N</th>
<th>pH</th>
<th>osmolality [mOsm/kg]</th>
<th>motility ap</th>
<th>eup</th>
<th>max. time active(^a) [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belar</td>
<td>3</td>
<td>7.6</td>
<td>330</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>Belton</td>
<td>2</td>
<td>6.5</td>
<td>530</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>E + B</td>
<td>2</td>
<td>6.5</td>
<td>270</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>P + M</td>
<td>2</td>
<td>7.0</td>
<td>310</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>Stevenson</td>
<td>2</td>
<td>6.0</td>
<td>110</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vago</td>
<td>3</td>
<td>4.6</td>
<td>280</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>Weevers</td>
<td>2</td>
<td>6.2</td>
<td>420</td>
<td>+</td>
<td>+</td>
<td>4-5</td>
</tr>
<tr>
<td>Wyatt</td>
<td>1</td>
<td>3.0</td>
<td>410</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dest.H(_2)O</td>
<td>1</td>
<td>6.5</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>hemolymph</td>
<td>1</td>
<td>6.5</td>
<td></td>
<td>+</td>
<td>+</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(+\) : motility; \(-\) : no motility observed

\(^a\) for eupyrene spermatozoa; motility weaker than normal

Table 8  The pH in different parts of the male and female reproductive system.

<table>
<thead>
<tr>
<th>part of system</th>
<th>N</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>testes</td>
<td>15</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>duplex</td>
<td>12</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>bursa copulatrix</td>
<td>12</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>spermatophore</td>
<td>5</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>receptaculum seminis</td>
<td>16</td>
<td>7.3 ± 0.5</td>
</tr>
</tbody>
</table>
Table 9 Literature on the different lepidopteran families in which apyrene spermatozoa have been described.

<table>
<thead>
<tr>
<th>Suborder</th>
<th>Family</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeugloptera</td>
<td>Micropterigidae</td>
<td>Friedländer 1983</td>
</tr>
<tr>
<td>Exoporia</td>
<td>Hepialidae</td>
<td>Friedländer 1983</td>
</tr>
<tr>
<td>Dilrysia</td>
<td>Bombycidae</td>
<td>Friedländer &amp; Gitay 1972;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shepherd 1974a; Katsuno 1977a-f, 1978;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osanai et al. 1987;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Miskimen et al. 1983;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Riemann &amp; Gassner 1973;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Henneberry et al. 1977;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LaChance et al. 1979;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nabi &amp; Harrison 1983</td>
</tr>
<tr>
<td></td>
<td>Crambidae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gelechidae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Geometridae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hesperiidae</td>
<td>Lai-Fook 1982a,b</td>
</tr>
<tr>
<td></td>
<td>Lyonetiidae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Noctuidae</td>
<td>Holt &amp; North 1970; Riemann &amp; Gassner 1973;</td>
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<td>Pair et al. 1977; Etman &amp; Hooper 1979a,b;</td>
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<td></td>
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<td>Benito-Espinal &amp; Laugé 1980;</td>
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<td>Henneberry &amp; Clayton 1984;</td>
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<td>LaChance 1984</td>
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<td></td>
<td>Papilionidae</td>
<td>Shepherd 1974a</td>
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<td>Plutellidae</td>
<td>Thibout 1977, 1981</td>
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<td>Pyralidae</td>
<td>Riemann &amp; Thorson 1971, 1976;</td>
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<td>Leviatan &amp; Friedländer 1979;</td>
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<td>Thorson &amp; Riemann 1982;</td>
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<td>Friedländer 1983</td>
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<td>Saturniidae</td>
<td>Shepherd 1974a, b, 1975;</td>
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<td>Sphingidae</td>
<td>Riemann &amp; Gassner 1973;</td>
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<td></td>
<td>Tortricidae</td>
<td>Ferro &amp; Akre 1975; Friedländer &amp; Benz 1981,</td>
</tr>
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<td>1982</td>
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Table 10 Timetable of eupyrene and apyrene spermatocyte meiosis in different lepidopteran species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Meiotic Divisions</th>
<th>Reference</th>
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<tr>
<td><em>Actias selene</em></td>
<td>early L5-? pupa-?</td>
<td>Friedländer et al. 1981</td>
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<td><em>Bombyx mori</em></td>
<td>early L5- only in pupa</td>
<td>Sado 1963</td>
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<td><em>Calpodes ethlius</em></td>
<td>early L4- adult</td>
<td>Lai-Fook 1982a</td>
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<td><em>Cydia pomonella</em></td>
<td>early L5- late L5- adult</td>
<td>Friedländer &amp; Benz 1981</td>
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<td><em>Ectomyelois ceratoniae</em></td>
<td>late L4- early pupa</td>
<td>Leviatan &amp; Friedländer 1979</td>
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<td><em>Ephestia kühniella</em></td>
<td>only in larva a</td>
<td>Riemann &amp; Thorson 1971</td>
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</table>

^ a not specified in which larval stage(s).
Fig. 1 Male reproductive system of *P. brassicae*. The accessory glands can be divided into two secretory sections: a proximal (p) and a distal (d). The ductus ejaculatorius simplex is divided into five secretory sections, numbered one through five (for further details see results 1.1.).
Fig. 2  Female reproductive system of *P. brassicae* (for further details of the bursa and the receptaculum seminis see Figs. 3 and 5, respectively).
Fig. 3  The bursa copulatrix shortly after copulation.
Fig. 4 The spermatophore of *P. brassicae* about two hours after copulation. The cap around the orifice has disintegrated and the imprint of the signa is clearly visible in the wall of the corpus.
Fig. 5  The spermathecal complex of *P. brassicae* (for detailed descriptions of the different parts see results 1.2.).
Fig. 6 Timetable of eupyrene and apyrene spermatogenesis and its relationship to the different stages of development.

*apyrene spermatids with micronuclei distributed along the length of the flagella*
Fig. 7 The activity of eupyrene and apyrene spermatozoa in male and female *Pieris brassicae*.
Fig. 8 Diagrammatic representation of the activity of apyrene spermatozoa from a fresh spermatophore in 10% glycerol E + B saline solution. Two kinds of activity patterns can be differentiated: circular rotation in middle of drop around eupyrene sperm bundles, and directional swimming to periphery. Six hours later (below) eupyrene bundles are disrupted and the free immobile spermatozoa transported towards the periphery of the drop.
CURRICULUM VITAE

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