Transfer, subsequent movement, and fate of sperm in the tobacco hornworm moth, *Manduca sexta*

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Abstract. During mating in the Lepidoptera (moths and butterflies), sperm are passed to the female via a copulation in which the male transfers a large and often complex spermatophore over the major part of an hour or more. Subsequently, over the course of an hour or often considerably more, the sperm exit the spermatophore and travel over a relatively complex route to the spermatheca, where the sperm are stored and then used as the eggs are laid. The process of spermatophore formation and migration of sperm in the female has been described in many Lepidoptera, but the mechanics involved have received less attention. Understanding these is important in discerning the relative roles of males and females in determining the outcome of matings. We describe how the spermatophore is formed, how the sperm migrate in the female, and the fate of the sperm in the spermatheca of the tobacco hornworm moth, *Manduca sexta*. We found that sperm movement from the spermatophore relied upon motility of the sperm, but further movement of the sperm to the spermatheca was dependent on female muscular action. After arriving in the spermatheca, the anucleate parasperm (apyrene sperm) separated into the lateral pouch of the spermatheca (lagena) and disappeared over 7 days, whereas the eusperm (eupyrene sperm) persisted in the central lumen of the spermatheca (utriculus). The relative persistence of these two sperm types could shed some light on what determines the proclivity of females to remate. Elucidation of these physiological mechanisms contributes to an understanding of the mechanisms of female choice and male competition in Lepidoptera.

Key words. Apyrene, Lepidoptera, *Manduca*, parasperm, sperm, sperm competition, spermatheca, spermatophore.

Introduction
Butterflies and moths (Lepidoptera) have some unusual reproductive traits that distinguish them from most other insects. First, females of all but the most phylogenetically basal lepidopteran families have two genital openings, one for copulation and the other for oviposition. Second, with the exception of the most phylogenetically basal genus (Sonnenschein & Häuser, 1990; Hamon & Chauvin, 1992), all Lepidoptera produce two types of sperm: one is the eusperm (or eupyrene sperm), a conventional insect sperm, but the other is the parasperm (or apyrene sperm). Parasperm lack nuclei and are usually smaller but are typically produced and transferred to the female in much larger amounts than are the eusperm (Swallow & Wilkinson, 2002). These features have apparently arisen monophyletically within the Lepidoptera (Friedländer, 1983; Friedländer et al., 2005; Mitter et al., 2017; Kawahara et al., 2019), and suggest Lepidoptera may have different physiological and behavioural reproductive mechanisms, especially relating to sexual conflict. Thus, the proximate mechanisms governing sperm translocation to and retention within the spermatheca (the final sperm storage organ in females) are of interest, especially the extent to which those mechanisms involve female versus male control.

Mating in Lepidoptera is initiated by a series of behavioural interactions between the male and female, followed by a
coupling event during which sperm are transferred from the male to the female in a spermatophore. Formation of the spermatophore typically involves the transfer of a considerable volume of accessory gland secretions from the male that form the capsule for the sperm and a copious seminal fluid (Meslin et al., 2018). Accomplishing this takes the major part of an hour or often more in most Lepidoptera. The male inserts his aedeagus (penis, intromittent organ) via the copulatory opening and deposits the spermatophore in the copulatory bursa (Fig. 1). The sperm must then undergo a relatively complicated migration from the copulatory pouch to the vestibulum of the oviduct, and then into a sperm storage organ, the spermatheca, where the sperm are held and then provided to the eggs as they are laid.

These processes of sperm transfer to and migration in the female have been described in a variety of Lepidoptera since the late 19th century, but there has been relatively little experimental analysis of the physiological mechanisms that effect these events and whether these processes are under male versus female control. Experimental studies of the role of active rather than passive female processes in sperm migration have shown that inhibition of muscular action causes at least partial inhibition of movement of sperm to the spermatheca (Stockel, 1973; Thibout, 1977; Tschudi-Rein & Benz, 1990; LaMunyon & Eisner, 1993). However, experimental studies of the role of sperm motility are lacking. In this study, we describe the processes of spermatophore formation, sperm migration, and storage in the adult female tobacco hornworm Manduca sexta. Then, we report the results of physiological experiments testing the relative roles of sperm motility and female muscular action, and therefore male and female control, in the migration of the sperm to the spermatheca.

Materials and methods

Experimental animals

M. sexta L. (tobacco hornworm: Sphingidae) came from a laboratory colony occasionally supplemented with animals from Carolina Biological Supply Co. Caterpillars were raised on an artificial diet (Bell & Joachim, 1976); all stages were kept at 22–26 °C on a 17 h light : 7 h dark cycle. Matings took place in 0.43 m³ cages under the same temperature and light conditions. As we found that more matings occurred at higher humidities, a
humidifier was operated during mating hours to keep the relative humidity above 60%. Moths were usually not fed, but were fully functional for 4 days or more. In the experiments counting spermatophore sperm 7 days after mating, females were fed to repletion on sugar solutions at least every other day. In some cases, tobacco plants were provided for oviposition.

Observation of spermatophore formation and sperm migration

**Coupling.** Mating was initiated when stationary female *M. sexta* began to release pheromone several hours after lights-off (Sasaki & Riddiford, 1984). When a male found a female, he engaged her posterior end with his terminal abdominal claspers, alighted beside her, and then turned to face away from her. He then inserted his aedeagus into her copulatory opening, which is ventral and anterior to the ovipore, to begin the process of spermatophore transfer to the female.

**Spermatophore formation.** We observed the process of spermatophore transfer in 10 pairs of moths by opening isolated abdomens of mating females coupled with males soon after initiation of mating; in two cases, the male’s head and thorax were also removed. The process of spermatophore formation in these preparations was then observed with a dissecting microscope. Manipulating pairs did not manifestly change their behaviour during copulation, and the duration of spermatophore transfer in these isolated abdomens was similar to that in intact animals.

**Sperm migration in the female.** Subsequent to uncoupling, we followed sperm migration in females by keeping 74 females caged as above and then dissecting them at intervals after uncoupling. As in many Lepidoptera (but not all), the spermatheca in *M. sexta* is divided into a principal lumen, the utriculus, the utriculus, connected to a long, tubular gland, and a lateral, blind-ended pouch, the lagena (Fig. 1). Of these 74 females, we made 30 qualitative assessments of sperm migration and motility in all parts of the female reproductive tract (spermatophore bulb, spermatophore stalk, seminal duct, vestibulum, and spermatheca) from 1.25 to 24 h after mating. Then, over a period of 9.5 h–7 days after mating, we counted sperm in both the utriculi and lagenae of another 44 females by dispersion and suspension of individualized sperm in physiological saline mixed with a drop of Tween-20 (a non-ionic detergent, Sigma Aldrich). The 44 females were divided into two groups for analysis: those dissected <24 h after termination of mating (*n* = 19) and those dissected ≥24 h after mating (*n* = 25). Three 1-μL samples were counted using dark-field optics in a compound microscope and then multiplied by the dilution factor. We could easily distinguish eusperm and parasperm because eusperm are twice as long as and conspicuously thicker and stiffer than parasperm, manifested by an obviously much longer arc of curvature, even if immotile.

**Sperm movement from isolated spermatophores.**

Thirty-four spermatophores were removed from female copulatory bursae (Fig. 1) 0–2 h after the end of copulation and incubated without saline on parafilm in an enclosed dish humidified with isotonic or slightly hypertonic saline. In 13 of these spermatophores, the abundant yellow secretion in which the beads or inactive sperm. Neutral red dye was used as a tracer in the females. Spermathecal of the moths were dissected and examined 20h later for the presence of the beads or inactive sperm.

**Statistical analysis.** Data were analysed in Program R, Version 3.4.2 (R Core Team, 2017). We used generalized linear models and package lme4 to examine changes in number of the two types of sperm in the lagena and utriculus of the spermatheca with time after termination of mating, using mean number of each sperm type as the response variable and time after mating as the explanatory variable (fixed effect). We specified the Poisson
distribution for the sperm counts (Crawley, 2017, p. 515). Figures were generated in ggplot2 (Wickham, 2009). Means are presented ± SE of the mean. Graphs of sperm counts used a jittered value so that overlapping points would be visible. Experimental results on translocation of sperm in females were analysed using the Fisher exact test.

**Results**

**Formation of the spermatophore**

After coupling, a male *M. sexta* generated and deposited a large spermatophore in the female’s copulatory bursa (average duration 3.61 ± 1.12 h, n = 16). Spermatophores were about 2.7% of the mass of a male (average spermatophore weight = 48 mg); about 30% of the spermatophore was spermatophore capsule and seminal fluid, the other 70% being a copious yellow secretion external to the capsule, originating from the posterior half of the male’s common duct (ductus ejaculatorius simplex). Over the first 2 h, the male ejaculated this yellow secretion; then a bulg of translucent grey secretion from the anterior half of the male’s common duct appeared at the base of the bursa and was inflated by sperm and secretions from the male’s seminal vesicles and accessory glands. Finally, in the last 10 min before separation of the pair, the bulb was rapidly pushed up into the anterior end of bursa, becoming surrounded by the yellow secretion (Fig. 1). This action revealed a long stalk attached to the base of the bulb that was formed in the male’s ejaculatory duct. The relatively hard bulg (final weight of bulb plus stalk: 14.2 ± 1.2 mg, n = 11) now sat embedded in the yellow secretion (33.5 ± 2.6 mg, n = 9), which at first was loose, but which partially hardened to a cheese-like consistency within a few hours after copulation ended. The bulg consists of two translucent layers, a thick outside one and a thinner inner one. Within the bulg, the parasperm were vigorously motile, whereas the eusperm were still conjoined in bundles of 256 and were not motile. The stalk, a hard, hollow tube filled with accessory gland secretion, extended posteriorly such that its end sat just anterior to the opening to the seminal duct (Fig. 1). Seminal vesicles of 11 *M. sexta* dissected within 1 h after mating contained only a few (0–685) remaining parasperm and no eusperm. In one additional case, there were several thousand parasperm and 10 eusperm bundles. Thus, at least in this species and under our environmental conditions, virtually the whole sperm complement from the seminal vesicles was transferred to the female at mating.

**Sperm migration in the female**

Parasperm were always motile in the spermatophore; individual eusperm only became slowly motile a few hours after dissociation of the bundles. Spermatophore stalks dissected 15–30 min after the pair separated were completely devoid of sperm, but they subsequently began to fill with highly motile parasperm. Then, over the next 17 h, most of the sperm exited the spermatophore, migrating via the seminal duct to the common oviduct, and then up the spermathecal duct into the spermatheca, where they remained pending fertilization and deposition of the eggs (Fig. 1). Initially, the sperm in the bursa gradually coagulated (a process that can be replicated in vitro: Shepherd & Bonk, 2021) with the accessory gland secretions and highly motile parasperm forming a dense mass at the base of the spermatophore at its junction with the stalk. After about 14 h, a clear solution of about 2–3 µL remained in the upper part of the spermatophore.

At about 3 h after mating ended, parasperm began traversing the seminal duct, and first entered the spermatheca at 4 h after mating ended. Their migration continued until most of the parasperm had reached the spermatheca about 15–17 h after the end of copulation (Fig. 2). Meanwhile, the eusperm bundles in the bursa dissociated over several hours into individual eusperm; these acquired a slow motility that appears to be enough to propel them through the spermatophore stalk and into the female ducts. However, these processes were sufficiently slow that the eusperm did not appear in the seminal duct until 11 h after the end of copulation. But they then rapidly accumulated in the spermatheca over the next 6 h, and became vigorously motile. Between 4 and 9.5 h, the parasperm had been slowly accumulating in the utriculus (not quantified). But simultaneously with the first appearance of the eusperm in the spermatheca at 11 h after mating, a new wave of parasperm began to accumulate in much larger numbers in the lagena, along with some eusperm. In three of eight females dissected at 13–20 h after mating (the time of maximum sperm migration), one or two eggs were present in the seminal duct, possibly blocking the duct. But like the other five females without such blockages, they already had large numbers of sperm in their spermathecae; apparently the eggs did not significantly inhibit sperm migration.

**Fate of sperm in the spermatheca**

Over 24–168 h after mating, the eusperm numbers decreased but they persisted in the utriculus (Fig. 3; GLZ: −0.003 ± 0.001, z = −8.1, n = 25, P < 0.001); they decreased exponentially to zero in the lagena (−0.030 ± 0.001, z = −20.2, n = 25, P < 0.001). Parasperm, initially equivalent to eusperm in abundance in the utriculus though massively exceeding them in the lagena, showed very strong declines in both the utriculus (−0.038 ± 0.001, z = −47.2, n = 25, P < 0.001) and the lagena (−0.067 ± 0.001, z = −58.5, n = 25, P < 0.001). This reflects a gradual separation of the eusperm into the utriculus and the parasperm into the lagena, but with a virtually complete disappearance of parasperm from both parts of the spermatheca within 7 days after their arrival (Fig. 3).
Transfer and fate of sperm in *Manduca sexta*

**Mechanics of sperm translocation in the female**

*Movement of sperm out of the spermatophore.* Fresh spermatophores dissected from the copulatory bursae of recently mated females (*n* = 34) exuded moderate to large masses of active parasperm in all but two cases. Eusperm also were extruded, though these were only occasionally motile, and of the 13 cases where the bulbs were incubated free of the yellow secretion, six showed few or no eusperm extruded. Because these preparations were obviously free of any muscular action by the female, the sperm exited the spermatophore due to their own motility, although some osmotic or chemical reaction within the spermatophore remains a possibility. No large amounts of accessory gland secretions were extruded.

*Role of sperm motility in evacuation of the spermatophore.* In 10 of 11 fresh spermatophores injected *in vivo* with spermicidal acetic acid or nonoxynol-9 within 1 h after termination of mating, sperm were found only in the spermatophore and not in the seminal ducts or spermathecae 20 h later. The acetic acid inhibited motility immediately and so sperm were found only in the bulb of the spermatophore. The nonoxynol-9 required 1–2 h to take effect, so 20 h later parasperm were found in the bulb and stalk of the spermatophore, but no further in the female reproductive tract. Eusperm were concentrated only in the bulb.

Fig. 2. Mean numbers of both types of *Manduca sexta* sperm in the utriculus (a, b) and the lagena (c, d) from 8.5 to 23.5 h after the pairs finished mating. Points were jittered to add a small amount of noise so that overlying points could be seen. Note that values vary on the *y*-axes.

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Fig. 3. Mean numbers of both types of *Manduca sexta* sperm in the utriculus (a,b) and the lagena (c,d) from 24 to 168 h after the pairs finished mating. Points were jittered (Program R) to add a small amount of noise so that overlying points could be seen. Note that graph d has a y-axis twice that of graphs a–c.

These sperm were immotile but appeared to have normal morphology. In one case, treated with nonoxynol, a few parasperm but no eusperm were found in the spermatheca; presumably the nonoxynol did not take effect before some of the parasperm had left the spermatophore. Injection of 3 μL saline into the spermatophore (*n* = 5) did not prevent normal migration of sperm to the spermatheca; nor did injection of the same volume of both spermicidal agents into the hemocoel near the bursa (each *n* = 5). The spermicidal treatments markedly reduced the migration of sperm out of the spermatophore compared with the combined saline-in-spermatophore and spermicide-in-hemocoel controls (Fisher exact test, *P* < 0.001).

Role of female muscular action in sperm migration through the seminal duct to the spermatheca. At the end of 20 h of anoxic anaesthesia by immersion in water, 12 mated females still had very active parasperm and somewhat active eusperm in their spermatophore bulbs but no sperm in their spermathecae. In two cases, there were small masses of parasperm in their seminal ducts. Another 10 females treated similarly but allowed to recover in air for an additional 20–24 h had large masses of sperm of both types in their spermathecae, with a variable number left in the spermatophore (anoxic treatment vs. re-oxygenated controls: Fisher exact test, *P* = 0.001). Evidently the sperm and the female were still normally functional. Similar results were obtained when eight mated females were held in a pure nitrogen atmosphere for 17 h: at the end of treatment, sperm were present only in the spermatophore. Four mated females treated the same way but then allowed to recover for 20–24 h had large numbers of sperm in their spermathecae (pre- and
post-recovery: Fisher exact test, $P = 0.002$). Muscle paralysis set in within 10 min in nitrogen but more slowly (up to 1 h) in water; paralysis was evidenced by weak or no visible heart-beat and complete lack of response to any disturbance (touch, jostling). When females recovered in air, they became responsive and active again though they often appeared incapable of flight. Sperm motility was unaffected by anoxia.

Transport of nonliving material to the spermatheca by female muscular action. Sephadex© beads ($n = 5$) or nonliving sperm ($n = 7$) injected into the bursal duct of freshly mated females were at least partially transported to the spermatheca within 20 h. Living sperm issuing from the spermatophore were blocked by the ligature below the spermatophore stalk. Because the injected material was labelled with neutral red dye and located immediately after injection only in the lowermost bursal duct, the injected material was labelled with neutral red dye and located immediately after injection only in the lowermost bursal duct and not the seminal duct, it appears that the pressure of injection was not the cause of translocation. As a control for any effects on muscular action of the agents used to kill sperm, Sephadex© beads were injected along with glacial acetic acid or nonoxynol-9 ($n = 5$ for each); this did not reduce translocation of the beads to the spermatheca.

Discussion

Evacuation of the sperm from the spermatophore

The processes of sperm transfer and migration in the Lepidoptera have been ably reviewed by Drummond (1984) with updates for Pieris brassicae by Tschudi-Rein & Benz (1990), Zygaena trifolii by Fänger & Naumann (1993), Pseudoletia separata by He et al. (1995), Spodoptera litura by Seth et al. (2002), two species of Choristoneura by Marcotte et al. (2003), and a general review for butterflies by Watanabe (2016).

Our observations on evacuation of the sperm from the spermatophore add a few details that may be more general for other Lepidoptera. The coagulation of parasperm and accessory gland secretions and subsequent very high parasperm motility that we observed at the base of the spermatophore soon after its deposition in the female, probably helps to channel the parasperm towards their path down the spermatophore stalk. The sperm then pass freely through thesemisolid but porous secretions in the core of the stalk, to emerge at the opening at the distal end of the stalk free of any large amount of accessory gland secretion.

In several lepidopteran species, escape of the sperm from the spermatophore has been attributed to its mechanical rupture, such as crushing the spermatophore, breaking the spermatophore stalk, or grating the spermatophore bulb with a toothed, heavily chitinized area (‘lamina dentata’ or ‘signum’) in the bursal wall that exists in most Lepidoptera, including M. sexta (Weidner, 1934; Srivastava & Srivastava, 1957; Watanabe, 2016). But the spermatophore stalk in at least most species of Lepidoptera studied, including M. sexta, has a preformed opening at its tip, and this seems a more parsimonious way to effect escape of the sperm. However, the possible function(s) of the chitinous structures remain contentious, and would profit from some more detailed anatomical examinations of how sperm escape from spermatophores (Sánchez et al., 2011; Watanabe, 2016; Meslin et al., 2018).

In some Lepidoptera, a firm mating plug is placed at the copulatory opening that blocks remating at least for a while (Dickinson & Rutowski, 1989), and would allow time for migration of the sperm into the seminal duct. In M. sexta, there is a loose portion of accessory gland secretion at the end of the fully-formed spermatophore stalk, but this seems unlikely to act as a significant block to remating.

Migration of sperm to the spermatheca

Our experiments show that active female muscular action must take over to convey the sperm through the seminal duct to the spermatheca in M. sexta. Some lepidopteran species have a conspicuous and usually muscular diverticulum (e.g. Choristoneura fumiferana: Outram, 1971) or expansion of the seminal duct that has been termed the bulla seminalis. It has been suggested that this may act as a pump, but there has not been any experimental validation of that hypothesis; this structure is lacking in many Lepidoptera including M. sexta. In Plodia interpunctella, Lum (1982) found that eggs lodged in the seminal duct blocked sperm migration. Though such eggs were not uncommon in our observations in M. sexta, we believe migrating sperm were not significantly blocked.

Sperm must then traverse the common oviduct to the spermathecal duct. Our observations only rarely showed any significant amount of sperm in the oviduct, suggesting that their transit is rapid and/or efficient. In many species of Lepidoptera, this region of the oviduct, termed the vestibulum, is somewhat expanded where the seminal duct and spermathecal duct open, these usually being close to each other; in some species there is a distinct channel between the two openings in the wall of the vestibulum (Miskimen et al., 1983). These features probably facilitate efficient transfer of sperm without dispersion up or down the common oviduct.

All reports on sperm migration in female Lepidoptera, including this report, indicate that parasperm reach the spermatheca in considerable numbers. Given the high motility of the parasperm immediately after deposition in the female in contrast to that of the eusperm, the parasperm might be expected to reach the spermatheca before the eusperm. That is indeed the case in M. sexta and has also been seen in other species of Lepidoptera (Tschudi-Rein & Benz, 1990; Watanabe et al., 2000; Marcotte et al., 2003). This may not be true in all lepidopteran species, though in the two noctuid species where the accumulation of the two sperm types has been reported to be contemporaneous, the migration (completed in just a few hours) is so rapid that any difference in accumulation might not have been detected (He et al., 1995; Yan et al., 2013, cf. Seth et al., 2002).

In M. sexta, our counts show that parasperm begin to enter the spermatheca just 4 h after the end of mating and continue until a massive second wave of many more parasperm arrive as the first eusperm arrive, starting at 11 h after the end of copulation (Fig. 2). Nearly all the parasperm in this second wave enter the lagena, which was largely vacant up to this time, whereas
the eusperm largely enter the utriculus (Fig. 1). The utriculus terminates in a long secretory gland at its distal end that is universally present in Lepidoptera, and is probably important in maintaining eusperm viability (see discussion below).

Ever since the first descriptions of the somewhat complicated journey of sperm inside the reproductive tract of female Lepidoptera, these reports (Michael, 1923; Weidner, 1934; Omura, 1938; Benz, 1977) have often been accompanied by suggestions of chemotaxis by sperm to the spermatheca, but this has never been verified. Using *Bombyx mori*, Weidner (1934) claimed chemotaxis based on simple choice experiments in which sperm accumulated in a capillary tube or a droplet containing a spermathecal gland, in preference to one without. This result could be attributable simply to a stimulation of motility, though this in itself is an interesting result. Subsequently, Behrenz (1952) reported that in a mated female *Lymantria dispar* whose spermathecal duct had been extirpated, sperm still reached the vestibulum, implying no chemotaxis to that point. Tschudi-Rein & Benz (1990) extirpated the spermathecal gland in young female *P. brassicae* before mating and found that after mating, sperm migration to the spermatheca occurred normally, though sperm motility, survival, and fertility were reduced. In a long migration path that is almost one-dimensional, there seems little need for a chemotactic stimulus to direct the sperm to the spermatheca. But one location where sperm might lose their way is when they cross the vestibulum. In spite of the openings of the seminal duct and the spermathecal duct being close together (Norris, 1932; Hewer, 1934), they apparently do not touch. Tschudi-Rein & Benz (1990) and Suzuki et al. (1996) describe sperm moving in a compact mass directly across the lumen of the vestibulum and Omura (1938) shows a photo of the same. One might assume that sperm move independently, but the relative dominance of viscous or viscoelastic forces at the microscopic dimensions of sperm means sperm can derive an energetic advantage by moving together in synchrony (Taylor, 1951; Tung et al., 2017). Bunching of the sperm probably increases the efficiency of their transport across the vestibulum, and perhaps some specific muscular action of the vestibulum enhances this efficiency.

**Role of sperm motility in sperm migration**

Because spermatophores that we isolated were obviously free of any muscular action by the female and inactivated sperm do not exit the spermatophore bulb, it seems most likely that the sperm exited the spermatophore due to their own motility. The very vigorous motility of the parasperm seen in the spermatophore of this species has been documented in many other reports in other species of Lepidoptera. As a result, it has often been suggested that the parasperm effect the translocation of the eusperm, whose motility is often seen as lacking or weak (Holt & North, 1970; Thibout, 1972; Etman & Hooper, 1979). But observations in this study and in several other species of Lepidoptera mention at least slow motility of eusperm in the spermatophore (Kinefuchi, 1977; Nabi & Harrison, 1983; Osanai et al., 1987; Tschudi-Rein & Benz, 1990; Konegaya et al., 2016). Given the relatively solid but porous matrix of male accessory gland secretions in the spermatophore stalk, this level of motility appears to be enough to propel the eusperm effectively. In other arthropod species, osmotic or chemical events in the spermatophore play a role in evacuation of the sperm (Khalifa, 1949; Feldman-Muhsam, 1967; Linley, 1981). But there is no direct evidence of this in Lepidoptera and it seems unlikely in *M. sexta*, particularly as the sperm leave the spermatophore unaccompanied by almost all of the copious accessory gland secretions present in the freshly-formed spermatophore bulb and stalk.

Our experiments show that subsequent translocation of sperm to the spermatheca requires female muscular action, though an accessory role of motility cannot be ruled out, particularly in crossing the vestibulum and in the ascent of sperm up the spermathecal duct to the spermathecal reservoirs.

**Role of female muscular action in sperm migration**

All histological studies to date in Lepidoptera have shown muscular layers around the bursa (especially at its base near the stalk), a thick layer around the seminal duct, and investments around the common oviduct and spermathecal duct leading to the spermatheca, the last having various thicknesses of muscle (Hewer, 1934; Weidner, 1934; Musgrave, 1937; Omura, 1938; Miskimen et al., 1983). Many reports describe contractions of the bursa wall after spermatophore transfer and we observed the same for 1–2 h after the end of copulation in *M. sexta*. However, these reports are often not clear about whether these contractions also preceded mating or not, or whether the act of dissection in saline might have triggered them. In any case, irrespective of mating, several other organs, for example, the nerve cord and hindgut, in the abdomen of adult Lepidoptera normally contract regularly, probably as a means to promote haemolymph circulation and perhaps air circulation in the tracheal system. It has often been suggested that the bursal contractions may play a role in evacuating the spermatophore of other lepidopteran species, and may indeed do so in those species that have soft spermatophores (Norris, 1932; Hewer, 1934; Weidner, 1934; Thibout, 1977). But the experiments reported above in *M. sexta*, which has a relatively incompressible spermatophore, show that contractions are unlikely to be important. Other investigators have had similar doubts for particular lepidopteran species that have hard spermatophores (Ferro & Akre, 1975; Proshold et al., 1975; Miskimen et al., 1983).

Our experiments show that muscular contractions are essential for movement of the sperm through the seminal duct. Exposing mated female *M. sexta* to anoxic conditions immediately after mating stops sperm migration at the base of the bursal duct by the opening to the seminal duct. As long as anoxia was not too prolonged (<17–20 h), the resumption of aerobic conditions allowed the resumption and completion of sperm migration. Similar experiments using anoxia or parathion to paralyse muscles in four other species of Lepidoptera have shown partial disruption of transfer to the spermatheca (Stockel, 1973; Thibout, 1977; Tschudi-Rein & Benz, 1990; LaMunyon & Eisner, 1993).
**Fate of sperm in the spermatheca**

At 24 h, the total number of both types of sperm in the spermathecae of female *M. sexta* approaches the total number transferred by the male (Shepherd & Bonk, 2021). In some other Lepidoptera, for example, the swallowtail butterflies *Papilio xuthus* and *Byasa alcinous*, only a small fraction of sperm transferred reach the spermatheca (Watanabe et al., 2000; Konegaya et al., 2016). Over the next 7 days in *M. sexta*, the eusperm remain intact without markedly diminishing in number (Fig. 3). By contrast, the parasperm disappear almost completely, especially from the lagena where the majority were stored. A similar trajectory of the parasperm has been reported in several other lepidopteran species (Holt & North, 1970; Riemann & Gassner III, 1973; Katsumo, 1977). However, in many other lepidopteran species, the spermatheca has only one reservoir (no lagena) and/or patterns of accumulation in the spermatheca vary considerably (Outram, 1971; Etman & Hooper, 1979; Tschudi-Rein & Benz, 1990; Watanabe & Hachisuka, 2005; Konegaya et al., 2016). In *P. xuthus* where parasperm do not disappear quickly, Watanabe & Hachisuka (2005) made the interesting observation that if oviposition is prevented, many more parasperm survive longer (up to 7 days) than if females oviposit normally. All of these observations rule out any generalizable hypotheses that parasperm always disappear or that the lagena is a site of parasperm degradation. However, it is generally agreed that even if they do not disappear, the parasperm become inactive and play no direct role in fertilization of the eggs.

In the basal half of the spermathecal duct leading to the vestibulum, Hewer (1934), Weidner (1934) and Omura (1938) apparently independently discovered that the duct bifurcates, forming a very narrow, pronounced, heavily chitinized channel in the wall of the main duct that spirals around the duct until it reaches the vestibulum. This has been called the ‘fertilization canal’ and several investigators assert that in various lepidopteran species, sperm do indeed travel in this groove during fertilization of the eggs prior to oviposition (Weidner, 1934; Omura, 1938; Callahan & Casio, 1963; Miskimen et al., 1983; Tschudi-Rein & Benz, 1990). The latter two reports also note that during oviposition in *Diaatraea saccharalis* and *P. brassicae*, a peristaltic contraction beginning in the utriculus pushes sperm into and down the spermathecal duct. Then, an occlusion of the main duct where the fertilization canal begins forces eusperm into the fertilization canal. Lum et al. (1981) have reported a detailed analysis of the opposite use of the main duct and fertilization canal in *P. interpunctella*, arguing that the conventional view of their use would not support the rapid oviposition rate in this species. Ultrastructural analysis of the spermathecal duct in females fixed during oviposition might resolve some of these issues about the mechanics of how lepidopteran eggs are fertilized. The fertilization canal also exists in *M. sexta* (unpublished histology), though we have not observed its function.

Another striking phenomenon, documented in electron micrographs, is a remarkable change in the morphology of the eusperm in the spermatheca just prior to fertilization: they shuck a substantial extracellular sheath, reducing the eusperm behind the nucleus to a simple axoneme plus a mitochondrial derivative (Riemann & Thorson, 1971; Friedländer & Gitay, 1972). The latter authors suggest this may begin during passage of the sperm through the seminal duct, though the former authors imply this occurs in the spermatheca prior to fertilization of the eggs. Further studies are needed to determine the site of this phenomenon and elucidation of its mechanism.

**Function of parasperm in the spermatheca**

The migration of the parasperm to the spermatheca in all species suggests one or more important functions for them in the spermatheca, and their subsequent demise or inactivation suggest that those functions have been executed. Three of the obvious changes of behaviour and physiology shown by mated female Lepidoptera are (i) a cessation of receptivity (e.g. by cessation of pheromone emission and/or onset of refractory behaviour), (ii) initiation or enhancement of egg maturation, and (iii) onset of oviposition behaviour.

It would seem adaptive for cessation of receptivity to begin as soon as possible after copulation and that suggests that the location for that would be the bursa, where the sperm first enter the female. That seems indeed to be the case in *M. sexta*: Riddiford and co-workers have shown via castration and other experiments that the presence of sperm (type not determined) and/or testicular secretions in the bursa results in a lasting cessation of calling (Sasaki & Riddiford, 1984; Stringer et al., 1985). In two other species, cessation of receptivity is induced or initiated simply by mechanical stimulation: by mechanical inflation of the female’s bursa by the spermatophore in *Pieris rapae* (Obara et al., 1975; Sugawara, 1979) and by contact of the male’s genitalia with the female’s genital tract in *L. dispar* (Giebultowicz et al., 1991). Measuring both pheromone content and calling behaviour, Giebultowicz et al. found an immediate and dramatic reduction of pheromone content in mated female *L. dispar* due to genital contact. However, sustained reduction of pheromone content and inhibition of remating necessitated the presence of the spermatheca, presumably containing sperm.

In many species of Lepidoptera, females mate with multiple males, though typically mated females are refractory to remating for at least several days before resuming receptivity (Wedell, 2005; Watanabe, 2016). Although there is apparently no information about mating frequency in wild populations of *M. sexta*, laboratory and semi-natural experiments indicate that females will become sexually receptive and remate, albeit with a long refractory period extending most of the normal lifetime of the female (Allen, 1955; Sasaki & Riddiford, 1984; Woods Jr. & Stevenson, 1996; Levin et al., 2016; personal observations). Even in females that mate relatively quickly after a prior mating, resumption of receptivity usually occurs well after the sperm have migrated from the spermatophore to the spermatheca, so if sperm have a role in the resumption of receptivity, it would seem likely to depend on sperm load in the spermatheca. Many reports have correlated sperm load in the spermatheca with receptivity (the latter usually measured by remating), but where studied, this has been attributed at least in part to reduced numbers of eusperm (Thibout, 1979; Proshold, 1995; Marcotte et al., 2006; Thorburn et al., 2018), or to fewer parasperm (He et al., 1995; Wedell, 2001; Lewis et al., 2013). Although decreasing numbers
of eusperm might seem to be the relevant factor in restoring receptivity, and parasperm appear to become inactive or actually disappear from the spermatheca, parasperm disappear slowly enough in many species to play a defining role in renewed receptivity (Cook & Wedell, 1999; Konegaya et al., 2016). Due to some ambiguities in some of the experiments cited above, we cannot infer whether it is the eusperm or parasperm that are the deciding factor in the resumption of receptivity, nor if there is indeed any pattern common to most Lepidoptera, leaving the matter in need of further study.

Many studies have shown that in most lepidopteran females, at least some eggs are mature and ready for fertilization and oviposition before mating. Although some species even eclose with a full complement of eggs (e.g. many saturniid moth species), mating markedly enhances the rate of egg maturation and/or oviposition in most species. By cutting the seminal duct of newly mated female M. sexta, Sasaki & Riddiford (1984) showed that sperm must progress beyond this point for normal maturation and oviposition of eggs. Though female B. mori eclose with a full complement of mature eggs, cutting the seminal duct reduces the number of eggs laid by mated females in this species as well (Karube & Kobayashi, 1999). One might suppose then that the locus of a stimulus to oviposition would be the spermatheca, but careful surgical experiments by the latter authors using B. mori located the critical stimulus to the vestibulum, the part of the common oviduct that the sperm pass through either going to or coming from the spermatheca. Earlier experiments by Sugai & Sugita (1976) using triploid male B. mori, which are deficient in eusperm but have a normal parasperm complement, found that females mated with such males had spermathecae filled with only parasperm but laid eggs only at the low rate of virgin females. Thus, Karube & Kobayashi (1999) conclude that it is eusperm in the vestibulum that are responsible for triggering rapid oviposition in B. mori. Other reports assert that it is eusperm in the spermatheca that trigger oviposition, but fail to either account for parasperm, make careful sperm counts, or experimentally analyse possible alternatives (Thibout, 1979; Marcotte et al., 2006). Thus, the functional role of parasperm in the spermatheca, if they have one, remains elusive.

Although the studies cited above point to sperm as causative agents in changing female behaviour after mating, we note that there is abundant evidence that male seminal fluid proteins or other molecules have direct roles in changing female behaviour in some species of insects, especially among the Diptera (reviewed by Avila et al., 2011). Male Lepidoptera transfer a large variety and amount of secretions along with the sperm, and likely have such effector molecules, but evidence for these is still sparse in this order.

Conclusions

Although not universal, it is often the case among insects, and most other animals that copulate, that spermatozoa are not deposited by the male directly into the female’s spermatheca or equivalent organ. In M. sexta, our observations and experiments support the idea that both sperm motility and female muscle action play essential roles in effecting the migration of the sperm to the spermatheca. We would surmise that sperm motility initially sorts out some nonfunctional sperm and that female muscular action provides an opportunity for female choice. The possession of a nonfertilizing class of sperm in the Lepidoptera, especially in such large preponderance over sperm capable of fertilization, is unusual but not unique in the animal kingdom. There is evidence that parasperm serve a role in sperm competition (Cook & Wedell, 1999) but also probably other more physiological roles, though these remain to be clarified (reviewed in Shepherd & Bonk, 2021).

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Author contributions

NLH for most of the experiments reported in the section on ‘Mechanics of sperm translocation in the female’, JLD for the data in ‘Fate of sperm in the spermatheca’, and the statistical analysis, and JGS for the remaining experiments. JLD and JGS wrote the paper.

Data availability statement

Data for Figures 2 and 3 are available at https://datadryad.org/stash/share/W48H7PvryXnFwRVNTciAHGeek5mnsCx4NoMht8UmRBw.

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