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ORIGINAL ARTICLE

The reproductive tract of the black soldier fly (*Hermetia illucens*) is highly differentiated and suggests adaptations to sexual selection

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Abstract

The reproductive biology of the black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), was investigated in males and females with a focus on how sperm is handled by males before – and by females after – copulation. As this insect has great economic importance for protein production from agri-food wastes, its reproduction needs to be precisely known. A detailed description of male and female reproductive organs was made, and sperm were counted in male and female storage organs. Measures of testis and sperm counts were performed in males of various ages and their dynamics was compared to the ovaries of females. Both male and female tracts were long, and consisted of various successive parts. In males, testes fill the vas deferens with mature sperm, that are tangled as 'windrows', and sperm pass successively through a 'studded pipe' and three phallic 'wands' before being transferred to females. In virgin males, mean (\pm SEM) sperm number increased from approximately 9700 (\pm 1400, $n=5$) to 39600 (\pm 3470, $n=13$) in 20 days, and testes gradually decreased in size, indicating continuous spermatogenesis. Sperm cells are long, more than 3 mm. During copulation, sperm are deposited by phallic 'wands' at the base of each spermatheca in a smooth tube followed by a 'fishnet canal' at the top of which they accumulate before complete storage. They then pass through a ringed canal, an elbow, and a rigid rod strewn with glands, before reaching the spermathecal reservoir. Females store approximately 680 spermatozoa after one mating in each spermathecal reservoir. Such complex sperm storage organs of females may indicate strong female post-mating selection, and reflect competition for long and numerous sperm in this species, in which female multiple matings are suspected. Besides its economical interest, BSF is an appealing model for further investigations on sexual selection.

KEYWORDS

black soldier fly, BSF, cryptic female choice, Diptera, ejaculate, *Hermetia illucens*, reproductive behaviour, sperm competition, sperm length, spermatheca, Stratiomyidae, vas deferens

INTRODUCTION

Black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), is a promising insect for conversion of agri-food wastes to high-quality proteins for feed. It is produced worldwide to transform larvae into very efficient protein foodstuff for fishes and in the near future for poultry and

pigs (Tomberlin & van Huis, 2020). To improve the management of mass cultures of this insect, a better knowledge of its biology is needed, especially of factors that can influence fertility and reproductive output, as this may directly improve yield and mass production.

Sperm management in males and females is a key factor in the success of reproduction (Parker, 1970). In insects, sperm

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are stored by females in the spermatheca. Spermathecae play a central role in reproductive processes, as they are considered as the main place for the control of paternity by females (Eberhard, 1996; Pascini & Martins, 2017). This part of the reproductive apparatus must be described with precision to understand post-copulatory sexual selection.

Sexual selection is a powerful source of selection that can lead to adaptations of reproductive tracts and gametes. Paternity biases after multiple mating of females with internal fertilization could be due to two non-exclusive mechanisms, namely cryptic female choice where females can manage bias paternity in favour of particular males, and sperm competition between spermatozoa of different males inside the female tract (Snook, 2005; Firman et al., 2017). Insects have been used extensively as models to test the occurrence of those two selective driving forces. It was proposed that sperm competition can select for various adaptations, for instance more numerous sperm when sperm competition is a fair raffle, or larger sperm to displace those of rivals, or even faster sperm to outcompete sperm of other males in a race to reach ova (Wigby & Chapman, 2004). Under this selection, metabolic resources are invested in sperm numbers and quality and testes are bigger (Lüpold et al., 2020). In the course of aging, the reproductive abilities of individuals may vary (Promislow et al., 2022), giving rise to more (Bressac et al., 2009) or less (Papanastasiou et al., 2011) fertile males. Male reproduction should therefore be investigated at different ages. Moreover, female cryptic choice is associated with differentiated female tracts, with diversified sperm storage organs. This allows them to select males after mating by their abilities to produce sperm of high quality (Higginson et al., 2012; Firman et al., 2017). Describing the reproductive organs and understanding reproductive physiology of both males and females can shed light on the potential for those postcopulatory mechanisms.

The reproductive behaviour of BSF is reviewed in Kortsmit et al. (2022) and Lemke et al. (2023). It begins with courtship by males which, if successful, is followed by copulation lasting about 30 min (Giunti et al., 2018). Both females and males can mate 2–3 days after emergence (Sheppard et al., 2002), illustrating a delayed sexual maturity. As yet, no multiple mating was directly observed in males or females, but multiple paternities were detected in iso-female lines (Hoffmann et al., 2021), suggesting polyandry in females.

Some traits of female and male tracts, and sperm ultrastructure, were described by Malawey et al. (2019). The male tract is well differentiated (Malawey et al., 2019), indicating that BSF males invest significantly in the production of their gametes. The female tract is complex, constituted of various parts, suggesting an evolution under selective constraints such as cryptic female choice after female multiple mating (Snook, 2005). Liu et al. (2020) published a clear picture of the female tract showing three spermathecae with their empty or filled reservoirs. Although having three sperm reservoirs is frequent in insects, those three spermathecae of BSF seem to have a common structure, unlike dimorphic spermathecae

which are typically present in species having more than one sperm storage organ (Pascini & Martins, 2017). Nevertheless, several features of the reproductive anatomy remain unknown, such as male sperm potential, sperm counts in both male and females, precise structures containing sperm in both sexes, and the mechanisms allowing sperm to be transferred from the male to the female apparatus.

In this experimental study, male and female genital tracts were described by optical microscopic observations. A method was developed to count sperm in males of various ages (virgin and mated) and in females after one mating. Spermatozoa of mature males were also measured. This information will be useful to evaluate the investment of both sexes in reproduction in the context of sexual selection as well as for control of reproduction for better mass production.

MATERIALS AND METHODS

Black soldier flies were reared under controlled conditions. The laboratory strain was provided by Innovafeed (Paris, France) in 2019 ('Texas' origin) and maintained under laboratory conditions since. Adults were hosted in 35 × 35 cm cages at 24 °C and were provided with a cotton ball saturated with water to maintain moisture and a sugar lump for feeding. The mass population consisted of about 500 individuals divided into two cages. Light–dark conditions were L12:D12 (light from 08:00 to 20:00 hours) with Philips TLD 36W-84 fluorescent tubes at 50 cm from the cages, providing 2000–6000 lux light. Females were provided with corrugated cardboard strips (10 × 2 cm) to lay their eggs. After collection, eggs and larvae were maintained in a climatic chamber at 27 °C in the dark, the developing substrate was the Gainesville diet (Tomberlin et al., 2002), and no additional moisture was added during development. Pupae were collected and maintained at 24 °C with sawdust until emergence. Emerging flies were collected and sexed daily for experiments.

To conform to published results, we used 3-day-old males and females for sperm transfer experiments (Tomberlin & Sheppard, 2002). In addition, males of different ages were investigated up to 20 days.

Female and male reproductive tract descriptions

Tracts and organs of virgin females and males were isolated by dissection after decapitation in tap water with fine forceps. Pictures were taken under a stereomicroscope with a Leica IC 80 HD camera (Wetzlar, Germany). Five female tracts were measured. Parts of the tract were isolated for microscopic observations and gently uncoiled. They were transferred to a glass slide in water, a drop of DAPI (4',6-diamidino-2-phenylindole) at 2 μg mL⁻¹ was added if needed, covered with a coverslip, and immediately observed. Light and phase contrast microscope pictures were taken under an Olympus BX51 microscope

(Tokyo, Japan) with a QImaging Micropublisher MP6 camera (Photometrics, Tucson, AZ, USA). Fluorescence pictures were taken with a CX40 Olympus microscope under UV excitation (495–521 nm filter) and a Moticom pro 205B camera (Micro Mécanique, Saint-Germain-en-Laye, France). Moreover, females from the mass culture were dissected at a young age (0–3 days), older (3–5 days), and after mating and egg laying (5 days and more) to observe the ovaries and glands under stereomicroscope. Between three and five females were considered in each category.

Sperm length measurements

Once the male's tract was observed and photographed, one of the vasa deferentia was gently opened with forceps and shaken onto the surface of the slide in PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride; pH 7.4) to separate some spermatozoa without breaking them. No fixation was made. The sperm apical end is well visible after DAPI labelling because it contains the nucleus (at this scale, acrosome could be neglected), but the distal end is not easy to reveal. We consider that a spermatozoon was unbroken when it showed only one continuous thread, and a thinned distal end (Dallai, 2014). Pictures showing multiple threads were discarded because they could result from broken membranes, releasing the axonem and the pair of mitochondrial derivatives separately. We measured only sperm that were clearly visible all along their whole length without any doubt, also excluding uncertain loops and crosses. In the end, only six reliable sperm measurements were obtained from four 3-day-old virgin males (some males had two spermatozoa which could be measured, others one). Sperm nuclei length ($n=12$, three nuclei per male) were measured from the same preparations after DAPI staining under a fluorescence microscope with a 40 \times objective.

Muscle labelling

Muscle labelling was done with Phalloidin linked to FITC (Bachem, Bubendorf, Switzerland; ref 4095643). Virgin male and female organs were isolated in PBS, then fixed in paraformaldehyde 4% for 15 min, rinsed in PBS, and incubated in FITC-Phalloidin at 50 $\mu\text{g mL}^{-1}$ for 30 min. After the last bath in tap water, organs were mounted in Immu-Mount (Eprelia, Portsmouth, UK), covered with a coverslip, and observed with an Olympus BX51 fluorescence microscope at 495–521 nm under a 20 \times objective. Organs from only one male and one female were photographed but observations were made on $n=5$ individuals of each sex.

Testis size and sperm counts in males

To investigate the dynamics of spermatogenesis, the width of one testis of virgin males of 0–8 days was measured,

giving a sample of 1–5 males for each day except days 6 and 7, which were not investigated (total $n=20$). Virgin males ($n=40$) from 0–20 days were investigated for sperm counts; males were sexed at emergence and maintained in groups of 5–10 in 15 \times 15 cm cages with a water-saturated cotton ball and a sugar lump at 24 °C under the same controlled conditions as adult flies (see above). Preliminary trials in BSF males showed that spermatozoa cannot be separated, as is the case in the vast majority of animals. In BSF, they are firmly intertwined and it is impossible to spread them as a monolayered smear for a classical cell count without breaking them. In order to count them, we uncoiled one of the vasa deferentia in PBS with fine forceps, and measured its whole length under a stereomicroscope. The preparation was then labelled with DAPI to reveal sperm nuclei, which have a typical filiform shape, and to count them in a section of the vas deferens under a fluorescence microscope (20 \times objective). The length of this section was also measured, and the ratio between the sperm counted-section and the whole vas deferens was calculated. Then, the number of sperm counted within the portion was multiplied by this ratio to estimate the entire content of the sperm in the vas deferens. This was then doubled for the total number of sperm of one male. We verified that sperm were evenly distributed along the vas deferens: this was checked by counting sperm at four positions along the vas deferens in five randomly drawn microscope slides among the 40. In 10 males of different ages, two vasa deferentia were investigated to check that the male tract was symmetrical. A correlation was made between male age and sperm count in vas deferens. The head width of each male was also measured as a proxy of body size (Jones & Tomberlin, 2021) to check the relationship between the male size and sperm production.

Sperm counts in females

Ten virgin females and 10 virgin males, both aged 3 days, were placed in a 15 \times 15 \times 15 cm cage under the same controlled conditions as adult flies, and continuously observed. After initial attachment for copulation was observed, the pairs were isolated until separation. Then, a sample of five females were dissected either after 2 and 30 min, to check the filling of spermathecal reservoirs; sperm were not counted, only the place of sperm masses in the tract was noted. Other mated females ($n=15$) were isolated for 1 day with sugar and water to ensure that the process of sperm storage was completed. Those once-mated females were then dissected and their spermathecal reservoirs were isolated on a microscope glass slide in a drop of PBS. Then, a drop of DAPI was added and a coverslip was pressed to squash the content of spermathecal reservoirs. As in males, sperm stay tangled and the ball-shape of the reservoir remains visible. DAPI fluorescence allowed to count sperm heads from each of the three reservoirs without mixing them. However, the initial place of each reservoir – either

left, central, or right – could not be assigned because the dissection needs to uncoil the whole spermatheca, and it results in a mixing of different sides of the tractus.

Data acquisition and statistical analysis

Measures were made with ImageJ v.1.53 (National Institutes of Health, Bethesda, MD, USA) after picture acquisition either under a stereomicroscope or a fluorescence microscope. Sperm counts / testis width of males depending on age and sperm counts / male head were tested as Spearman rank correlations after checking for

their non-normality by Shapiro-Wilks tests. Sperm numbers contained in the three reservoirs of females were tested by an ANOVA after checking the normality of the distribution. Statistical analyses were performed with R v.4.0.3 or R Studio v.1.3.1093, and graphs were produced with the package ggplot2.

RESULTS

Parts of the male and female reproductive tracts (Figures 1 and 2) that were not previously described in the literature are named by us; these are in quotes.

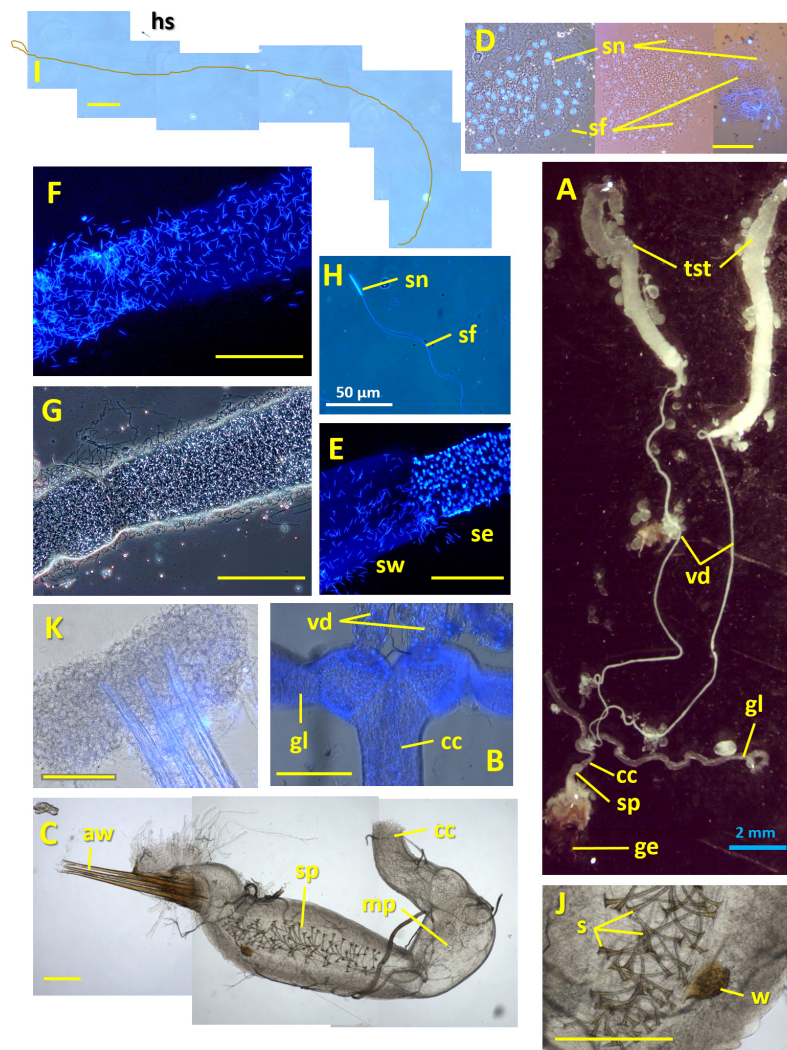
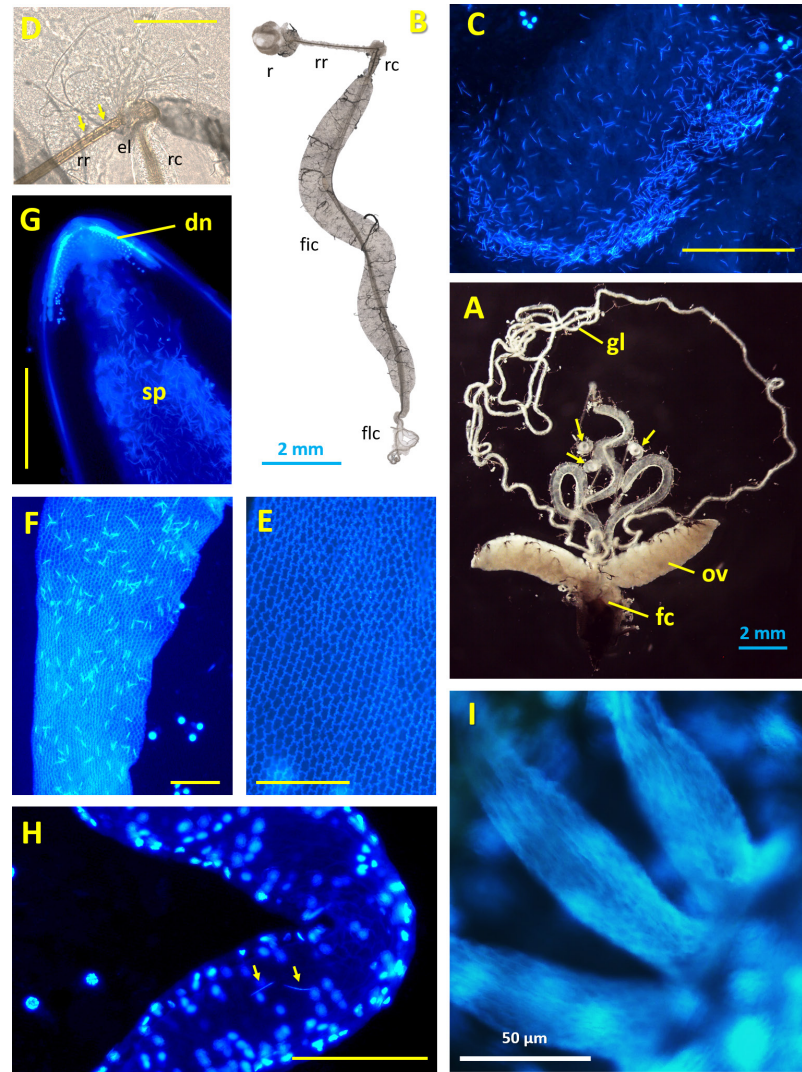


FIGURE 1 Black soldier fly male reproductive tract. (A) Whole tract uncoiled; testes (tst), vasa deferentia (vd), glands (gl, cut off for the picture), common canal (cc), sperm pump (sp), genitalia (ge). (B) Junction between vasa deferentia (vd), glands (gl) and common canal (cc). (C) Whole view of the sperm pump under phase contrast, common canal (cc), muscular pump (mp), 'studded pipe' (sp), three aedeagal 'wands' (aw). (D) Spermatid bundles from the testis showing DAPI stained nuclei (sn) in blue and growing flagella (sf). (E) Part of one vas deferens labelled with DAPI; single layered epithelium (se) and sperm window (sw). (F) Part of one vas deferens showing sperm window without surrounding epithelium labelled with DAPI; only sperm nuclei are visible. (G) Same as (F) under phase contrast showing sperm flagella. (H) Apical part of one sperm labelled with DAPI; sperm nucleus (sn) and sperm flagellum (sf). (I) One uncoiled spermatozoon made of seven successive pictures captured at 10x magnification; whole spermatozoon was redrawn in yellow, the minute line represents a human sperm (hs) at the same scale. (J) Part of the 'studded pipe' under phase contrast showing studs (s) and the sole 'wart' (w). (K) Sperm escaping from aedeagal 'wands' under phase contrast. Scale bar is 200 μm except in (A) and (H).

FIGURE 2 Black soldier fly female reproductive tract. (A) Whole tract; arrows indicate sperm reservoirs; ovaries (ov), glands (gl), fertilization chamber (fc). (B) One isolated spermathecal reservoir (r), rigid rod (rr), ringed canal (rc), fishnet canal (fic), flexible canal (flc). (C) DAPI staining of one sperm mass released from the spermathecal reservoir; only nuclei are visible; note that the ovoid shape of the reservoir is preserved. (D) Rigid rod (rr) showing holes (arrows), elbow (el), and ringed canal (rc). (E) Fishnet canal empty. (F) Fishnet canal with spermatozoa labelled with DAPI. (G) Upper part of the fishnet canal with sperm (sp) and a layer of dense nuclei (dn), the ringed canal is disconnected. (H) Flexible canal labelled with DAPI, showing epithelial cells nuclei (round) and sperm nuclei (filiform, arrows). (I) Connection between spermathecae and fertilization chamber; the wall is thicker near the base, suggesting muscles. Scale bar is 200 μm except in (A), (B), and (I).



Male tract description

The male tract is composed, from testis to copulatory apparatus, of one pair of testes, each prolonged by one vas deferens joining at the base of glands (Figure 1A). The tract is a single tube, composed of a common canal, a muscular pump, a 'studded pipe', and three aedeagal (or phallic) 'wands' (Figure 1B, C).

The testes are tubular shaped, connected with many tracheas and fat globules. They are full of immature sperm at different stages, from their apex to their base. Germinal cells are recognizable by the number of nuclei in cysts, the shape of nuclei, and the growth of flagella. The apex of the testis contains spermatocytes, the middle part elongating spermatids, and the base free spermatozoa (Figure 1D).

Vasa deferentia are undifferentiated tubular reservoirs, surrounded by a single-layered epithelium. They contain free spermatozoa tangled as 'windrows', keeping their tubular shape when the epithelium is peeled (Figure 1E). Sperm nuclei are not concentrated or oriented in any particular organization, but distributed all along the vas deferens

(Figure 1F), and flagella are tangled without any apparent stratification (Figure 1G). Spermatozoa have a filiform shape (Figure 1H, I). Their full length is 3.60 ± 0.27 mm (mean \pm SD; $n=6$) and the length of the nuclei is 16.2 ± 1.04 μm ($n=12$). Sperm do not separate and do not exhibit any obvious motility, neither in water nor in saline solution (PBS). However, some motility of parts of flagella was occasionally visible in PBS, as propagating waves.

The base of the tract is a single tube beginning with an undifferentiated canal layered by a thick wall along its main length (Figure 1B). A muscular part precedes a kind of labyrinth made of appendage-like spicules, clearly chitinized and fixed in the inner side of the wall (Figure 1J). We called this part the 'studded pipe'. A 'wart' is also visible near the base of this actually uncommon organ. The wall of this canal is highly muscular with only circular surrounding fibres (Figure 3A). Among experimental males, no spermatozoa were observed in this part of the male tract, after the vas deferens. The aedeagus – or phallic complex – linked with muscles to the exoskeleton of the male, is composed of three individualized 'wands' transferring spermatozoa from the male to the female (Figure 1K).

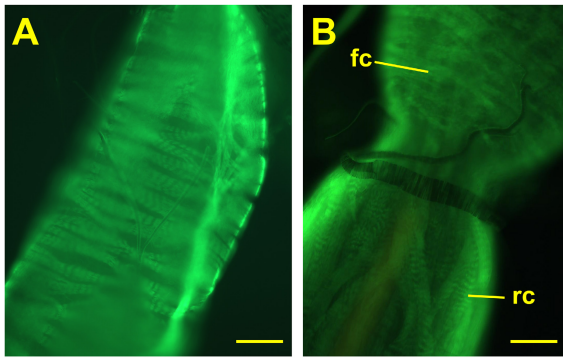


FIGURE 3 Part of the male and female black soldier fly reproductive tracts labelled with phalloidin-FITC to reveal actin F filaments of muscles. (A) Male 'studded pipe' showing circular muscles. (B) Female external part of the fishnet canal (fc) showing circular muscles, and ringed canal (rc) showing longitudinal muscles. Scale bar is 200 μm .

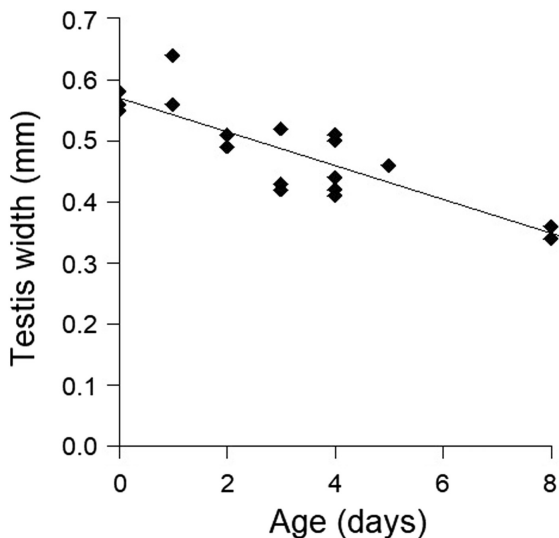


FIGURE 4 Testis width (mm) of virgin black soldier fly adult males according to their age ($n=20$). Each point represents one individual male, day 0 is the day of emergence. The line is based on linear regression (Spearman $\rho=0.87$, $P<0.001$).

Within the abdomen of the male, before dissection, testes are elongated in lateral positions, and both glands and vasa deferentia are tangled in the center of the abdomen. These elongated organs are never bound nor knotted but are kept coiled by numerous tracheas, which makes them tricky to disentangle.

Testis size and sperm counts in males

The width of the testis decreases over time (Figure 4), and this decrease is linear during 8 days in virgin males. Sperm are present in vasa deferentia of males immediately after emergence (Figure 5). Sperm counts steadily increase in virgin males, at least until 20 days. The minimum sperm

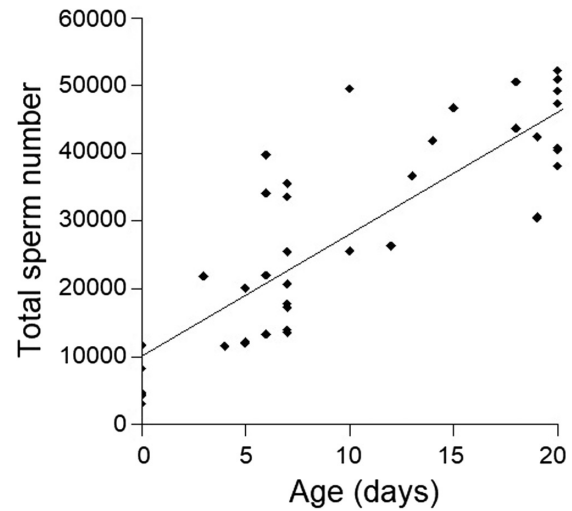


FIGURE 5 Sperm number in vasa deferentia of virgin black soldier fly adult males according to their age ($n=40$). Each point represents one individual male. The line is based on linear regression.

count is 3125 for a 0-day-old male, and the maximum is 52183 for a 20-day-old male. The number of sperm in vasa deferentia is highly correlated with age (Spearman $\rho=0.85$, $P<0.001$), but not related to head width (Pearson $r=0.27$, $P=0.12$). The rate of sperm production is 1741 sperm per day, which is the slope of the regression line ($n=40$).

The female tract

The female tract is composed of two ovaries, two glands, and three spermathecae. All of these organs are connected at the base of the tract, close to the beginning of the ovipositor (Figure 2A). The glands are 38.2 ± 1.72 mm long (mean \pm SD), they are filled with a white liquid, and come out at the very end of the tract, lower than spermathecal ducts. Their shape and content apparently do not change, neither during the ageing of females nor between maturation of the ovaries.

The spermathecae are three similar and complex tubular structures (Figure 2B), each ending with an ovoid reservoir (0.50 ± 0.04 mm long, 0.37 ± 0.04 mm wide) containing spermatozoa (Figure 2C). The reservoir wall is translucent and shows no visible cuticle. Sperm appear immobile through the wall of the reservoir. When removed, sperm are still tangled in a ball, with their nuclei more or less orientated to the outside part of the ball (Figure 2C). As in males, a weak motility can be observed. The outside of the spermathecal capsule is covered with tracheas, giving the impression of being streaked. Just under the reservoir is a 'rigid rod' (mean \pm SD = 2.15 ± 0.10 mm), coloured and pierced with small holes (Figure 2D). It ends with an 'elbow', followed by a 'ringed canal' (0.70 ± 0.05 mm), apparently flexible. This latter is connected with a canal adorned as a 'fishnet' (Figure 2E), itself included inside a wide and smooth tube (6.08 ± 0.27 mm long). This 'fishnet canal' was

full of sperm, 2 min after one copulation (Figure 2F), and the three canals appeared equally filled. Thirty min after copulation, sperm were concentrated at the top of the 'fishnet canal', just below the entrance of the flexible 'ringed canal' (Figure 2G), and look as pressed against a rim of dense cells. After 24 h, 'fishnet canals' were empty of sperm, and spermathecal reservoirs were full. Before the 'fishnet canal' is a 'flexible canal' (3.28 ± 0.42 mm), with no obvious peculiarities (Figure 2H). The three spermathecae do not join at any place, they are individually connected at the base of the female tract by a thin canal (Figure 2I); the end part has a thicker wall that seems to be muscular. Spermathecae bases do not reach the ovaries or the glands. All along the tract, muscles are visible; they are transversal along the 'fishnet canal', allowing circular contractions or peristalsis,

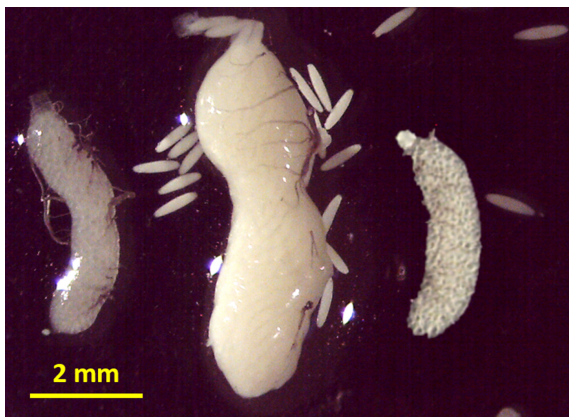


FIGURE 6 Ovaries of black soldier fly females. Left, soon emerged; center, 3-day-old ready to oviposit (some mature oocytes escaped); right, after egg-laying.

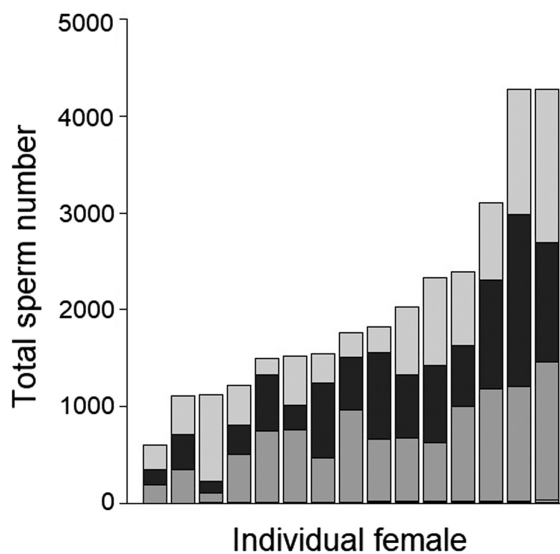


FIGURE 7 Sperm counted in the three spermathecal reservoirs of once-mated 3-day-old black soldier fly females with a 3 day-old virgin male. Each bar indicates an individual female ($n = 15$), and gray scale shows the three reservoirs. Females are ranked by sperm stock.

and longitudinal around the 'ringed canal', which could thus contract like an accordion (Figure 3B).

Ovaries (Figure 6) can have three shapes, depending on the stage of oocyte maturation. In young females (0–3 days), they are white with round cells, looking like a bunch of grapes. In older females (>3 days), oocytes are shaped like rice grains, and the whole ovary looks like a bunch of bananas, entirely white. In much older females, when eggs were laid, ovaries appear translucent and yellow, oocytes being replaced by apparently empty balls, the whole ovary looks like a sponge and no new oocytes are developing.

In the abdomen of the female, spermathecal reservoirs are close to the sternites, in ventral position. They are first visible when the cuticle is removed, with 'rigid rods' that are brown and easy to detect among white fat and organs. Note that they are not easy to collect because they are attached by many tracheas. Naturally, spermathecae are S shaped. A first half turn is the 'elbow', and a second is in the middle of the 'fishnet canal'.

Sperm counts in females

After one mating with a 3-day-old virgin male, all three spermathecal reservoirs contained sperm, and in most females in balanced proportions (ANOVA: $F_{2,42} = 0.13$, $P = 0.88$) (Figure 7). A female stored 2025 ± 280 (mean \pm SD; $n = 15$) sperm as a whole (i.e., the sum of the three counts), and 100–1772 in one individual reservoir. Minimum and maximum total sperm stocks were 601 and 4250, respectively; the median number was 1744.

DISCUSSION

In BSF, both female and male reproductive tracts are complex, showing successive organs that may play a role in sperm migration along with copulation and fertilization. Some parts are useful for taxonomy, as they serve as diagnostic for the description of species, such as the triphid aedigium (Rozkosny & Kozánek, 2005).

The male tract has only one way to travel for sperm, from spermatogenesis to ejaculate. The pushing mechanism could be ensured by sperm production itself, which progressively fills the vas deferens. For sperm transfer during copulation, the sperm pump at the male side is before the 'studded pipe'. It is not actually an ejaculatory bulb because sperm are not accumulated inside it before copulation. The 'studded pipe' may be the organ allowing the compact sperm windrow to be divided into three equal parts during sperm transfer to the female. Its high circular musculature may play a role in such sperm unravelling. Additionally, the anatomy of the sperm pump may be used as a phylogenetic diagnosis in insects (Hünefeldt & Beutel, 2005), but that of BSF bears no resemblance to the

sperm pump described in other Diptera, which suggests the action of specific evolutionary forces.

The female tract does not show any bursa copulatrix receiving male ejaculate as is described in many insects (Pascini & Martins, 2017). It can be proposed that sperm are deposited by males at the base of the spermathecae, directly by the triple aedigial 'wands', with each of the 'wands' entering the base of one spermatheca, and being maintained by ring muscles of the female during copulation. After transfer of sperm, a pump may be constituted by muscles surrounding the wall of the 'fishnet canal' and the ringed canal; however, no muscle surrounds the ending spermathecal reservoirs. After one mating, the three reservoirs contain spermatozoa. It was hypothesized in insects with complex spermathecae that a differential filling after mating could allow females to sort between males, and choose one over others for fertilization (Taylor et al., 2001; Pascini & Martins, 2017), but this was clearly not observed in BSF. We found no differential filling of the three spermathecae, this was also observed in other experiments on controlled mating in BSF (Liu et al., 2020).

We have not seen any mobility in sperm, i.e., progressive movements, only slight motility was visible in some parts of flagella, confirming ultrastructural observations showing that flagella are organised as being able to beat (Malawey et al., 2019). One hypothesis on sperm movement is that both studs of 'studded pipe' in males, and 'fishnet' structure in females, allow sperm self progression, as is supposed for giant sperm of *Drosophila bifurca* (Patterson & Wheeler), in the roller male organ (Joly et al., 2003). In BSF, those organs, where spermatozoa only transit, have a muscular wall. It suggests that sperm migration is due to a combination of both sperm movements, allowing sperm sneaking, and contractions of organs. After copulation, spermatozoa are visible in the flexible canal, and as a compact mass at the top of the fishnet canal. The mechanisms by which females control the number of spermatozoa inside the spermathecae are not clear. Sperm flagella could not be detected inside the female tract, unlike nuclei, which can be easily labelled with a vital dye such as DAPI. We can only imagine how sperm go up in the female tract after copulation and down for fertilization. In any case, the organization of muscles along the female tract, forming mobile walls and sphincters, are reliable characteristics to understand the management of spermatozoa during storage and fertilization (Pascini & Martins, 2017). During oviposition, all eggs are laid in a few minutes (Tomberlin & Sheppard, 2002), suggesting that sperm are released rapidly from the reservoirs of the spermathecae. In addition to migration, sperm detangling is also a mystery, as some upper parts of the female tract ('rigid rods') do not allow a passage of thick sperm balls as they are observed in the fishnet canal or the spermathecal reservoir. Spermatozoa have to be uncoiled and unraveled inside aedeagal 'wands' in males, and in females in the 'ringed canal', 'elbow', and 'rigid rod'. Moreover, in females, this mechanism occurs at least twice, when sperm are stored, and when they are released. Just before spermathecal reservoirs, 'rigid rods' have a cuticular structure which is pierced with holes. It may be an area of secretions of small

glands that are arranged along this 'rigid rod'. In BSF, no differentiated spermathecal gland was observed around the reservoir, as it is in many insects (Pascini & Martins, 2017), but those small glandular units scattered along the 'rigid rod' and 'elbow' may provide secretions. It would be similar to *Drosophila*, where small glands open directly into the spermathecae (Filosi & Perotti, 1975). Mechanisms of sperm movements in very thin and elongated parts of the male tract were investigated in Coleopteran species (Matsumura et al., 2019), suggesting that sperm motility and tract contractions could not be separated. The mechanisms of sperm transit in male and female deserves to be described, to understand the control of oocyte fertilization within the female tract. It was recently shown that some BSF females lay clutches of eggs with at least two paternities, evidencing polyandry (Hoffmann et al., 2021). Such female multiple mating has not been directly observed (Giunti et al., 2018), but has been demonstrated in laboratory experiments (C Bressac, unpubl.). No information is available about female mating rates in natural populations.

Observation on ovaries confirmed that oocytes are produced in synchrony and that females are not able to produce any more gametes after their single egg-laying event. In BSF, ovarian synchronicity was observed previously (Tomberlin et al., 2002), and this trait was maintained in our laboratory strain. Generally, the number of ovarioles is used to describe ovarian dynamics in insects (Church et al., 2021). This trait is not consistent in BSF because ovaries are constituted of only one compartment, and multiple ovarioles are not documented in this insect. The synchrony of ovaries is opposite to the continuous filling of the vasa deferentia by testes. However, spermatogenesis is not actually infinite because testis width decreased with age, evidencing progressive exhaustion of sperm in males following successive copulations. The increase of sperm in aging males, from ca. 10000 to 50000 in 20 days, is consistent with male multiple mating, i.e., polygyny, which is suspected in this male-lekking insect (Tomberlin & Sheppard, 2002; Giunti et al., 2018), but, as for females, not clearly demonstrated. Investigations on the mating abilities and competitiveness of older males would be of interest to show whether they, despite their greater sperm stock, are more apt to mate than younger ones, unlike in the medfly *Ceratitis capitata* (Wiedemann) (Papanastasiou et al., 2011).

Upon emergence, males have from ca. 3000–11000 sperm in their storage organs. Despite the presence of spermatozoa in the vasa deferentia, males do not mate before the 2nd day after emergence (Sheppard et al., 2002; Giunti et al., 2018). It suggests that the presence of mature sperm, in sufficient numbers for transfer, is not itself a stimulus for male mating behaviour, but it confirms the observations of Malawey et al. (2019) on the presence of free spermatozoa at emergence. However, it is not in the order of magnitude of some hundreds of sperm counted by automatic techniques in BSF males (Malawey et al., 2021). It shows that the sperm of many insects cannot be investigated by the same techniques as those developed in vertebrates. This amount in males is greater than the average of sperm stored in spermathecae

of females (i.e., 2000) and much more than what is needed for egg fertilization, i.e., 360–620 eggs laid (Tomberlin et al., 2002). In many insects, the efficiency of sperm stored in females is high and, with a ratio of four stored to one fertilizing sperm, BSF are no exception (Sivinsky, 1980; Bressac et al., 1994; Chevrier & Bressac, 2002). Considering sperm production, no relationship was shown with male size, contrary to age. This observation is consistent with controlled experiments (Jones & Tomberlin, 2021), showing that small males mate successfully with females, even when competing with bigger males, despite some disadvantages if the ratio between male and female sizes is too high. From an industrial perspective, the control of male sexual maturity is an interesting zootechnical trait. If the small males have a fertility equivalent to the large ones, they would be more interesting to raise because they would have consumed less nutritional resources (Jones & Tomberlin, 2021).

The whole sperm content varies between females with a seven-fold difference in sperm stored, despite controlled conditions of mating, and limited genetic diversity. It suggests a control of sperm transfer by females during copulation, the alternative being that males could adjust their sperm transfer to a perceived quality of the mate, therefore sperm stored may result from a choice of females between males, or males between females. The filiform shape of the male tract, with no ejaculatory bulb, is in favour of a continuous sperm transfer during copulation instead of a time-limited ejaculation. In that case, the duration of copulation may allow regulation of sperm transfer, as in some tephritid flies (Perez-Staples & Abraham, 2023). In BSF it is from 20 to 40 min (Giunti et al., 2018), indicating variability, but not in the same order of magnitude as sperm stocks in females. In the house fly, *Musca domestica* L., which is like BSF authorized as feed-stuff, smaller males induce a shorter period of non-reception of females, suggesting reduced sperm transfer compared to larger males (review in Kortsmmit et al., 2022). Further investigations are needed to assess which sex can control copulation duration in BSF and consequently sperm transferred, which could be a major determinant of sperm storage.

Spermatozoa are more than 6× longer than the average of animals performing internal fertilization (Kahrl et al., 2021). Moreover, sperm are produced in large amounts by BSF males, the reproductive tract is well developed, and counts of sperm are not reduced to <100 as in giant-sperm insects (Bressac et al., 1994). In general, sperm length is assigned to both sperm competition between rival males and cryptic female choice inside the female tract (Snook, 2005). For a male, the risk is the displacement of its sperm in favour of another. It is exemplified when the female storage organ has a fixed volume and is filled after only one mating (Pascini & Martins, 2017). It was proposed that sperm length was selected by the sperm storage organ of females, leading to co-evolution of these two structures, but only in species having an elongated sperm storage organ (Joly & Bressac, 1994; Higginson et al., 2012). In BSF, the reservoir has an ovoid shape, but the four successive canals reaching it are elongated. Our observations in BSF show that sperm of 3 mm are shorter than the 'fishnet canal'

(ca. 6 mm), but are comparable to fine structures connected to the reservoir, i.e., 'rigid rod' plus 'ringed canal' being ca. 3 mm. This peculiar set, fine and rich in glands, may be a candidate for sperm length selection. This hypothesis could be tested by comparisons with other close relatives, the genus *Hermetia* having almost 80 species (Rozkosny & Kozánek, 2005), with only one – BSF – being domesticated.

To conclude, BSF males produce long sperm in large quantities and females have a highly differentiated sperm storage organ. Further studies are needed to investigate the complete physiological context allowing reproduction in BSF males, including behaviour and mating potential (Kortsmmit et al., 2022). Actually, investigations on sperm precedence after female multiple mating are needed to demonstrate whether and how such uncommon reproductive anatomy allows female control of paternity, and thus genetic benefits for the next generation.

AUTHOR CONTRIBUTIONS

Paul Munsch-Masset: Conceptualization (equal); formal analysis (equal); investigation (lead); methodology (equal); validation (equal); visualization (equal). **Carole Labrousse:** Conceptualization (equal); data curation (equal); investigation (lead); methodology (equal); supervision (equal); validation (equal); visualization (equal). **Laureen Beaugeard:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal). **Christophe Bressac:** Conceptualization (lead); formal analysis (lead); investigation (equal); methodology (equal); project administration (lead); supervision (lead); validation (equal); visualization (equal); writing – original draft (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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