Sperm production and allocation respond to perceived risk of sperm competition in the black soldier fly *Hermetia illucens*

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Abstract

In polyandrous species, competition between males for reproduction goes on after copulation via the competition of their ejaculates for the fertilisation of female's oocytes, it is called sperm competition. Different models of sperm competition predict adaptative plasticity of males in the production and allocation of their spermatozoa. These predictions were tested in the black soldier fly (BSF) *Hermetia illucens*, a farmed insect whose biology is little known despite its economic interest for bioconversion and as an animal feed. Two manipulations were carried out to modify the risks of sperm competition perceived by the males. The first consisted of placing adult males in different social contexts (alone or in groups of 10) and then measuring their sperm production. The second took place at the beginning of the copulation; pairs were transferred to different contexts of risk of sperm competition (empty cages, cages containing 10 males or cages containing 10 females), then the spermathecae of the females were collected in order to count the number of spermatozoa allocated by the males. Males in groups of 10 showed more spermatozoa in their seminal vesicles than males alone. Regarding sperm allocation, spermathecae of females in groups of 10 males, as well as those in groups of 10 females, had more spermatozoa than those placed in empty cages. We discussed this last result as a possibility that BSF males are not able to recognize the sex of their conspecifics. Copulation duration was not affected by these treatments, but was affected by the pair age. These manipulations of sperm competition risk showed that sperm production and allocation are dependent on social context in BSF. Males respond to the risks of sperm competition by a greater investment in sperm production and transfer. The existence of these mechanisms and their effects on reproduction underline the importance of studying the biology of farmed insects, for which fertility is essential.

Keywords: Reproduction, Farming insect, Social context, Sexual selection, Copulation, *Spermatheca*

Introduction

The struggle for reproduction is an important selective pressure leading to many evolutionary adaptations which is particularly typified by the competition between males of polyandrous species (Andersson, 1994). Fifty years ago, Parker (1970) theorized that intrasexual competition between males could be expressed both before and after copulation, as it could continue within female reproductive organs, in the form of sperm competition - i.e. 'the competition within a single female between the sperm from two or more males over the fertilization of the ova'.

Many physiological (Pizzari & Parker, 2009; Godwin et al., 2017), morphological (Córdoba-Aguilar et al., 2003) and behavioural (Alcock, 1994; Cueva del Castillo, 2003; Barbosa, 2012) traits have been interpreted In light of this paradigm shift (Parker et al., 1998; Wigby and Chapman, 2004). For example, longer spermatozoa swimming faster, or mate-guarding strategies are selected by sperm competition as they maximize male's fertilization success in the competition (Alcock, 1994; Godwin et al., 2017). Among these traits, plasticity in sperm production (i.e. spermatogenesis), as well as the sperm allocated to particular copulation events have been the subject of many predictions (Parker, 1970; Parker et al., 1997; Parker & Pizzari, 2010). Based on the costs to males of spermatozoa and seminal fluid content (Dewsbury, 1982), theoretical models predict fitness benefits when males are able to assess the risks of sperm competition - i.e. the probability that the sperm of a male will compete with the sperm of other males for fertilization of a defined set of ova (Parker 1998) - and optimize their ejaculate size accordingly (Parker et al., 1997; Engqvist & Reinhold, 2005).

The predictions of sperm competition models have been successfully tested in many organisms, including rodents, fish, and many insects (delBarco-Trillo, 2011). For example, in Drosophila melanogaster, sperm production increases when males are housed with other males for a long period of time - mean risks of sperm competition - (Moatt et al., 2014). Moreover the arrival of rival males during copulation - immediate risks of sperm competition - , induces focal males to transfer more spermatozoa to the female (Garbaczewska et al., 2013).

The quantity of sperm produced or allocated is not the only component of copulation modified in the context of sperm competition. For instance, the duration of copulation is particularly studied as it can be considered as a proxy for the amount of sperm allocated (Bretman et al., 2009; Barbosa, 2011), although it is not always true (see Weggelaar et al., 2019). Regardless of the sperm allocation, copulation duration is also predicted to vary with sperm competition risks (Alcock, 1994). By copulating longer, males undertake mate guarding thus preventing the female from remating (Alcock, 1994), a widespread behavior in insects (Lorch et al., 1993; Cueva del Castillo, 2003; Barbosa, 2011).

In this study, we aimed to test the predictions of the sperm competition theory in the black soldier fly Hermetia illucens (BSF), a species of great interest for mass-rearing and organic waste bioconversion (Tomberlin & van Huis, 2020). Despite its economic interest, studies on adults BSF and their reproductive biology are scarce. Giunti et al., (2018) reported a high prevalence of same-sex sexual behaviors in adults BSF, which can be associated to a high degree of polygyny in other species (MacFarlane et al., 2010). Multiple matings have been reported (Permana et al., 2020; Hoffmann et al., 2021) and morphological traits including complex spermathecae, long and numerous spermatozoa, large testes (Munsch-Masset et al., in press) imply post-copulatory sexual selection pressures in this species. Here we experimentally manipulated the risks of sperm competition to examine the phenotypic plasticity in ejaculate expenditure. First, we tested whether long-term exposure to other males could affect sperm production (mean risks of sperm competition) in males' seminal vesicles. Secondly, we assessed if the sudden appearance or disappearance of rivals (immediate risks of sperm competition) coupled with different mean sperm competition treatments could affect the duration of copulation and sperm allocation in females spermathecae.

Materials and methods

Rearing conditions

The individuals used in this study were reared under controlled conditions. Adults were hosted in 50x50x50 cm cages at 24°C and were provided with a cotton ball saturated with water to maintain

moisture. They were exposed to a 12 hours day/night regimen with Philips TLD 36W-84 fluorescent tubes positioned at 10 cm from the cages and providing 2000 to 6000 lux. After collection, eggs and larvae were maintained at 27°C, the developing substrate was the Gainesville diet (Tomberlin & Sheppard, 2002), 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. Females were isolated in 15x15x15cm cages in groups of 20 females per cage, as for males, they were isolated differently depending on the treatment (see below).

Production of spermatozoa

A group of males was maintained under conditions supposed to simulate a low risk of sperm competition (n = 19). These individuals were single, placed in individual 120 mL plastic containers preventing any visual or physical contact with other males and limiting olfactory cues. The second treatment consisted in placing ten males in a 960 mL plastic container allowing physical, visual, and chemosensory contacts, to simulate a high risks of sperm competition (n = 24).

Allocation of spermatozoa

As BSF will not initiate copulations when a single pair is placed in a cage (Personnal observations), the first step for the experiment on the immediate risks of sperm competition involved transferring 20 virgin males from both treatments (10 single males and 10 grouped males) to 15 cm3 cages containing 20 virgin females. Individuals remained in contact for 5 hours and fourteen replicates were performed.

Once copulations began, each mating pair was gently placed on the lid of a petri dish and transferred in a cage of similar size containing either no individuals to simulate low immediate risks of sperm competition (n = 38), 10 males to simulate high immediate risks of sperm competition (n = 38), or 10 females to test the ability of males to recognise genuine competitors (n = 24). Time was recorted once copulations were completed to evaluate duration, and pairs were kept together in petri dishes within which there is not enough space for extra copulations to occur (see Munsch-Masset et al., in press), until dissection of the female reproductive tracts.

Dissections and collection of data

Since age can affect the number of spermatozoa in seminal vesicles (Munsch-Masset et al., in press), we dissected males of similar ages (n = 5 males of 5 days, n = 28 males of 6 days and n = 10 males of 8 days, and we controlled the age in the statistic models, see below). Dissections were performed under a stereomicroscope in PBS saline buffer using fine forceps. For all males, the abdomen was opened after decapitation to collect seminal vesicles which were then placed on a slide and gently uncoiled with fine forceps. The seminal vesicles were photographed and their whole length was measured with ImageJ. A drop of DAPI was then applied to the preparation to label the nuclei of the spermatozoa for counting in a section of one of the two seminal vesicles using a fluorescence microscope (x20 objective) as in Munsch-Masset et al., (in press). The length of this section was also measured to obtain the ratio between the sperm-counted-section and the whole seminal vesicles. Then, this ratio was multiplied to the number of sperm counted within the portion to obtain the total number of spermatozoa in the seminal vesicles. Finally, this was doubled for the total number of sperm of one male.

The dissection of females took place the day after copulation. The two individuals of a pair were photographed to measure the head width using ImageJ. This measure can be considered as a good proxy of the size of the individuals (Munsch-Masset et al., in press). For all individuals, the abdomen was opened to collect the three spermathecae which were then placed on a slide. Before crushing them with a microscope slide to release the spermatozoa, a drop of DAPI was applied to the spermatheca to mark the nucleus of the spermatozoa which were counted under a fluorescence microscope (x20 objective).

Statistical analyses

To test our hypotheses, linear mixed models (LMM) were used with the « lmer » function in the « lme4 » package in R (Bates et al., 2015). The response variable was either the number of spermatozoa in the seminal vesicles or the number of spermatozoa counted in the female's spermathecae. Regarding the spermatozoa in the seminal vesicles, mean risks of sperm competition treatment was included in the model with the head size, the size of the seminal vesicles and the age of the male. As for the number of

spermatozoa in the spermathecae, age, sizes of the male and the female, copulation duration, mean sperm competition risks treatment and immediate sperm competition risks treatment were included in the model. To study the copulation duration, we used cox proportional hazard model with the « coxph » function in the « survival package » in R (Therneau, 2019). In the same way, age, sizes of the male and the female and both sperm competition risks treatments were included as fixed effects in the model.

The day of sampling was included as a random effect to account for variability inherent to each series in the sperm count in the models. The fixed effects in our models were tested using the « lmerTest » package (Kuznetsova et al., 2017), with type III ANOVA F statistics using Satterthwaite approximations for the linear mix models and with type III ANOVA Chi statistics for the survival model. The assumptions of the linear mixed model, including normality of residuals, constant variance, and absence of multicollinearity among the independent variables were checked graphically. As heteroscedasticity in the models was deteted, a logarithmic transformation was applied on the response variables. We also assessed the proportional hazards assumption of the cox model using Schoenfeld residuals and found no significant violations of this assumption.

All statistical analyses were performed using R version 4.0.2 (R Core Team, 2020). The significance level was set at alpha = 0.05 for all tests. Quantitative data are presented as means ± standard errors (SE) and hazard ratios (HR) are reported for cox models.

Results

On average, 5 hours of contact at this population density – 20 females and 20 males - allowed 7.64 ± 0.87 mating to occur. Grouped males copulated significantly more than single males (Fisher's exact test: P = 0.01). In total n = 64 copulations from grouped males and n = 36 copulations from single males were observed.

Production of spermatozoa

The number of spermatozoa found in the seminal vesicles of the males was neither related to their size ($F_{1,37} = 0.56$; $P = 0.46$) nor with their age ($F_{2,37} = 0.34$; $P = 0.71$) nor with the length of their seminal vesicles ($F_{1,37} = 1.83$; $P = 0.18$). However, the two treatments of mean risks of sperm competition showed a significant effect on the number of spermatozoa in the seminal vesicles of males ($F_{1,37} = 7.75$; $P < 0.01$; full model $R^2 = 0.20$) (Fig.1). Males kept in groups had a mean 43 % increase in the number of spermatozoa (mean ± SE : 15578 ± 1105, n = 24) in their seminal vesicles compared to males raised alone (mean ± SE : 10920 ± 1200, n = 19).

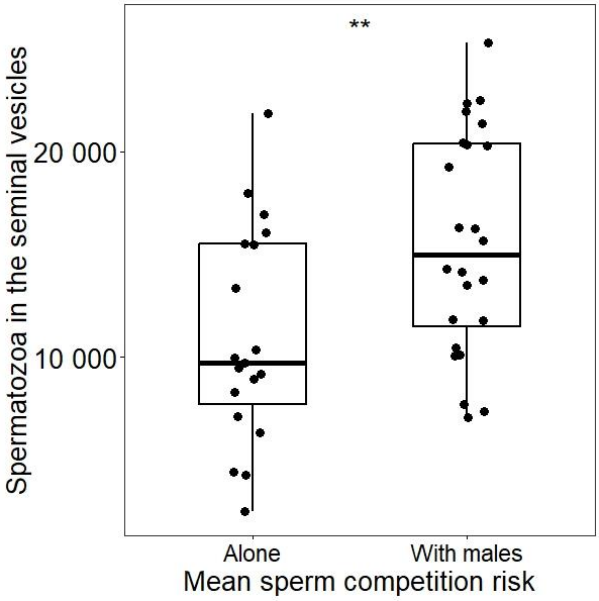

Figure 1 - The number of spermatozoa in the seminal vesicles of males according to the mean sperm competition treatment (either the male alone or the male within a group of 10 males) Box plots show median (horizontal bars), upper, and lower quartiles (borders of the box). Whiskers extend from the 10th to the 90th percentiles.

Allocation of spermatozoa

The number of spermatozoa found in the female's spermathecae was neither related to the size of the male ($F_{1,87.84}$ = 1.40 ; P = 0.24), nor with their age ($F_{1, 10.19}$ = 0.24; P = 0.86), nor to the copulation duration ($F_{1,83.10}$ = 0.75 ; P = 0.39) (Fig.2). However, the number of spermatozoa found in the female's spermathecae was related to immediate sperm competition risks treatment ($F_{2,84.07}$ = 8.49; P < 0.001; full model R^2 = 0.39) (Fig.3). There was no significative difference (t = -1.10, P = 0.27) between the content of spermathecae of females mated with males in the 10 males treatment (mean ± SE : 4943 ± 420, n = 38) and those in the 10 females treatment (mean ± SE : 6287 ± 376, n = 24). Females mated with males in groups of males or females had a mean 60 % increase in the number of spermatozoa (mean ± SE : 5464 ± 292, n =62) compared to the males mating alone (mean ± SE : 3406 ± 268, n = 38).

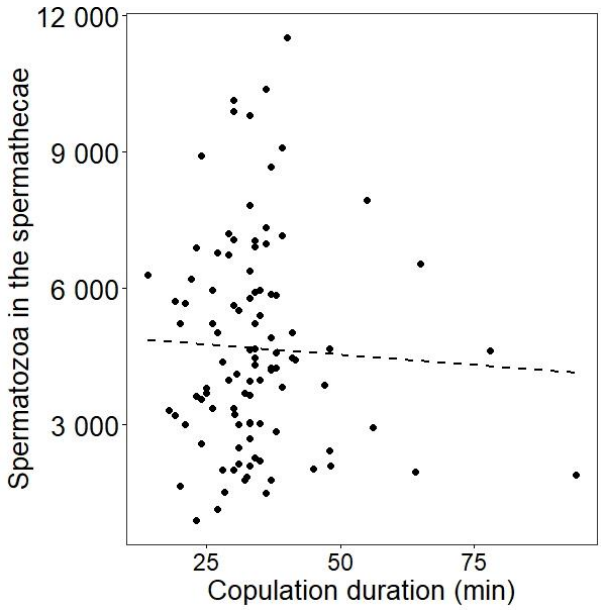

Figure 2 - The number of spermatozoa in the spermathecae of females according to the copulation duration. Each point is an individual female, n = 100. The dashed line represents a non significant relationship between these two variables, linear regression: R^2=0.002).

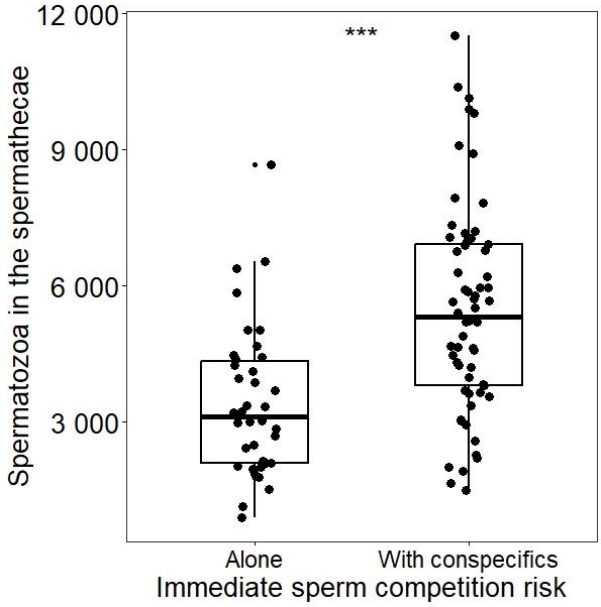

Figure 3 - The number of spermatozoa in the spermathecae of females according to the immediate risk of sperm competition (either the pair mating alone or with 10 conspecifics independantly of their sex). Box plots show median (horizontal bars), upper, and lower quartiles (borders of the box). Whiskers extend from the 10th to the 90th percentiles.

Copulation duration

Immediate sperm competition risks (χ^2 = 3.71 ; P = 0.16) and mean sperm competition risks treatments (χ^2 = 0.55 ; P = 0.46) showed no effects on the copulation duration. Both the size of the female (χ^2 = 4.17 ; P = 0.04) and the size of the male (χ^2 = 4.30 ; P = 0.04) were related to copulation duration, with bigger females and smaller males copulating longer (HR ± SE = 0.38 ± 0.47 for female size and HR ± SE = 2.79 ± 0.49 for male size) (Fig.4a and Fig.4b). However, these relationships were mainly driven by one extreme copulation duration implying a small male and a big female and disappeared when this pair was removed from the model (χ^2 = 3.16 ; P = 0.07 for female size and χ^2 = 2.82 ; P = 0.09 for male size). Age of the mating pair (χ^2 = 7.79 ; P = 0.05) marginally influenced copulation duration, older individuals copulating for a shorter time (HR ± SE 4 days = 1.86 ± 0.30 ; 5 days= 1.76 ± 0.29 ; 6 days = 4.94 ± 0.67) (Fig.5). The mean times of copulation for each age categories were 39.34 ± 3.01 minutes for 3 days old individuals, 33.30 ± 1.38 minutes for 4 days old individuals, 32.78 ± 1.88 minutes for 4 days old individuals and 26 ± 3.79 minutes for 4 days old individuals.

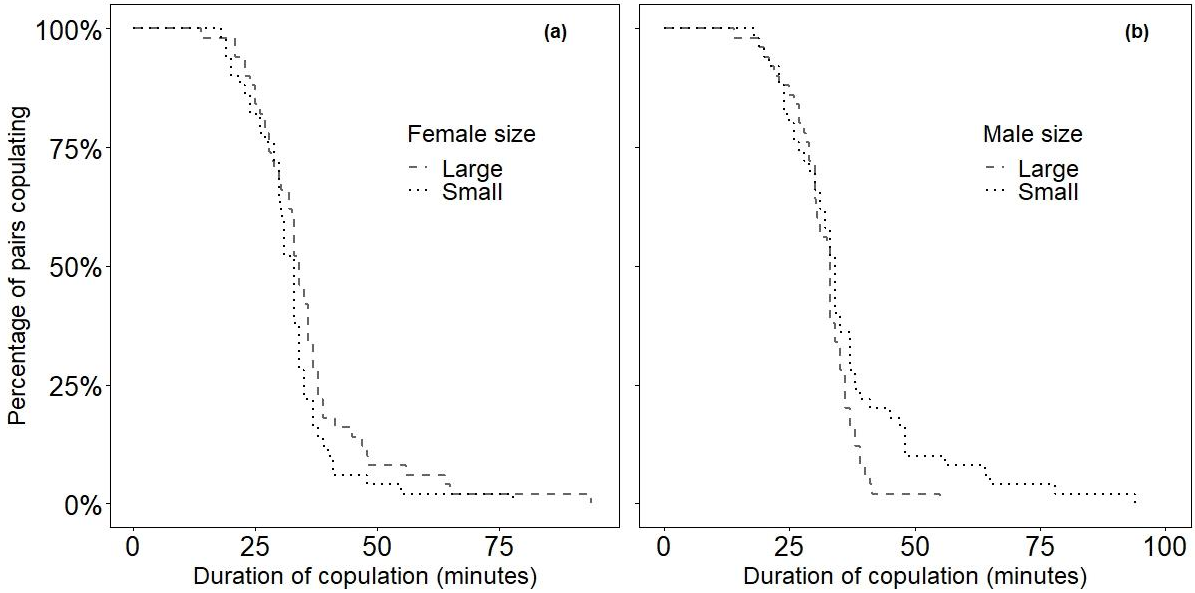

Figure 4 - Occurrence of copulation endings according to (a) the female size and (b) the male size. For representation purposes we categorised large (n = 50) and small (n= 50) females/males as individuals being either larger or smaller than the median value for the relevant sex size (4.14 mm for females and 3.74 mm for males).

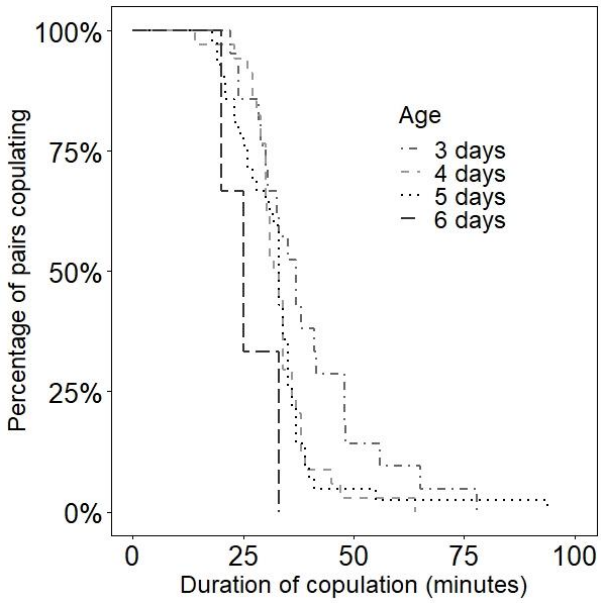

Figure 5 - Occurrence of copulation endings according to the age of the pair mating.

Discussion

Males BSF had more sperm in their seminal vesicles when they were grouped, and females of pair mated with conspecific stored more sperm in their spermathecae. In line with the predictions of the sperm competition theory, the males of the BSF respond, on one hand, to mean risks of sperm competition (long-term exposure to rivals) by producing more spermatozoa in their seminal vesicles and on the other hand, to the immediate risks of sperm competition (sudden exposure to rivals) by allocating more spermatozoa in a copulation. In contrast, copulation duration was neither related to sperm competition risks treatments, nor to the number of transferred spermatozoa, but was age-dependent.

Regardless of sperm competition risks, it has been shown that ejaculate expenditure could be condition dependent (Perry & Rowe, 2010; Kaldun & Otti, 2016; Wylde et al., 2020), or sometimes associated with secondary sexual signals (Mautz et al., 2013; Polak et al., 2021). It seems not to be the case in the BSF where it has already been shown that male size does not affect sperm production (Jones & Tomberlin, 2021; Munsch-Masset et al., in press). In the same way, we show here that it does not affect sperm allocation. Interestingly, it appears that males producing more spermatozoa (reared under high risk of sperm competition) do not transfer more sperm to females. Although it is not the case, one would expect that the amount of spermatozoa available to males might be partly determinant of the amount allocated to a copulation (Engqvist & Reinhold, 2005). Meanwhile, we observed more copulations from grouped males than single males during our experiments (see results). We could hypothetise that besides stimulating spermaotozoa accumulation males reared in high risks of sperm competition also increase mating attempts (see the male mating rate hypothesis in Vahed & Parker, 2012), while allocating the same amount of spermatozoa at each mating. To investigate further this hypothesis would require a different experimental set up allowing for multiple copulations per treatments.

In contrast to some other species (Martin & Hosken, 2002; Engqvist & Sauer, 2003), copulation duration is not related to the amount of sperm transferred by the male in the BSF. Sperm transfer dynamics that do not follow a linear relationship with time are not rare and this indicates that complex physiological mechanisms may be at work (Weggelaar et al., 2019). Here, the duration of copulation was not different in the three treatments. Apart from sperm transfer dynamics, plasticity in copulation duration when males are exposed to rivals can be associated with active mate guarding (Lorch et al., 1993; Alcock, 1994; Cueva del Castillo, 2003), a behavior that BSF males do not appear to exhibit (Giunti et al., 2018), as confirmed by our three treatments.

The duration of copulation marginally varied with the age of the mates, with younger flies copulating longer. Since both individuals in the pair were the same age, this effect may be linked to the age of the male, the female, or both. Age affects the characteristics of the ejaculate as well as the outcome of sperm competition or female choosiness in Drosophila melanogaster and Dermestes maculatus (Mack et al., 2003; Jones et al., 2007). Whether this relationship between copulation duration and age is due to physiological constraints or adaptive strategies by one sex or the other is unclear and, so far, whether a specific sex has any control over the duration of copulation remain unexplored although males have hooks that appear to hold the female during copulation (personal observations). Moreover, bigger females and smaller males copulated for a longer time even though the latter transferred the same number of spermatozoa as bigger males. However, this relationship between individuals sizes and copulation duration may be driven by one extreme value in our experiment (see results, Fig.4) and should therefore be further investigated. The duration of copulation for single pairs was the same as for pairs in the presence of a conspecific. Thus, it would appear that once copulation has begun, surrounding males lose interest in the pair, unlike during courtship when other males may pounce on the pair attempting copulation (Julita et al., 2020).

Numerous cues can be used by males to assess the risk of sperm competition. For example, another Diptera, Drosophila melanogaster uses combinations of cues as diverse as visual, contacts, chemosensory, and sounds to detect rivals (Bretman et al., 2011). It has been suggested that BSF uses acoustic signals to identify conspecifics without differentiating females from potential rivals (Giunti et al., 2018), leading to a lot of same-sex sexual behaviors. These behaviors are observed with males displaying aedaegus eversion (Personnal observations, Giunti et al., 2018), which may indicate that males of the BSF attempt to copulate indifferently with males and females. Interestingly, we found that BSF males appeared to adjust the number of spermatozoa allocated in a copulation when they were with conspecifics, regardless of whether these were males or females. This sperm adjustment is in line with a potential absence of sex recognition in BSF.

Like many aspects of BSF biology, pre-copulatory sexual selection processes in this species are not precisely known. Sexual dimorphism is low and preliminary results indicate that male size does not play a role in female's mates selection (personnal observation). BSF was described as using leks to mate (Tomberlin & Sheppard, 2001). Those structures are defined as aggregated males display sites that females attend primarily for the purpose of fertilization (Höglund & Alatalo, 1995). Supposedly aggressive intrasexual interactions were also observed but females were said to be 'similarly greeted' than males in the supposed lek sites, except that these interactions ended in copulation (Tomberlin & Sheppard, 2001). We did not notice any aggregating area akin to a lek in our rearing conditions (Benelli et al., 2014),

furthermore the notable lack of sex recognition may question the hypothesis of the BSF actually being a lekking species.

Previous studies have demonstrated the occurrence of multiple mating in BSF (Permana et al., 2020; Hoffmann et al., 2021). Consistently with sperm competition theory, our findings suggest that males invest more in sperm production and allocation as a strategy to overcome rivals in this competitive reproductive environment. However, a bet hedging strategy is not evidenced here because males copulating in the presence of virgin females do not spare their sperm reserves in the perspective of the insemination of a maximum number of mates. Besides sperm competition, the complexity of female spermathecae in this species (Munsch-Masset et al., in press) strongly suggests that post-copulatory intersexual selection mechanisms are at work, such as cryptic female choice (Pascini & Martins, 2017).

BSF is a species that is of great economic interest in animal production for its potential as a feed source (Tomberlin & van Huis, 2020). The strategy of sperm production and transfer is a key factor that should be integrated in the future to control reproduction and genetics.

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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, scripts, code, and supplementary information availability

Analyses reported in this article can be reproduced using the data and script provided by Frédéric Manas (2023) (https://zenodo.org/record/8058417). https://doi.org/10.5281/zenodo.8058416

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