Age-related sperm production, transfer, and storage in the sweet potato weevil, cylas formicarius (fabricius) (coleoptera: Curculionidae)

Authors: Satoshi Hiroyoshi, Tsuguo Kohama, and Gadi V. P. Reddy

The final publication is available at Springer via http://dx.doi.org/10.1007/s10905-016-9590-0.

Hiroyoshi, Satoshi, Tsuguo Kohama, and Gadi V P Reddy. "Age-related sperm production, transfer, and storage in the sweet potato weevil, cylas formicarius (fabricius) (coleoptera: Curculionidae)." Journal of Insect Behavior (November 2016). DOI:https://dx.doi.org/10.1007/s10905-016-9590-0.

Age-Related Sperm Production, Transfer, and Storage in the Sweet Potato Weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Curculionidae)

Satoshi Hiroyoshi[1,2] · **Tsuguo Kohama**[1,3] · **Gadi V. P. Reddy**[4]

[1] Okinawa Prefectural Fruit-fly Eradication Program Office, 123 Maji, Naha, Okinawa 902-0072, Japan

[2] 7-12-203 Kotobukiso, Nishikawacho, Itoman, Okinawa 901-0304, Japan

[3] Okinawa Prefectural Agricultural Research Center, 820 Makabe, Itoman, Okinawa 901-0336, Japan

[4] Western Triangle Ag Research Center, Montana State University, 9546 Old Shelby Rd., P. O. Box 656, Conrad, MT 59425, USA

Abstract The relationship between sperm production, insemination rate, and sperm transfer were studied in the sweet potato weevil, *Cylas formicarius*. Older adult males retained more sperm in the testes-seminal vesicle complex (TSC) and thus more was ejaculated into females at first mating. Number of matings per day for males was relatively constant across different ages, and frequent mating resulted in a reduced amount of sperm transferred to females, especially in young males. Young virgin males had a relatively small ejaculate, and almost all sperm transferred to females was stored in the spermatheca, whereas older virgin males transferred a larger amount of sperm to females, in whom sperm was found in both the spermatheca and post-spermathecal organs (PSO) after mating. The number of sperm in the PSO decreased markedly within 24 h after mating, but amounts in the spermatheca remained the same. Just where the sperm in the PSO went is a point that remained undetermined. The amount of sperm in the spermatheca was reduced more rapidly in females that laid eggs than in females that did not, although sperm reduction occurred even in the latter. Insemination of this weevil corresponded with the volume of the spermatheca, and the amount of sperm stored in the TSC was determined by the age and mating history of the males.

Introduction

Matings, especially repeated or multiple matings, affect male reproduction by reducing ejaculate size. Although the effect of male ejaculate size on reproduction has not been well documented, it seems highly likely that ejaculate size affects reproduction rates of both sexes. Males try to defend their ejaculates to guarantee their own offspring by mate guarding (Thornhill and Alcock 1983), transferring large ejaculates (He and Tsubaki 1991), expelling the ejaculates of the rival males (Waage 1979; Yokoi 1990), and through sperm displacement (de Villiers and Hanrahan 1991; Gack and Peschke 1994Such behaviors are thought to have evolved as reproductive strategies.

In insects, the morphology of female reproductive organs and the number of spermathecae varies among species (Calder 1990; Pascini et al. 2013; Souza et al. 2014). Some insects, such as the tephritid fly *Anastrepha ludens* (Loew), have additional organs (called seminal receptacle) in addition to their spermathecae for sperm storage (Thomas et al. 2014). Some species have a hard spermathecae, which may prevent the entry of excess sperm. In general, male ejaculate, including the sperm and accessory gland substances, changes with age, mating history and temperature, and other factors (Simmons 2001). How males manipulate ejaculate size is unclear. It is hypothesized that males try to deliver large ejaculates to avoid sperm competition (Eberhard 1991; Birkhead and Moller 1998). If ejaculate size is small, male reproductive success may be diminished, because females would mate with other males, resulting in sperm competition. Thus even a small butterfly such as *Leptophobia elodia* (Boisduval) invests a relatively high percentage of its resources to ejaculate production (Caballero-Mendieta and Cordero 2013), probably to avoid sperm competition.

Sperm transfer from males to females has been examined in various insects. Females, especially those of monandrous species, must avoid sperm-depleted males (Steiner et al. 2008; Kant et al. 2012) because mating with these individuals results in reduced number of offspring. Multiple mating behavior thus ensures fertilization (Parker 1984). On the other hand, multiple male matings have a detrimental effect on female fitness due to male sperm depletion (Elzinga et al. 2011). Females mating with an already-mated male acquire less sperm, fewer nutrients in the nuptial gift, require prolonged copulation, and suffer an increased risk of not being fertilized, and generally experience shorter longevity, although males can allocate similar numbers of sperm at successive mating in *Anastrepha obliqua* (Perez-Staples and Aluja 2006) and the last females have slightly longer life spans than the first females to mate with males in *A. obliqua* (Perez-Staples et al. 2008). In general, females mated with previously mated males have a shorter refractory period than those mated with virgins (Marcotte et al. 2006; Abraham et al. 2011), which would be responsible for remating. Females have an incentive therefore to avoid sperm and/or accessory gland substance-depleted males. The ability of males to produce ejaculate seems to relate to mating ability because ejaculate size should be determined by spermiogenesis and accessory gland substances synthesis. Moreover, ejaculate, which contains the sperm and additional substances like nutrients or chemical compounds, is related to female somatic maintenance and egg production.

The sweet potato weevil, *Cylas formicarius* (Fabricius), is a serious pest of sweet potato, *Ipomoea batatas* L., in tropical and subtropical countries. In Japan, this weevil was first recorded as an introduced pest on subtropical Okinawa Island in 1903, and since then it has spread over the neighboring islands (Kohama 1990). In Okinawa Prefecture, an eradication program against this pest, using a combination of a synthetic female sex pheromone and sterile male releases, has been undertaken (Yasuda 1995; Moriya 1997; Moriya and Miyatake 2001), with initial success on Kume Island (Haraguchi et al., unpublished data). To successfully expand this eradication program to other islands, basic information about the reproduction and mating ability of released sterile males is essential. Males of this weevil mate multiple times, while females remate for up to 40 days after eclosion (Sugimoto et al. 1996). Thus, at least a proportion of females of this weevil are polyandrous. The aim of this study is to clarify the males' reproductive ability, especially in terms of sperm production and insemination. The stage of active spermiogenesis and the amount of sperm ejaculated and stored in the female's reproductive organs are less understood. Thus, we conducted this study to determine the relationships between sperm production, sperm transfer by males and the sperm storage capacity of the spermatheca of females in the sweet potato weevil.

Materials and Methods

Insects

The culture used in this study was established with *C. formicarius* adults collected from Yomitan, Okinawa Island in October 1992, that had been successively reared on sweet potatoes at 25 ± 1°C and a 14:10 h L:D photoperiod. Experiments were carried out from 1997 to 1999. The weevils used in all the experiments were kept at the same environmental conditions as mentioned above.

Approximately 500 female and male adult weevils, 2- to 6-weeks old, were reared in plastic containers (14.5 l). Sweet potatoes (600 g per cage) were provided as food and an oviposition substrate for producing the next generation and changed twice a week. By four weeks after eggs were laid on sweet potato tubers, most weevils had reached the pupal stage. Pupae were collected by crushing sweet potato tubers, removing intact pupae, and placing them in plastic cups for adult emergence. Since the pupae of this weevil are very soft, some of them were damaged (abnormal eclosion) and discarded from the experiments. The day of adult emergence is designated as 0-days-old. Newly emerged weevils with similar body size were sexed within two days of emergence and held separately in groups of approximately 20 individuals with a piece of sweet potato (about 40 g), which was changed twice a week. *C. formicarius* is nocturnal and mating takes place at night (Sakuratani et al. 1994).

Mating

Pairs of weevils (one male and one female) were confined in a glass Petri dish (9 cm diameter) during the dark half of the photophase, and approximately 16 h later the females were dissected to check their mating status based on the presence of sperm. *C. formicarius* females have only one spermatheca, and the presence of sperm in the

spermatheca indicates the female has mated. However, as will be mentioned later, the presence of sperm in the vagina, burtha copulatorix, or the spermathecal duct (post-spermathecal organs: PSO) alone was in some cases also taken as an indication of mating, even if no sperm was found in the spermatheca.

Sperm Counts

Sperm counts were made according to the methods by Yamagishi and Tsubaki (1990), Tsubaki and Yamagishi (1991) and K. Okumura (Per. Com.). *C. formicarius* males have a pair of testes and a seminal vesicle (testes-seminal vesicle complex: TSC), both of which contain free sperm. The male reproductive systems of mated or unmated individuals were removed by dissection in 0.9% salt solution, immersed in 0.1% triton X solution, and their fat body carefully removed with a piece of tissue paper and forceps. The TSC was transferred into 500 μl deionized water on a glass Petri dish and torn into small pieces. The water containing the free sperm was stirred 20 times to homogenize the sample, and then 10 μl of solution was taken from the sample with a microsyringe and spread onto a slide glass to air dry. Sperm counts were made with a video apparatus (VM-60, Olympus, BR-S925, Victor and VY-VP20, Hitachi) attached to a microscope, and the value obtained was multiplied by 50 to determine the total sperm per individual. The reproductive organs of females from tests were removed by dissection in 0.9% salt solution, and the ovaries and lateral oviducts were discarded. The spermatheca and remaining organs of each female were separated and placed individually in 100 μl of deionized water. In some experiments, sperm counts were made for the spermatheca only, leading to underestimation, as will be mentioned later.

Exp. #1. Relationship between Age and Mating Rate

In Exp. #1, two trials were run in order to examine the effects of adult age and sex on mating rate. First, virgin males of various ages (0, 1, 3, 5, 10, 20, 30, 40 or 50-day-old) were paired with 20-day-old or older (21- to 30-day-old in most cases) virgin females (referred to as sexually mature females hereafter) individually. Second, virgin females at various ages (0, 1, 3, 5, 10, 20, 30, 40 or 50-day-old) were paired with 20-day-old or older (21- to 30-day-old in most cases) virgin males (referred to as sexually mature males hereafter). Forty individuals were used for each combination.

Exp. #2. Effect of Male Age on Sperm Production

In Exp. #2, the relationship between male age and sperm production was assessed by counting the number of sperm in the TSC of virgin males at various ages (0, 1, 3, 5, 10, 20, 30, 40 or 50-day-old). Twenty males were examined for each age.

Exp. #3. Effect of Male Age on Sperm Transfer

In Exp. #3, the relationship between male age and sperm transfer was examined by pairing virgin males of various ages kept in Petri dishes with sexually mature females (20-day-old or older (21- to 30-day-old in most cases) virgin females). The number of sperm in the spermatheca of each female was counted 16 h later (early photophase). In

a separate experiment, virgin males of various ages (1, 2, 3, 4, 5, 7, 10, 15, 20, 30,40 or 50-day-old) were paired with sexually mature females or, for the 10, 30-.or 50-day-old controls, kept virgin for one night, and their respective TSCs were then dissected to compare the number of sperm between the mated and virgin males. The number of sperm in the TSC of mated males subtracted from that of virgin males was considered to be the ejaculated sperm number. Twenty male adults were used for each age group.

Exp. #4 Mating Frequency of variously Aged Males

In Exp. #4, virgin male of various ages (10, 15, 20, 30, or 60–day-old) were held overnight with 10 mature females (20-day-old or older (21- to 30-day-old in most cases) virgin females) per male, and the following day females were then dissected to observe the presence of sperm in the spermatheca to determine the mating frequency. In our preliminary observations, mated females did not attract males and thus assume that mated females did not engage in further mating during the observation period. Therefore, we considered the number of mated females to be the mating frequency of the males. The abundance of sperm found in these females was categorized as 0) no sperm, 1) a few (less than 100), 2) common (sperm occupied less than half the volume of the spermatheca), or 3) many (sperm occupied more than half the volume of the spermatheca). Twenty male adults were tested for each age group.

Exp. #5 Effect of Consecutive Matings on Sperm Storage in TSC

In Exp. #5, 20-day-old virgin males were paired individually, each with five virgin, sexually mature females kept in Petri dishes to examine the relationship between the number of matings and the amount of sperm that remained in the male's TSC. We observed mating behavior directly to count the number of matings by each male, using the method described below. Males were then dissected and the sperm present in the TSC were counted after mating. Control sperm counts were those taken from 20-day-old virgin males. Data from eight to 15 male adults was collected for each mating frequency.

Exp. #6 Effect of Female Age on Sperm Storage

In Exp. #6, virgin females of two age groups (15- to 17-day-old and 44- to 50-day-old, respectively) were individually paired with one 20-day-old virgin male kept in Petri dishes to examine the effect of female age on insemination. After being held with the male overnight, females were dissected to count the sperm present in the spermatheca. Twenty adult females were of each age group were tested.

Exp. #7 Effect of Male Age on Copula Duration

In Exp. #7, the effect of male age on the duration of copulation was examined. Virgin males 10-, 30- or 50-day-old were paired individually with sexually mature females in a polystyrene tube (1 cm diameter × 5 cm height) that was used to observe many samples for a short time. Mating behavior was observed at 24 ± 2°C under dim light conditions using a flashlight covered with a piece of red cellophane, producing a light wavelength

to which the weevils are not sensitive. The time when a male inserted its penis into a female's genital organ was considered as the beginning of copulation. Whether a male actually inserted the penis into the female's genital organs was confirmed under a dissecting microscope attached to an illumination apparatus covered with red cellophane. Because mating checks were made at approximately 5 min intervals, the onset of copulation recorded was a maximum of 5 min later than the actual onset. Separation of the genitalia in the pair was considered the end of copulation. Twenty male adults were used of each age group.

Exp. #8 Sperm Dynamics during and after Copulation

In Exp. #8, the effect of male age on sperm dynamics in the female's reproductive organs was examined. A 10-, 30- or 50-day-old male and a mature female were confined in a polystyrene tube (same as above) to observe mating behavior. Groups of 20 mated females were dissected 0, 15, 30, or 60 min after the onset of mating or immediately after mating and the number of sperm in the spermatheca and PSO of each female counted. Except for females dissected during mating, those females in which no sperm was found in the reproductive organs were discarded as unmated.

Exp. #9 Effect of Oviposition on Sperm Dynamics

In Exp.# 9, the effect of ovipositing activity on the number of sperm in the female's reproductive organs was examined. A series of 20-day-old virgin females were paired individually with a single sexually mature male during the first four hours of the photophase in a vial, and, after mating, the male was removed and a piece of sweet potato put in the vial. During the photophase of the next day, the sweet potato pieces were examined for the presence or absence of eggs. The number of sperm in the spermatheca and PSO were separately determined in these females and compared between females that oviposited (n = 10) and those that did not (n = 17). Mating status was confirmed for all females by both direct behavioral observations and dissection.

Results

Exp. #1 Relationship between Age and Mating Frequency

When males of various ages were paired with sexually mature females or vice versa, mating rates increased with age until the insects were 10 days old in males and 20 days old in females, and leveled off thereafter (Fig. 1). There was a significant difference in mating rates between males and females (Chi-square, T-value = 123.971, df = 20, $P < 0.0001$).

Exp. #2 Sperm Production

Free sperm was absent in the testes of males at adult emergence. On the 5th day, almost all males (95%) had free sperm in the testes and the number of sperm stored in the TSC

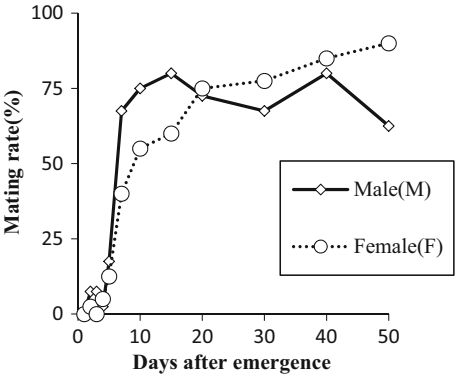


Fig. 1 Changes in the mating frequency of *Cylas formicarius* females and males after adult emergence ($n = 4$ per group)

increased linearly with age thereafter (y = 1.7828 × −2.7086, $r = 0.986$, $P < 0.0001$) (Fig. 2).

Exp. #3 Relationship between Male Age and Insemination

When sexually mature females mated with a young (1–5-day-old) male, the number of sperm found in the spermatheca after mating was small (<300) except for one female that copulated with a 4-day-old male. In females mated with 5- to 15-day-old males, the number of sperm increased up to approximately 5000 on average and remained unchanged thereafter (Fig. 3a). There was a positive regression relationship between male age and the number of sperm (y = 107.096 × −552.18, $P = 0.0009$). However, the number of sperm ejaculated by males, as determined by the difference in the number of sperm in the TCS between mated and virgin males, increased from 7636 on day 10 to 61,720 on day 50 (Fig. 3b), and these values were much greater than those found in the spermatheca of females (Fig. 3a). This indicates that males transferred excess sperm for

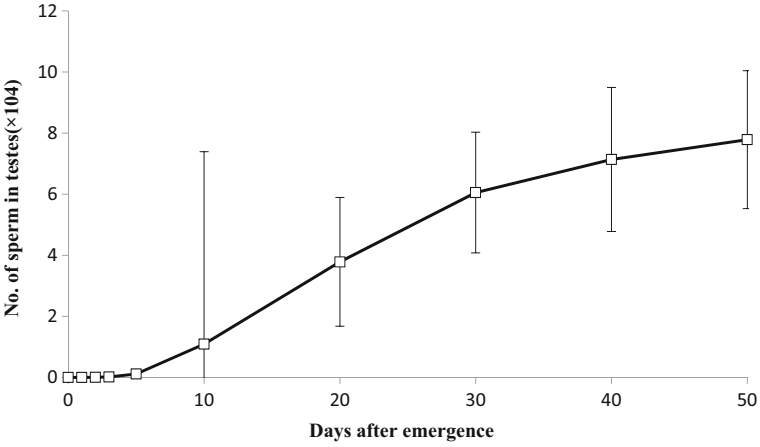


Fig. 2 Changes in the number of sperm in the testes-seminal vesicle complex (TSC) with age of *C. formicarius* virgin male adults (mean ± SD) ($n = 20$ per point)

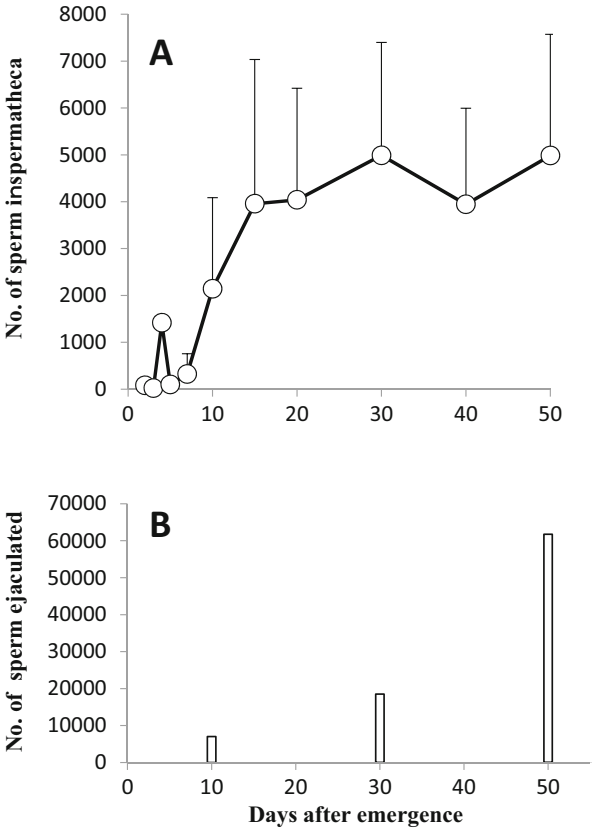


Fig. 3 Changes in the number of sperm in the spermatheca (**a)** and in the testes-seminal vesicle complex (**b)** with age of *Cylas formicarius* adult males after mating (mean ± SD) (n = 20 per point)

the spermatheca. Probably, males might ejaculate all the sperm stored in the seminal vesicles, and thus they cannot regulate sperm transfer at first mating. Moreover, this weevil stores an amount of sperm in the testes so that the sperm in the testes remains after ejaculation.

Exp. #4 Effect of Male Age on Mating Frequency and Insemination

When individual virgin males of various ages were allowed to mate with 10 sexually mature females overnight, males mated 4 or 5 times on average and no significant difference was found among males of different ages (Scheffe's method after ANOVA, n = 240, $P > 0.05$) (Table 1). However, the total number of sperm found in the spermatheca was higher in females mated with older males (F = 4.952, df = 5204, error = 204, $P = 0.003$). The number of sperm in the spermatheca per mating was also higher with older males (F = 39.03, df = 5204, error = 203, $P < 0.0001$). Frequent mating often resulted in a reduced number of sperm being transferred, especially in young males. In contrast, older males could inseminate several females with many sperm in the spermatheca (F = 27.912, df = 5204, $P < 0.0001$, Scheffe's method after ANOVA).

Table 1 Mating times and ejaculation pattern at each age fo *Cylas formicarius*

Age at mating	No of insects used	Mating duration			
		Mean duration ± SD	Duration relative to sperm number in spermatheca		
			less	common	many
10	40	4.2 ± 2.1^{a}	2.3 ± 1.5^{a}	1.8 ± 1.3^{a}	0.3 ± 0.5^{a}
15	40	4.5 ± 2.4^{a}	1.8 ± 1.7^{a}	2.3 ± 1.7^{a}	1.1 ± 0.7^{b}
20	40	4.8 ± 2.8^{a}	1.7 ± 16^{ac}	2.7 ± 1.6^{a}	1.5 ± 0.9^{bd}
30	40	5.5 ± 2.5^{a}	1.5 ± 1.4^{ac}	2.7 ± 1.7^{a}	1.9 ± 0.9^{cd}
60	40	4.4 ± 2.9^{a}	0.7 ± 1.3^{bc}	1.8 ± 1.3^{ac}	2.6 ± 1.3^{cd}
90	40	4.5 ± 2.7^{a}	0.5 ± 0.9^{bc}	1.3 ± 1.3^{bc}	3.3 ± 1.6^{c}

Different letters indicate significant differences

Exp. #5 Effects of Mating Frequency on the Number of Sperm in the TSC

The number of sperm in the TSC tended to decrease with mating frequency (Fig. 4). There were significant differences between the control (no mating) or once- and twice- or thrice-mated male adults ($F = 14.827$, $df = 3$, $P < 0.05$ by Tukey's method after ANOVA). These results indicate that mating frequency affected the number of sperm remaining in the TSC after mating.

Exp. #6 Effect of Female Age on Insemination

The number of sperm stored in the spermatheca after mating was similar between young (4174 ± 2914 (SD), $n = 7$) and old females (3983 ± 2319, $n = 20$) (Mann-

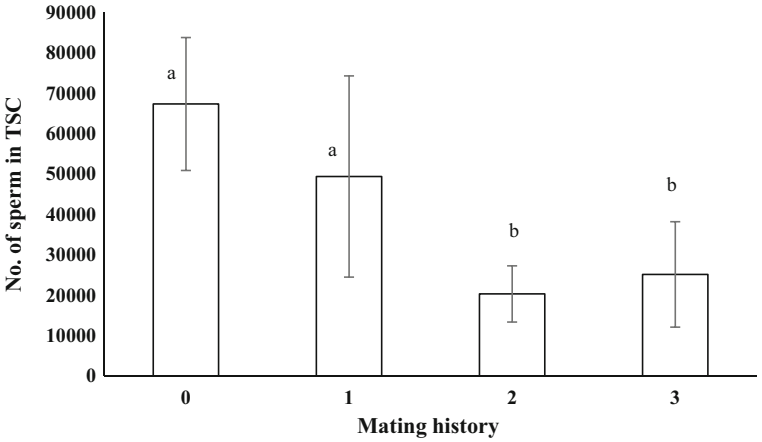


Fig. 4 Duration of copulation in 10-, 30- or 50-day-old *Cylas formicarius* males that were allowed to mate with sexually mature females (mean ± SD) ($n = 20$ per point)

Whitney's U-test, $P > 0.05$) (data not shown). This result indicates that female age did not affect male insemination efficiency.

Exp. #7 Effect of Male Age on the Length of Copulation

When 10-, 30- and 50-day-old males copulated with sexually mature females, copulation lasted 50.7, 73.7, or 78.3 min on average, respectively (Fig. 5). Copula duration in 10-day-old males was significantly shorter than that in 30- and 50-day-old males ($F = 16.545$, $df = 2$, $P < 0.01$, Scheff's method after ANOVA). Short copulations (less than 30 min) were common in 10-day-old males.

Exp. # 8 Sperm Dynamics during and after Copulation

During copulation, sperm in the seminal vesicles moved into the vas deferens, ejaculatory duct, and penis of the male, and then entered the vagina, bursa copulatorix, spermathecal duct, and spermatheca of the female weevil. The sperm in the female reproductive organs during copulation was very motile and those in the spermatheca actively rotated. However, their motility was much reduced the day after mating and thereafter.

When females mated with 30- or 50-day-old males, sperm in the PSO was abundant both during and immediately after mating (Fig. 6), and was significantly more abundant than in females mated with younger (10-day-old) males, both globally (F = 5.4,74

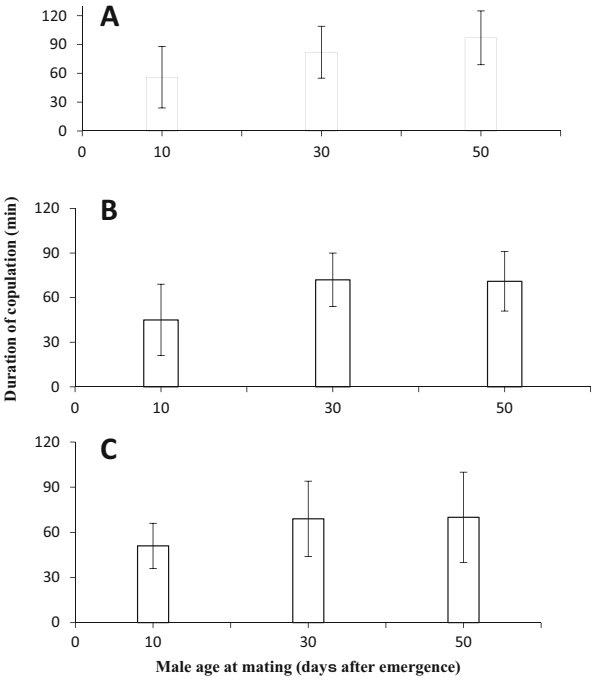


Fig. 5 Change in sperm number in TSC of males of *Cylas formicarius* after consecutive matings (mean ± SD) (n = 8 to 15)

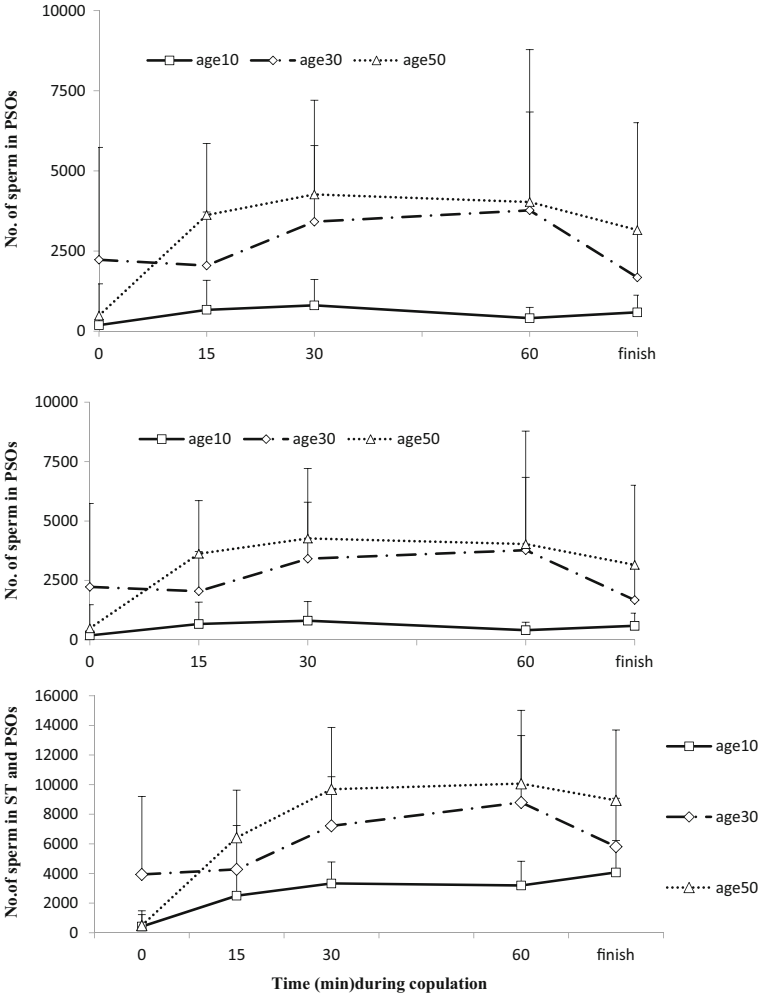
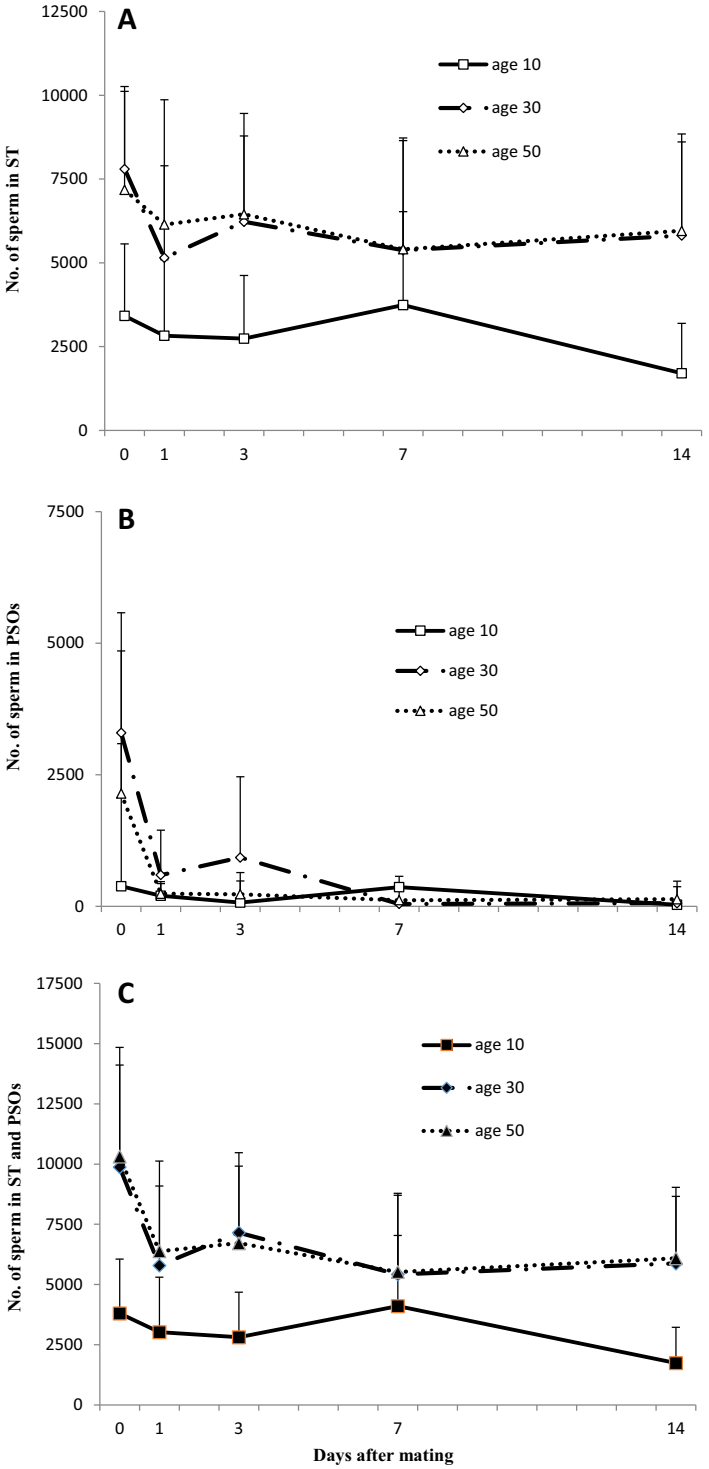


Fig. 6 Changes in the number of sperm in the spermatheca or post-spermathecal organ (PSO) in *Cylas formicarius* females mated with 10-, 30- or 50-day-old males (mean ± SD) (n = 20 at each point)

df = 2, $P < 0.05$, n = 300, Scheffe's method after ANOVA) and at four time points following mating (at mating: F = 5.481,df = 2, error = 57, $P = 0.067$; 15 min after onset of mating: F = 15.272, df = 2, error = 57, $P < 0.0001$; 30 min: F = 13.087, df = 2, error = 57, $P < 0.0001$; 60 min: F = 7.639, df = 2, error = 57, $P = 0.012$; and "after mating": F = 7.336, df = 2, error = 57, $P = 0.015$), except for females examined immediately after mating with 50-day-old males. On the other hand, the number of sperm in the spermatheca increased significantly with copula duration (F = 7.903, df = 2, error = 57, $P < 0.001$), regardless of male age at mating. This indicates that sperm was transferred to females gradually over the entire period of mating.

The number of sperm in the spermatheca was relatively constant over the first 14 days after mating (Fig. 7). During this time the females probably laid several eggs. Male age at mating affected the number of sperm transferred to the PSO. The number of sperm in the PSO was consistently low in females that had mated with 10-day-old


A
No. of sperm in ST
12500
10000
7500
5000
2500
0
age 10
age 30
age 50
0
1
3
7
14
B
No. of sperm in PSOs
7500
5000
2500
0
age 10
age 30
age 50
0
1
3
7
14
C
No. of sperm in ST and PSOs
17500
15000
12500
10000
7500
5000
2500
0
age 10
age 30
age 50
0
1
3
7
14
Days after mating

◀ **Fig. 7** Changes in the number of sperm in the spermatheca (**a)** and in the post-spermathecal organs (**b)**, and the pooled number (C; ST and PSOs) of *Cylas formicarius* females mated with 10-, 30- or 50-day-old males (mean ± SD). PSOs and ST indicate post-spermathecal organ and spermatheca, respectively (n = 20 at each point)

males, but was considerably higher in females that had mated with 30- or 50-day-old males, especially immediately after mating, after which it reduced markedly. There were significant differences in females mated with 30- and 50-day-old males between day 0 and day 1 or later ($P < 0.05$, Tukey's method after ANOVA).

Exp. #9 Effect of Oviposition on Sperm Storage in Females

No significant differences were observed in the number of sperm in the spermatheca between females that had laid eggs the day after mating and those that had not (Fig. 7) (Mann-Whitney's U-test, W Value = 132.0, n = 27, $P > 0.05$), while a significant difference between these groups was found in number of sperm in the PSO (Mann-Whitney's U-test, W-Value = 742.5, n = 27, $P < 0.05$). However, irrespective of the presence or absence of oviposition activity, sperm number decreased significantly in the PSO the day after mating (Mann Whitney's U-test, $P < 0.05$).

Discussion

The life stage in which sperm formation occurs differs among insect species. In *C. formicarius*, spermiogenesis occurred in the adult stage and the number of sperm stored in the testes-seminal complex (TSC) increased with adult age (Fig. 2). Apparently, this is directly related to the high level of mating capability maintained during the adult stage except for the teneral period (Fig. 1). The number of sperm remaining in the TSC after mating decreased with mating frequency (Fig. 5). It appears that mature male adults of this weevil can allocate the sperm in the TSC to several virgin females (Table 1). Although multiple matings by this weevil may deplete the sperm in the TSC, they compensate to some extent by continuing to produce sperm. Interestingly, they use sperm from both the testes and the seminal vesicles during ejaculation in consecutive matings (Hiroyoshi, unpublished data). Since older males produce and store more sperm in the TSC, the number of sperm transferred to females increases with male adult age.

Sperm Number and Temporal Dynamics

Mating activity in this weevil was maintained at a high level after the teneral period. However, insemination size tended to increase with the age of virgin males (Fig. 3b). On the other hand, the number of sperm found in the spermatheca after mating was not affected by male age at mating (Fig. 3a), suggesting that not all sperm ejaculated by males are transferred to the spermatheca, but only that of older males. Because the spermatheca of this weevil is sclerotized, it has little ability to change its size and shape in response to the volume of sperm transferred, leading us to assume that excess sperm fails to enter the spermatheca. This occurs in *Coroica* species (Diptera: Sphaeroceridae), in which excess sperm remaining

in the vagina appears to be digested or expelled (Lachmann 1997). In the red flour beetle (*Tribolium castaneum*) the number of sperm stored in the spermatheca exhibits an 8.5-fold increase between 0 and 60 min after mating, but the majority of sperm appear to be expelled from the bursa shortly after mating (Bloch Qazi et al. 1996), as we assumed was the case for *C. formicarius* in the present study. Similar results were obtained in the noctuid moth *Helicoverpa armigera* (Yan et al. 2013). In *Drosophila* flies, females store sperm in two distinct storage organs, the seminal receptacle and the paired spermathecae (Manier et al. 2013b); sperm from the first mating enters the seminal receptacle, while sperm from the next mating enters the spermathecae. Manier et al. (2013a) showed that females after the second mating displaced resident first-male sperm by ejection from the seminal receptacle to the bursa. Sperm partition of this weevil depends on the ability of active adult spermiogenesis and sperm transfer because these weevils can supply sperm transferred into females from the TSC (Hiroyoshi, unpublished data).

Sperm dynamics within the female reproductive organs of *C. formicarius* was influenced by male age (Fig. 6). When females mated with young virgin males with a relatively small number of sperm in the TSC and a small ejaculate, most sperm ejaculated entered the spermatheca. On the other hand, when females mated with older virgin males with a large number of sperm in the TSC and large ejaculate, many sperm were found in the spermatheca and PSO immediately after mating. The amount of sperm in the PSO dropped sharply thereafter, but that in the spermatheca remained unchanged (Fig. 7), indicating that sperm in the PSO did not move to the spermatheca after mating. These observations are consistent with the above assumption that the spermatheca has a fixed capacity to accommodate sperm.

The most likely cause for the reduction of sperm in the PSO of *C. formicarius* after mating could be ioviposition, which is stimulated by mating. In fact, females that had laid eggs shortly after mating had significantly fewer sperm in the PSO than did similarly mated females that had not laid eggs (Fig. 8). However, the latter group of females also experienced a reduction in the number of sperm after mating. Therefore, the loss of sperm is not fully explained solely by use of sperm to support oviposition.

It has been demonstrated in several species (Fowler et al. 1968; Gilbert 1981; Eady 1994; Yuval et al. 1996; Price et al. 2009; Pérez-Staples et al. 2010; Thomas et al. 2014) that only part of the sperm ejaculated by males can enter the spermatheca. For example, excess sperm is ejected by females in *Locusta migratoria* (Reinhardt and Meister 2000) and *Chorthippus* grasshoppers (Reinhardt 2000). Moreover, repeated mating causes the expulsion of the sperm from the previous matings in *D. melanogaster* (Scott and Richmond 1990). In female damselflies of *Ischnura senegalensis*, the bursa copulatrix and spermatheca have different sperm storage roles (Nakahara and Tsubaki 2007), and it has been hypothesized that the spermatheca is used for long-term storage and the bursa copulatrix for short-term storage. In *D. melanogaster*, sperm dumping occurs to eliminate dead, useless sperm (Snook and Hosken 2004). In *C. formicarius*, females have four ovarioles and produce relatively large eggs, but the number of eggs produced each day is only 4 or 5 at most with an average of 1 to 2 per day (Sugimoto et al. 1996). Thus, the number of sperm used for fertilization would be very small and even if only a small proportion of sperm remaining in the PSO of this weevil was used for fertilization, most sperm present in the PSO appear to be wasted. However, we cannot exclude the possibility that excess sperm and/or other ejaculate is used for certain reproductive-related events in females, such as sperm transfer, sperm activation, modification of

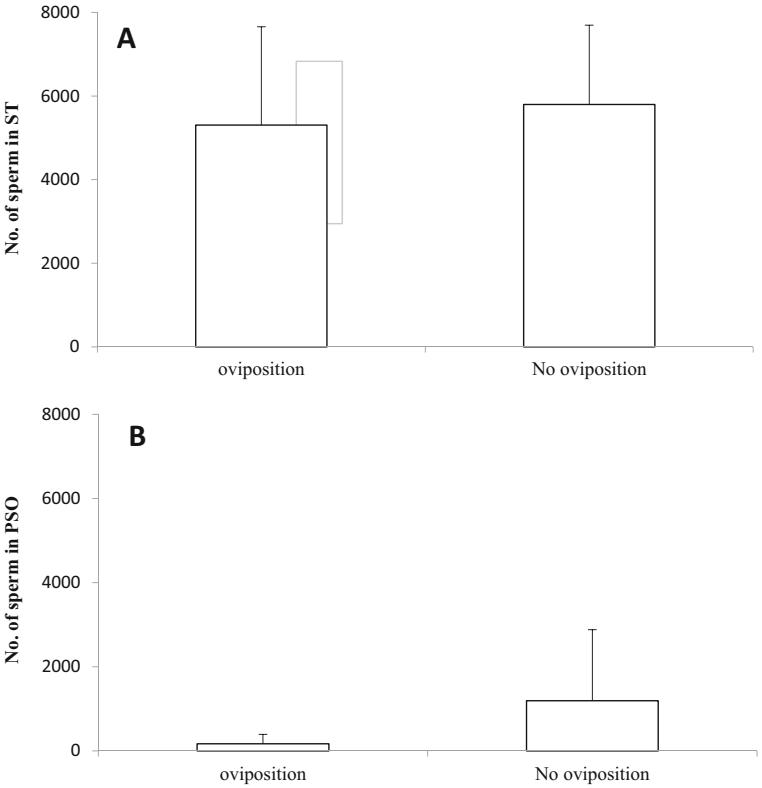


Fig. 8 Comparison of the number of sperm in the spermatheca (**a**) or the post- spermathecal organs (**b**) between *Cylas formicarius* females that had laid eggs (n = 10) and those that had not (n = 17) (mean ± SD). PSOs (post-spermathecal organs) and ST (spermatheca) indicate post-spermathecal organ and spermatheca, respectively

behavior, inhibition of female remating, nutrition for egg development, or pheromonostatis (cessation of pheromone production) (Leopold 1976; Chen 1984; Gillott 2003). In the present study, whether or not excess sperm were actually expelled from females was not determined.

Factors Influencing Ejaculate Size

Although insemination has been examined in various insects (Fowler 1973; Smith et al. 1988; Ward 1993; Gack and Peschke 1994; Snook and Markow 1996; Otronen et al. 1997; Snook 1998; Cook 1999; Reinhardt 2001; Schaus and Sakaluk 2002), these studies observed females only. To evaluate ejaculate size, both females and males need to be examined. For example, if all the sperm ejaculated could not enter the spermatheca, examination of the spermatheca alone could underestimate the male ejaculate size. In the present study, the number of ejaculated sperm of *C. formicarius* was examined by dissecting both sexes. As a result, we demonstrated that the number of ejaculated sperm estimated in the male was much greater than that reaching the female spermatheca and that male age affected ejaculate size. Therefore, either not all the sperm ejaculated by males are transferred to females or some sperm are lost shortly

after mating. The same results have been reported for the red flour beetle and the fly *Dryomyza anilis,* in which the majority of sperm transferred to females are expelled (Bloch Qazi et al. 1996; Otronen 1997). In the tiger beetles *Pseudoxychila tarsalis* and *P. bipustulata,* one or two spermatophores are ejected during or after mating (Rodriguez 1998).

Mating Failure

Mating failure is known to occur in more than 100 species of insects (Rhainds 2010). Kuriwada et al. (2013) reported that more than half the females of *C. formicarius* that had copulated had no sperm in their spermatheca, especially when females walked during mating. However, as far as we observed, any male *C. formicarius* that inserted its penis into the female certainly succeeded in inseminating them. This discrepancy was likely due to the difference in the size of the test arena used for experiments. Kuriwada et al. (2013) used a 24-well multiplate, whereas we used Petri dishes or polystyrene tubes, although mating rate in Petri dishes (9 cm dia.) was higher than that in polystyrene tubes (1 cm dia.). Less space would lead to mating failure, probably because females did not emit the pheromone attracting the males in narrow space.

For the SIT (Sterile Insect Technique), males irradiated at various dose of Co60 (Kumano et al. 2008) had a high inseminating ability for approximately 10 days. These results suggest that released sterile males into fields would find and inseminate several females, resulting in a decline of wild virgin females. Thus apparently there is low infertility of males. Remating of females in mass-reared conditions would further decrease the possibility of no sperm being transferred.

Male Mating History

The number of sperm transferred by male insects during mating can be influenced by age, the quality of the mating partner, mating experience, feeding status of the male, time of day, temperature, handling of weevils by researchers, or the insect's strain. For example, in the leafhopper *Balclutha incisa*, the number of sperm transferred to females increases exponentially with male age (Baily and Nuhardiyati 2005). It is thus important to identify the factors affecting sperm transfer. This study demonstrated that the number of sperm in the TSC or the number of sperm transferred to females by insemination by *C. formicarius* males was determined by the interaction of adult age and mating history (Table 1 and Fig. 5). The older males produced and transferred more sperm. This phenomenon is important to consider when investigating the effects of mating on reproduction. In fact, male ejaculate size even affects the re-mating and ovipositing behaviors of *C. formicarius* females (Hiroyoshi, unpublished data). Large ejaculate delayed re-mating of females and extended the female's oviposition period. On the other hand, small ejaculate did not prevent the re-mating of females and even stopped oviposition. Sugimoto et al. (1996) also reported that females that re-mated within 30 days after their first mating stored significantly more sperm than those that did not re-mate. Female polyandry in this weevil allows it to avoid interruption of oviposition due to sperm depletion.

The present study demonstrated that males ejaculate excess sperm into the spermatheca of females. This implies that a proportion of sperm ejaculated by males is not

used for fertilization. We observed that the sperm in the seminal vesicle of *C. formicarius* males rotates vigorously during mating, although this phenomenon was not observed in unmated males. The significance of sperm rotation is unclear. We speculate that the outside of the sperm mass rotating in the seminal vesicle may be the first to enter the spermatatheca. If so, active sperm is the first to enter the spermatheca, while inactive sperm may not be ejaculated at all, possibly supporting the sexually selected sperm hypothesis (Bocedi and Reid 2014). Therefore, clarifying the dynamics of sperm within the male's reproductive organs is important to understanding sperm competition and requires further study.

Acknowledgments We would like to thank Dr. S. Tanaka of National Institute of Agrobiological Sciences for critical reading of the manuscript. Thanks are also due to Dr. S. Moriya, Dr. K. Tateishi and Mr. K. Takasino for sending the materials.

References

Abraham S, Goane L, Rull J, Cladera J, Willink E, Vera MT (2011) Multiple mating in *Anastrepha fraterculus* females and its relationship with fecundity and fertility. Entomol Exp Appl 141:15–24

Baily WJ, Nuhardiyati M (2005) Copulation, the dynamics of sperm transfer and female refractoriness in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae). Physiol Entomol 30:343–352

Birkhead TR, Moller AP (1998) Sperm competition and sexual selection. Academic Press, New York

Bloch Qazi MC, Herbeck JT, Lewis SM (1996) Mechanisms of sperm transfer and storage in the red flour beetle (Coleoptera: Tenebrionidae). Ann Entomol Soc Am 89:892–897

Bocedi G, Reid JM (2014) Evolution of female multiple mating: a quantitative model of the "sexually selected sperm" hypothesis. Evolution 69:39–58

Caballero-Mendieta N, Cordero C (2013) Male mating costs in a butterfly that produces small ejaculates. Physiol Entomol 38:318–325

Calder AA (1990) Gross morphology of the soft parts of the male and female reproductive systems of Curculionoidea (Coleoptera). J Nat Hist 24:453–505

Chen PS (1984) The functional morphology and biochemistry of insect male accessory glands and their secretions. Annu Rev Entomol 29:233–255

Cook PA (1999) Sperm numbers and female fertility in the moth *Plodia interpunctella* (Hübner) (Lepidoptera; Pyralidae). J Insect Behav 12(6):767–779

de Villiers PS, Hanrahan SA (1991) Sperm competition in the Namib desert beetle, *Onymacris unguicularis*. J Insect Physiol 37:1–8

Eady P (1994) Sperm transfer and storage in relation to sperm competition in *Callosobruchus maculatus*. Behav Ecol Sociobiol 35:123–129

Eberhard WG (1991) Copulatory courtship and cryptic female choice in insects. Biol Rev 66:1–31

Elzinga JA, Chevasco V, Grapputo A, Mappes J (2011) Influence of male mating history on female reproductive success among monandrous Naryciinae (Lepidoptera: Psychidae). Ecol Entomol 36:170–180

Fowler GL (1973) Some aspects of the reproductive biology of *Drosophila*: sperm transfer, sperm storage, and sperm utilization. Adv Genet 17:293–360

Fowler GL, Eroshevich KE, Zimmering S (1968) Distribution of sperm in the storage organs of the *Drosophila melanogaster* female at various levels of insemination. Mol Gen Genet 101:120–122

Fricke C, Martin OY, Bretman A, Bussière LF, Chapman T (2010) Sperm competitive ability and indices of lifetime reproductive success. Evolution 64:2746–2757

Gack C, Peschke K (1994) Spermathecal morphology, sperm transfer and a novel mechanism of sperm displacement in the rove beetle, *Aleochara curtula* (Coleoptera, Staphylinidae). Zoomorphology 114: 227–237

Gilbert DG (1981) Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster*. J Insect Physiol 27:641–650

Gillott C (2003) Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu Rev Entomol 48:163–184
He Y, Tsubaki Y (1991) Effects of spermatophore size on female remating in the armyworm, *Pseudaletia separata*, with reference to larval crowding. J Ethol 9:47–50
Kant R, Trewick SA, Sandanayaka WRM, Godfrey AJR, Minor MA (2012) Effects of multiple matings on reproductive fitness of male and female *Diaeretiella rapae*. Entomol Exp Appl 145:215–221
Kohama T (1990) Invasion and colonization of the sweet potato weevils in Okinawa and current problems for their control. Shock 44:115–117 (In Japanese)
Kumano N, Haraguchi D, Kohama T (2008) Effect of irradiation on mating ability in the male sweetpotato weevil (Coleoptera: Curculionidae). J Econ Entomol:1198–1203
Kuriwada T, Kumano N, Shiromoto K, Haraguchi D (2013) Female walking during copulation reduces the likelihood of sperm transfer from males in the sweet potato weevil, *Cylas formicarius*. Entomol Exper Appl 147:225–230
Lachmann AD (1997) Sperm transfer during copulation in five *Coproica* species (Diptera: Sphaeroceridae). Eur J Entomol 94:271–286
Leopold RA (1976) The role of male accessory glands in insect reproduction. Annu Rev Entomol 21:199–221
Lewis SM, Austad SN (1990) Sources of intraspecific variation in sperm precedence in red flour beetles. Am Nat 135(3):351–359
Manier MK, Belote JM, Lüpold S, Berben A-HOL, Collins WF, Pitnick S (2013a) Rapid diversification of sperm precedence traits and processes among three sibling drosophila species. Evolution 67:2348–2362
Manier MK, Lüpold S, Pitnick S, Starmer WT (2013b) An analytical framework for estimating fertilization bias and the fertilization set from multiple sperm-storage organs. Am Nat 182:552–561
Marcotte M, Delisle J, McNeil J (2006) Impact of male mating history on the postmating resumption of sexual receptivity and lifetime reproductive success in Choristoneura rosaceana females. Physiol Entomol 31: 227–233
Moriya S (1997) Is it possible to eradicate the two weevil pests of sweet potato, *Cyalas formicarius* and *Euscepes postfasciatus* from Japan? Bull Okinawa Agri Exp Stat 18:19–27
Moriya S, Miyatake T (2001) Eradication programs of two sweet potato pests, *Cylas formicarius* and *Euscepes postfasciatus*, in Japan with special reference to their dispersal ability. JARQ 35:227–234
Nakahara M, Tsubaki Y (2007) Function of multiple sperm-storage organs in female damselflies (*Ischnura senegalensis*): difference in amount of ejaculate stored, sperm loss, and priority in fertilization. J. Insect Physiol 53:1046–1054
Otronen M (1997) Sperm numbers, their storage and usage in the fly *Dryomyza anilis*. Proc R Soc Lond B 264:777–782
Otronen M, Reguera P, Ward PI (1997) Sperm storage in the yellow dung fly *Scathophaga stercoraria*: identifying the sperm of competing males in separate female spermathecae. Ethology 103:844–854
Parker GA (1984) Sperm competition and the evolution of animal mating strategies. In: Smith RL(ed) sperm competition and the evolution of animal mating system. Academic Press, London, pp. 2–60
Pascini TV, Ramalho-Ortigão JM, Martins GF (2013) The fine structure of the spermatheca in *Anopheles aquasalis* (Diptera: Culicidae). Ann Entomol Soc Am 106:857–867
Perez-Staples D, Aluja M (2006) Sperm allocation and cost of mating in a tropical tephritid fruit fly. J Insect Physiol 52:839–845
Perez-Staples D, Aluja M, Marćas-Ordó ñ ez R, Sivinsky J (2008) Reproductive trade-offs from mating with a successful male: the case of the tephritid fly *Anastrepha obliqua*. Behav Ecol Sociobiol 62:1333–1340
Pérez-Staples D, Weldon CW, Radhakrishnan P, Prenter J, Taylor PW (2010) Control of copula duration and sperm storage by female Queensland fruit flies. J Insect Physiol 56:1755–1762
Price T, Lewis Z, Wedell N (2009) Sperm dumping as a defense against meiotic drive. J Biol 8:6–8
Rhainds M (2010) Female mating failures in insects. Entomol Exper Appl 136:211–226
Reinhardt K (2000) Variation is sperm precedence in *Chorthippus* grasshoppers (Caelifera: Gomphocerinae). Physiol Entomol 25:324–329
Reinhardt K (2001) Determinants of ejaculate size in a grasshopper (*Chorthippus parallelus*). Behav Ecol Sociobiol 50:503–510
Reinhardt K, Meister J (2000) Low numbers of sperm retained in the spermatheca may explain the high values of sperm precedence in the migratory locust, *Locusta migratoria* (Latr.). J Insect Behav 13:839–849
Rodriguez S, RL (1998) Mating behavior of two *Pseudoxychila* beetles (Coleoptera: Cicindelidae). Can Entomol 130: 735–750
Sakuratani Y, Sugimoto T, Setokuchi O, Kamikado T, Kiritani K, Okada T (1994) Diurnal changes in micro-habitat usage and behavior of *Cylas fromicarius* (FABRICIUS) (Coleoptera: Curculionidae) adults. Appl Entomol Zool 29:307–315

Schaus JM, Sakaluk SK (2002) Repeatability of sperm number across multiple matings in three cricket species, *Gryllodes sigillatus*, *Gryllus veletis*, and *Gryllus texensis* (Orthoptera: Gryllidae). Can J Zool 80: 582–585

Scott D, Richmond RC (1990) Sperm loss by remating *Drosophila melanogaster* females. J Insect Physiol 36: 451–456

Simmons LW (2001) Sperm competition and its evolutionary consequences in the insects. Princeton University Press, New Jersey

Smith PH, Browne LB, van Gerwen ACM (1988) Sperm storage and utilization and egg fertility in the sheep blowfly, *Lucilia cuprina*. J Insect Physiol 34:125–129

Snook RR (1998) Sperm production and sterility in hybrids between two subspecies of *Drosophila pseudoobscura*. Evolution 52:266–269

Snook RR, Hosken DJ (2004) Sperm death and dumping in drosophila. Nature 428:939–941

Snook RR, Markow TA (1996) Possible role of nonfertilizing sperm as a nutrient source for female drosophila pseudoobcura FROLOVA (Diptera: Drosophilidae). Pan-Pac Entomol 72:121–129

Souza GK, Pikart TG, Zanuncio TV, Wilcken CF, Serrão JE, Zanuncio JC (2014) Reproductive tract histology of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae). Ann Entomol Soc Am 107:853–857

Steiner S, Henrich N, Ruther J (2008) Mating with sperm-depleted males does not increase female mating frequency in the parasitoid *Lariophagus distinguendus*. Entomol Exp Appl 12:131–137

Sugimoto T, Sakkuratani Y, Fukui H, Kiritani K, Okada T (1996) Estimating the reproductive properties of the sweet potato weevil, *Cylas formicarius* (F$_{\text{ABRICIUS}}$) (Coleoptera, Brentidae). Appl Entomol Zool 31:357–367

Thomas DB, Leal SN, Conway HE (2014) Copula duration, insemination, and sperm allocation in *Anastrepha ludens* (Diptera: Tephritidae). Ann Entomol Soc Am 107:858–865

Thornhill R, Alcock J (1983) The evolution of insect mating system. Harvard University Press, Cambridge, Massachusetts

Tsubaki Y, Yamagishi M (1991) "longevity" of sperm within the female of the melon fly, *Dacus cucurbitae* (Diptera: Tephritidae), and its relevance to sperm competition. J Insect Behav 4:243–250

Waage JK (1979) Dual function of the damesfly penis: sperm removal and sperm transfer. Science 203:916–928

Ward PI (1993) Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria* (L.). Behav Ecol Sociobiol 32:313–319

Yamagishi M, Tsubaki Y (1990) Copulation duration and sperm transfer in the melon fly, *Dacus cucurbitae* C$_{\text{OQUILLETT}}$ (Diptera: Tepharitidae). Appl Entomol Zool 25:517–519

Yan S, Li H, Zhang J, Zhu J, Zhang Q, Liu X (2013) Sperm storage and sperm competition in the *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 106:708–715

Yasuda K (1995) Mass trapping of the sweet potato weevil *Cylas formicarius* (Fabricius)(Coleoptera: Brentidae) with a synthetic sex pheromone. Appl Entomol Zool 30:31–36

Yokoi N (1990) The sperm removal behavior of the yellow spotted long horn beetle *Psacothea hilaris* (Coleoptera: Cerabycindae). Appl Entomol Zool 25:383–385

Yuval B, Blay S, Kaspi R (1996) Sperm transfer and storage in the Mediterranean fruit fly (Diptera: Tephritidae). Ann Entomol Soc Am 89:486–492