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Sperm Supply from the Testes to the Seminal Vesicle over Consecutive Matings in the Sweetpotato Weevil, *Cylas formicarius* ($F_{ABRICIUS}$) (Coleoptera: Curculionidae)

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Abstract: The sweetpotato weevil, *Cylas formicarius,* stores free sperm in both the testes and the seminal vesicle, even though the primary function of the testes is sperm production. Here, it was investigated sperm storage in the testes in relation to mating frequency. In particular, it was examined the effect of the positional relationship between the testes and seminal vesicle, on sperm storage by counting the number of sperm in those organs. After mating, not only were the number of free sperm in the seminal vesicle reduced, but also was the number in the testes, suggesting that free sperm stored in the testes moved into the seminal vesicle during or immediately after mating. Since this weevil seems to not regulate the number of sperm in the seminal vesicle for ejaculation, the sperm supply system of the testes may contribute ejaculate to support consecutive, multiple matings by this weevil.

Keywords: Insemination, *Ipomoea batatas*, Reproduction, Spermatogenesis, Sperm Supply System, Sweet Potato

1. Introduction

The morphology of the male reproductive organs differs considerably among insect species [1]. The function of the male reproductive organs, however, is similar in all insect species, in that the testes produce sperm and accessory gland synthetizes and secretes substances, and then an ejaculatory gland and an aedeagus are used to transfer the sperm into females at mating. The testis is a sperm-producing organ, while the seminal vesicle is a sperm storage organ. The positional relationship between the testis and the seminal vesicle, however, differs among species. For example, in the melon fly, *Dacus cucurbitae* (Coquillett) (Diptera: Tephritidae), the seminal vesicle is next to the testis, enclosed in the same membrane [2], while in the dung beetle *Onthophagus catta* (Fabricius) (Coleoptera: Scarabaeidae) there is no seminal vesicle [3].

To the best of our knowledge, few studies have examined the positional relationship between testes and seminal vesicles and the influence of their arrangement on sperm storage. Usually, the testes are connected with the seminal vesicle via the vas deferens. The length of the vas deferens differs in species, resulting in the differences of distance between the testes and the seminal vesicle. In the sweetpotato weevil, *Cylas formicarius* Fabricius) (Coleoptera: Curculionidae), both the testes and the seminal vesicle are very close to each other and both store free sperm [4] The vas deferens of this weevil is connected with ejaculatory duct. The positional relationship between the testes and the seminal vesicle, however, is unclear.

The sweetpotato weevil is a serious pest of the sweet potato, *Ipomoea batatas* (L.) Lam. (Convolvulaceae), in tropical and subtropical countries [5]. This weevil was first

recorded as an introduced pest on subtropical Okinawa Island, south of Japan in 1903, and since then it has spread throughout the neighboring islands (Kohama 1990). In Okinawa and Kagoshima Prefectures, two eradication programs against this pest using a combination of a synthetic female sex pheromones and sterile insect releases has been undertaken respectively [6, 7, ,8], and recently succeeded in Kume Island (Haraguchi et al. unpublished data). To successfully expand this eradication program to other islands, basic information about the male reproduction is essential.

Both sexes of *C. formicarius* mate multiple times, although females are unwilling to remate [9], depending on the volume of ejaculates by males after mating. This weevil is polyvoltine and reproduce all the year except for winter (Kohama 1990). Male adults, if given 10 virgin female adults, mate on average 4 or 5 times per night, irrespective of the male's age [4]. Mean copula duration of 10-day-old male adults (51 min) is significantly shorter than that that of 30-day-old (74 min) and that of 50-day-old (79 min) male adults [4]. Young 10-day-old males ejaculates 7636 sperms on average while 50–day-old adults ejaculates do 61720. The total number of sperm in both testes and the seminal vesicle increases with adult age [4]. As the mass of free sperm in the seminal vesicle increases with adult age (Hiroyoshi, unpublished data), the number of free sperm in the seminal vesicle appears to increase until there is no more capacity. The number of sperm ejaculated increases with adult age at first mating [4]. This suggests that old male adults transfer large numbers of sperm along with accessory gland substances to females, whereas young males transfer fewer sperm. Why the testes store free sperm as well as the seminal vesicle in some insects is generally unknown. Thus, the present study investigated the possibility that male adults of this weevil can supply the free sperm from the testes to the seminal vesicle at consecutive matings, thus allowing them to store more sperm in the testes, and support more matings, than would otherwise be possible.

2. Materials and Methods

2.1. Source and Rearing of Experimental Insects

The colony of sweetpotato weevil used in the present study was established with adults originally collected in the sweet potato field of Yomitan Village (26.23°N, 127.44°E), Okinawa Island (Japan) in October 1998, which had been successively reared on sweet potato tubers at 25 ± 1 °C and a 14:10 h L:D photoperiod. Adults of this weevil feeds the leaves and tubers of sweet potato or other wild host plants, whereas larvae feeds tubers only. Experiments were conducted in 1999.

Approximately total of 500 female and male adults, 2- to 6-weeks old, were reared in plastic containers (14l). Sweet potatoes (Bise variety) (600 g per container), were provided as food and as an oviposition substrate and changed twice a week. Five weeks after removal of sweet potatoes from the oviposition container, eggs had hatched and progeny weevils had reached the pupal stage. Pupae were then collected by dissecting sweet potato tubers carefully, removing intact pupae, and placing them in plastic cups for adult emergence. Since the pupae of this weevil are very soft, some emergent adults were damaged and were discarded. The day of adult emergence was designated as 0-day-old. Newly emerged weevils with similar body size were separated by sex 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.

2.2. Procedure to Obtain Matings

To obtain matings for experimental purposes, a pair of a 15-day-old virgin males and five 20-day-old or older virgin females were placed into a petri dish (dia. 9 cm) 30 minutes before the dark portion of the photoperiod. Mating rate of females and males peak at 10 and 20 days old and level off thereafter [4]. To determine the effect of mating on sperm number, a similar number of unmated males were used as a control. *C. formicarius* is nocturnal and mating takes place at night [3].

To confirm mating, we observed weevils in the darkened room 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 the beginning of copulation. Whether a male actually inserted its penis into the female's genital organs was confirmed under a dissecting microscope attached to an illumination apparatus covered with red cellophane. After one or two times mating, males and females were removed for dissection. The female's reproductive organs were dissected out from each individual in 0.9% salt solution, and the spermatheca was transferred to separate 100 µl of deionized water to check the mating status of each weevil. Females have only one spermatheca, and the presence of sperm in the spermatheca indicated the female had mated.

2.3. Sperm Counts in Male Organs

Sperm counts in the seminal vesicle and testes were made according to the following methods [10,11]. Male *C. formicarius* has a pair of two testes and one seminal vesicle (totally 4 testes and 2 seminal vesicles), all of which may contain free sperm (See Figure. 1), which were then placed in separate dishes containing 500 µl of deionized water and each tissue was torn up in water into small pieces. The water containing free sperm was stirred 20 times with to homogenize the sample. The testes and seminal vesicle dissected out in 0.9% salt solution was immersed in 0.1% triton X solution and the fat body was carefully removed with a piece of tissue paper and a forceps using the method by K. Okumura (Per. Com.). A ring of a less than 1 cm string taken from strings was made using a pair of forceps under a microscope. The testes and seminal vesicle were ligated by a

ring of a string, and separated. Ten µl of solution was collected with a microsyringe and spread onto a glass slide and allowed to dry. Sperm counts were made at magnitude ×100 using a counter on 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 number of sperm per half testes versus the seminal vesicle.

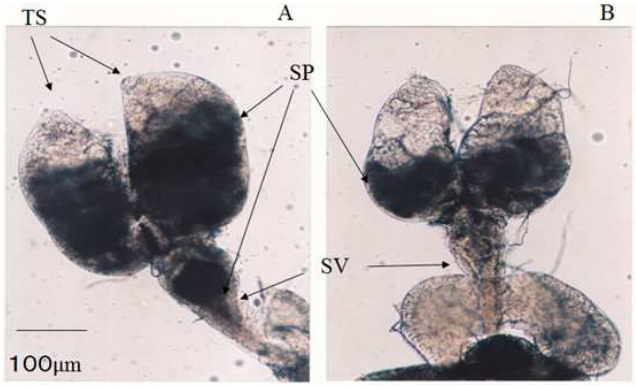

Figure 1. *Photographs of the testes and seminal vesicle in a virgin male adult Cylas formicarius (A) and a once mated male adult (B). SP, TS and SV indicate sperm, testis, and seminal vesicle, respectively.*

2.4. Statistical Analyses

We compared the number of sperm in the testes and the seminal vesicle in relation to mating times (0-2). As we used the same individuals for examining the number of sperm in the testes and the seminal vesicle, sample size was the same. Data on the number of sperm in the testes and the seminal vesicle were compared by mating times analyzed by Kruskal-Wallis method after ANOVA.

3. Results

The number of sperm in both the testes and the seminal vesicle in a male that mated once (Figure 1B) was greatly reduced compared with that in a virgin male (Figure 1A). The sperm in the seminal vesicle was almost empty. This was confirmed by dissection (Figure 2 and Figure 3). The number of free sperm found in testes decreased significantly with the number of matings, and there was a significant difference between virgin males and males that had mated twice ($H = 53.50961$, $df = 29$, $P<0.0001$) (Figure 2). The male seminal vesicle had significantly smaller numbers of free sperm after the first one or two matings compared to virgin males ($H = 50.480764$, $df = 29$, $P<0.001$) (Figure 3).

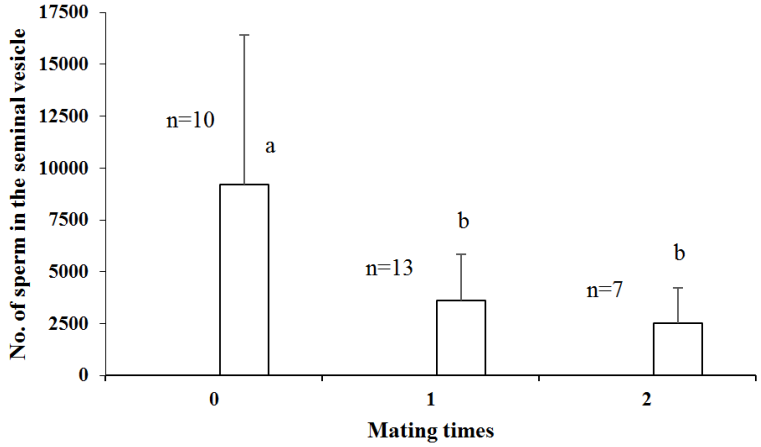

Figure 2. *Comparison of the number of free sperm in the testes between virgin and mated Cylas formicarius. The data was represented as mean±SD. Different letters indicate significant differences.*

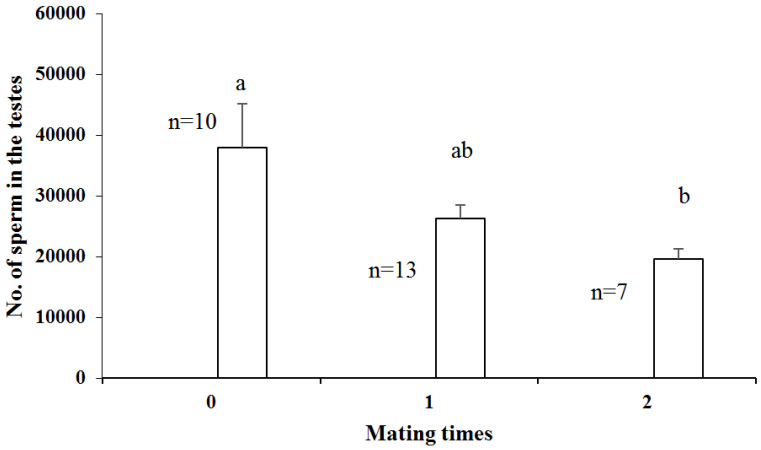

Figure 3. *Comparison of the number of free sperm in the seminal vesicle between virgin and mated Cylas formicarius. The data was represented as mean±SD. Different letters indicate significant differences.*

4. Discussion

A reduction in the number of sperm in the seminal vesicle following mating could undoubtedly be due to ejaculation. The presence of small numbers of sperm in the seminal vesicle after mating strongly suggests the possibility that sperm in the testes moved into the seminal vesicle during or immediately after mating and that these sperm would be used in subsequent mating, confirmed by the fact that the number of free sperm in the testes also decreased following mating.

We observed the influx of sperm from the testes to the seminal vesicle *in vitro*, although this might be an artificial phenomenon. Such a phenomenon, however, was not observed in virgin (control) males. This sperm supply mechanism seems to be useful to avoid sperm depletion in the seminal vesicles, allowing for successful consecutive matings. The phenomenon that sperm from both the testes and the seminal vesicle were used for ejaculation is the first such report. At least for this species, the testes appear to have the additional function of sperm supply during consecutive matings.

Hiroyoshi et al. [4] determined that the older males were at first mating, the greater the number of ejaculated sperm. On the other hand, because *C. formicarius* females' spermatheca has only a limited ability to accommodate sperm, excess sperm cannot enter the spermatheca and are expelled from the reproductive organs after mating [4]. As suggested in the present study, the number of sperm in the seminal vesicle decreased considerably after mating. These results, together with the results that ejaculated sperm number increased with age at first mating, suggest that male *C. formicarius* cannot regulate the number of sperm ejaculated. As the testes are right next to the seminal vesicle, the testes can supply sperm into the seminal vesicle immediately, suggesting that the positional relationship between the testes and the seminal vesicle is very important in this weevil. Although the research on spermatogenesis and morphology of male reproductive organs has been proceeding in the last two decades [12, 13, 14, 15, 16], in the present study, it has been highlighted on the positional relationship and its function between the testes and the seminal vesicle for the first time, and many unknown phenomenon, like sperm reflux [17], remains to be found on male reproduction.

5. Conclusion

The Sweetpotato weevil *Cylas formicarius* males store the free sperm in both testes and seminal vesicles, both of which are connected to each other directly. At consecutive matings, the sperm in the testes migrate to the seminal vesicle, used for the next mating. The positional relationship between the testes and seminal vesicle is very important for sperm transfer.

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