Appl Entomol Zool (2012) 47:245–254
DOI 10.1007/s13355-012-0116-8

ORIGINAL RESEARCH PAPER

The significance of multiple mating and male substance transferred to females at mating in the white grub beetle, *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae)

Ken-ichi Harano · Makoto Tokuda · Toyomi Kotaki · Fumiko Yukuhiro · Seiji Tanaka · Nao Fujiwara-Tsujii · Hiroe Yasui · Sadao Wakamura · Atsushi Nagayama · Yasuhiro Hokama · Norio Arakaki

Received: 20 April 2012 / Accepted: 7 June 2012 / Published online: 8 July 2012

Abstract Males of the white grub beetle, *Dasylepida ishigakiensis* Niijima et Kinoshita, transfer a large amount of a colloidal substance to females during mating. In this study we investigated the effect of the male substance on the reproductive performance of mated females and the significance of multiple mating of this beetle. Females artificially separated from the males 5 min after the start of mating produced fewer eggs than those separated after 30 min or those that were undisturbed and separated spontaneously, suggesting that the male substance is used as a nutrient for egg production by the females. When females were allowed to mate with 1–4 males, multiple mating had no clear effect on reproductive performance. The amount of male substance stored in the bursa copulatrix (BC) was not significantly increased by a second mating. The functions of multiple mating of this species may be to provide a chance for females to obtain sufficient amounts of male substance when the first male to mate has only small amounts of this substance, and to increase the genetic heterogeneity of the progeny. The presence of a serine proteinase and its possible involvement in the dynamics of the BC contents are reported.

Keywords Male reproductive accessory gland · Multiple mating · Nuptial gift · Paternal investment · Proteinase

K. Harano · M. Tokuda · T. Kotaki · F. Yukuhiro · S. Tanaka · N. Fujiwara-Tsujii · H. Yasui · S. Wakamura
Kebuka Project Team, National Institute of Agrobiological Sciences at Ohwashi (NIASO), Tsukuba, Ibaraki 305-8634, Japan

K. Harano (✉)
Honeybee Science Research Center, Tamagawa University, Machida, Tokyo 192-0364, Japan
e-mail: kharano@lab.tamagawa.ac.jp

M. Tokuda
Laboratory of System Ecology, Faculty of Agriculture, Saga University, Saga, Saga 840-8502, Japan

S. Wakamura
Faculty of Bioenvironmental Sciences, Kyoto Gakuen University, Kameoka, Kyoto 621-8555, Japan

A. Nagayama · Y. Hokama · N. Arakaki
Okinawa Prefectural Agricultural Research Center (OPARC), Itoman, Okinawa 901-0336, Japan

Introduction

Insect males often transfer not only sperm but also secretions of the reproductive accessory glands to females during mating. Such substances may have a variety of effects on the physiology and behavior of the females (Gillott 2003). For example, biologically active agents contained in the male secretion reduce the mating acceptability and stimulate oviposition in females (Yamane et al. 2008a, b; Yamane and Miyatake 2010). Females may use male secretion as a nutrient for egg production and other activities. It has been reported that molecules of male-derived material deposited in the female during mating are incorporated into oocytes developing in her ovaries, and into her somatic cells (Rooney and Lewis 1999; Hayashi and Kamimura 2002). In some insects, male-derived substances are harmful to females. Males of the fruit fly *Drosophila melanogaster* Meigen deposit secretions which kill sperm from other males and reduce the life span of females (Partridge and Farquhar 1981; Fowler and Partridge 1989; Chapman et al. 1993, 1995). Males of the white grub beetle *Dasylepida ishigakiensis* Niijima et Kinoshita, a serious sugarcane pest in the Miyako Islands, Okinawa, Japan (Sadoyama et al. 2001), transfer a large amount of

secretion to females during mating (Tanaka et al. 2008). The function of the secretion is unknown.

The life cycle of *D. ishigakiensis* is semi-voltine and seems to create special problems, i.e. limited energy reserves and mating activity, which might affect the reproductive strategy of this beetle. Larvae feed on roots and underground stems of sugarcane (Oyafuso et al. 2002). They reach the last (3rd) stadium in the autumn of the first year and stop feeding by early summer of the second year when summer diapause is induced (Oyafuso et al. 2002; Tanaka et al. 2008). They terminate diapause and pupate in response to the low temperature in the autumn. Newly emerged adults enter a brief reproductive diapause that is terminated by low temperatures in the winter (Tanaka et al. 2008). The gonads of the insects develop slowly during the winter and the adults emerge from the soil for mating in February. Because feeding ceases early in the summer, they spend a total of approximately 9 months without feeding before mating at a mean temperature of 25.5 °C. Furthermore, this beetle does not feed as an adult (Arakaki et al. 2004) and its reproductive activity must depend almost entirely on the energy resources sequestered during the larval stage. Females captured at mating had many immature eggs in the ovaries, indicating that a substantial proportion of eggs develop after mating (Tanaka et al. 2008). These observations may suggest that any additional nutrient given by the males during mating is likely to enhance egg production by the female.

In this study, we examined the possibility that *D. ishigakiensis* females use the male substance as a nutrient. A previous study showed that only a small amount of secretion was transferred from males to females during the first 5 min of mating (Harano et al. 2010b). We varied the amount of male secretion transferred to females by interrupting their mating at different times and tested the effects of different amounts of male secretion on fitness-related characteristics including fecundity, longevity, and progeny body size in the females.

Multiple mating of females has previously been reported for this beetle (Harano et al. 2010b). Some mating pairs are accompanied by an extra male which remain on the back of the mating female. This extra male mated with the female soon after the first male withdrew its copulatory organ from the female genitalia. There are mating pairs accompanied by 2 extra males, and it is suggested that females may mate with 3 males in one evening, although mating activity seems to be limited to a few hours in the evening. The total amount of male substance received by a female may be increased by successive mating with several males (Harano et al. 2010b). We thus examined the effect of multiple mating on female reproduction in the present study.

The male substance transferred to females is stored in the bursa copulatrix (BC). It has been observed that BC contents decreased after mating of females. To clarify the time course of this decrease, BC contents of mated females was quantified at different times after mating. In addition, mated females were observed to determine how the reduction in BC contents was brought about. One possibility is expulsion of the male substance from the BC by the female. Another possibility is enzymatic degradation of the male substance. Most of the male-derived material transferred to females is proteinaceous (Gillott 2003) and females possibly degrade the material with a proteinase. In this study we investigated these possibilities. Proteinase activity in the BC, spermatheca, and spermathecal glands of females and that in the reproductive accessory glands of males were examined. The spermathecal gland is a small sac-like organ situated at the medial oviduct adjacent to the BC. Its function is unknown in *D. ishigakiensis*.

Materials and methods

Experiment 1: manipulation of the quantity of male secretion transferred to females by separation of mating pairs

To examine the effects of different amounts of male substance transferred to the females on the reproductive performance of *D. ishigakiensis* females, mating was interrupted at different times by separating mating pairs. Adults were obtained by rearing individuals captured as 3rd (last) instars on Miyako Island (24°47′N; 125°17′E), Okinawa, Japan, in February 2009. The larvae were individually housed in plastic cups containing humus and fed a piece of sugarcane stem every 3 weeks, as described elsewhere (Harano et al. 2010a). Adults were kept at 20 °C for 2 months after eclosion for sexual maturation (Tanaka et al. 2008). They were then transferred to L:D 8:16 (L 08.00–16.00 h) at 20 °C to acclimatize them to the light–dark cycles for 3 days before experiments.

Approximately 10 pairs of virgin males and females were held in a plastic container (41 × 25 × 11 cm) and allowed to mate at low light intensity (approximately 0.03 W/m^2) and room temperature (ca. 26 °C). Immediately after mating started, the pairs were individually transferred to a 9-cm Petri-dish and each pair was separated 5 or 30 min later by gently tapping their bodies. A previous study showed that only partial transfer of male substance occurs during the first 5 min of mating (BC contents, 1.1 mg) and that 30 min is required for complete transfer (BC contents, 5.4 mg) (Harano et al. 2010b). Some mating pairs were left undisturbed as controls until they separated spontaneously.

Mated females were individually kept in plastic cups with moist humus in darkness at 20 °C and checked for

oviposition every day until they died. Dead females were removed and dissected to determine the number of eggs left in the ovary. Deposited eggs were counted and kept under the same conditions until hatching. Hatched larvae were weighed with an electric balance and removed from the cup to prevent cannibalism within 24 h of hatching. Larvae that have started feeding can be recognized by the presence of food in their digestive tracts, because of their semi-transparent body; such larvae were not included in the analysis. For each female adult the pre-oviposition period (time from mating to oviposition), longevity after mating and after oviposition, numbers of eggs deposited and those left in the body at death, total numbers of eggs produced, proportion of eggs deposited to total number of eggs produced, and hatchability were determined.

Experiment 2: mating with different numbers of males

To determine the effects of multiple mating on the reproductive performance of females, the observations described above were made and compared among female adults mated with 1–4 males. Female adults were hand-collected as mating pairs in sugarcane fields near the Miyako-jima Branch of Okinawa Prefectural Agricultural Research Center (MB-OPARC) from 19.00 to 20.00 h in February 2010. The pairs were placed individually in small plastic cups and brought to MB-OPARC. Soon after mating ended, the males were removed from the cups to prevent re-mating. We believed that this was the first mating of these females because (1) only virgin females appear on the ground for mating (Arakaki et al. 2004); (2) they start mating at approximately 18.50 h; (3) copulation lasts for 1–3 h (Arakaki et al. 2004); and (4) a 2nd mating may occur but only after 21.00 h (Harano et al. 2010b).

Mated females were either kept individually in cups with moist humus, to enable oviposition, or subjected to additional matings. A 2nd mating in the same evening was made by introduction of two sexually active males to naturally mated females in cups. After pairing with one male, the other male was removed from the cup. Some females were allowed to mate with 3rd and 4th males within 5 days of first mating. The males used for multiple mating were obtained by use of a funnel trap baited with (*R*)-2-butanol, a female sex pheromone of this insect (Wakamura et al. 2009a, b), in sugarcane fields on the day of mating trial or a few days earlier. The mating pairs were left undisturbed and the male was removed soon after copulation ended. The same males were not used more than once. The females that mated with 1–4 males were individually housed in cups with humus and transported by air to the Tsukuba laboratory. They were kept at 20 °C in darkness and the same observations were made as described in experiment 1.

To investigate the effects of multiple mating on the amount of male substance stored in the female BC, virgin females were allowed to mate with one or two males and dissected at MB-OPARC approximately 1 h after the last mating. The BC was removed from each female and weighed. BC contents were calculated by subtracting the weight of the BC tissue after squeezing the contents out, by use of a pair of tweezers, from the weight of the BC plus contents (Harano et al. 2010b).

Experiment 3: outdoor observations of multiple mating

We have previously reported that a small proportion of *D. ishigakiensis* females mated with 2 males successively in one night (Harano et al. 2010b). In that work we observed some females mating with a male were accompanied by 2 extra males on the back of the mating female (see Fig. 4d in Harano et al. 2010b for a photograph of a mating pair with 2 extra males). However, we did not observe whether the last (3rd) males successfully mated with the females. In this study, we observed mating pairs with extra males in sugarcane fields to determine whether females mated with 3 males during the same night and, if so, how often that occurred in the field. After beetles formed mating pairs on sugarcane plants at approximately 19.00 h, we counted the number of mating pairs with and without extra male(s) in a sugarcane field of 800 m^2 on 7 and 10 February 2010. A piece of numbered vinyl tape was placed on a leaf near a mating pair for identification. The mating pairs were observed continuously or every 10–20 min for mating with extra males. We observed each mating pair until all extra males mated, or the female or all males disappeared from the plant.

Experiment 4: BC contents at different times after mating

Sexually mature virgin adults reared as described above were transferred to 25 °C and L:D 8:16 (L 04.00–12.00 h) more than 1 day before experiments to acclimatize them to the light–dark cycle. Individual pairs of a female and a male were placed in small plastic cups at room temperature (ca. 26 °C) shortly before light off (12.00 h) and covered with a light-proof cloth to stimulate their mating activity. Mating pairs were left in the cups until they spontaneously separated. Females were then transferred to another cup with moist humus and kept at 25 °C. They were killed to determine the BC contents 1, 6, 9 and 24 h after mating.

Experiment 5: observations to monitor expulsion of male substance by mated females

The possibility that the decrease in the amount of male substance stored in the female BC is a result of expulsion of the substance by females was tested. Mated females were obtained as in experiment 1 and held individually on filter paper (Whatman no. 1, Springfield Mill, UK) placed on the floor of 9-cm Petri dishes immediately after mating ended. The females were maintained at 20 °C for 7 days after mating, then the filter paper was checked for expulsion. If expulsion of male substance occurred, it should be detectable as blots on the filter paper.

Experiment 6: detection of proteinase activity

To understand how the amount of male substance stored in the BC is reduced in female *D. ishigakiensis*, proteinase activity was examined in the female reproductive organs by use of gelatine-coated plastic film (MMP in-situ Zymo-Film; Wako Pure Chemical Industries, Osaka, Japan). Here we call this method "film zymography (FZ)"; it is a modification of film in-situ zymography described in a previous paper (Kotaki 2005). The BC, spermatheca, and spermathecal glands were removed from females which had not mated (virgin females) or which had ended copulation 2, 24, or 48 h before dissection. Reproductive accessory glands were also removed from sexually mature virgin males. Each organ was placed in a 1.5-ml tube with 20 µl extraction buffer (0.1 M Tris–HCl, pH 8.5, containing 0.25 M NaCl, 0.1 % Triton-X 100) and homogenized with a plastic pestle. After centrifugation of the homogenates with a portable centrifuge (Chibitan; Merck Millipore, USA) at approximately 2000 x G for 3 min, 1 µl supernatant was applied to the gelatine-coated film and the film was kept for 2 h under humid conditions in a small box at 30 °C. The gelatine on the film was then stained with a Biebrich scarlet staining solution (Wako). Because proteinases, if any, digest the gelatine layer, proteinase activity can be detected as a white spot with a red background on the film.

The family of proteinases found in females was then investigated by examining the effect on enzymatic activity of different proteinase inhibitors. Leupeptin (cysteine and serine proteinase inhibitor), chymostatin (chymotrypsin inhibitor), AEBSF (serine proteinase inhibitor), and cathepsin G inhibitor were dissolved in DMSO, and soybean trypsin inhibitor was dissolved in Milli-Q water, and 100 µg/ml solution was obtained for each inhibitor. E-64 (cysteine proteinase inhibitor) was dissolved in DMSO to give 1 mM solution. One microliter inhibitor solution or DMSO was mixed with 1 µl supernatant taken from homogenate of female spermathecal glands before being applied to the film. The activity of the proteinase was then examined for each treatment as described above.

Statistics

In experiments 1 and 2, each value was compared among females in different treatments by use of a Kruskal–Wallis test followed by the Steel–Dwass multiple comparison test at the 5 % significance level. The same statistical tests were used to evaluate the change in mass of the BC contents of females after mating (experiment 4). In experiment 2 the effect of multiple mating on BC contents was tested by comparing BC contents between females mated once and those mated twice, by use of the Mann–Whitney *U* test at the 5 % significance level.

Results

Experiment 1: effects of separation of mating pairs

Early separation of mating pairs affected oviposition, egg production, and the number of hatched larvae (Fig. 1a). Females separated from the male 5 min after the start of mating deposited 15.6 ± 6.6 eggs (mean ± SD) and this value was significantly increased when separation was delayed until 30 min (mean ± SD, 23.7 ± 9.7). No further increase was observed even when mating remained undisturbed (mean ± SD, 27.6 ± 7.2). The same pattern of differences was found for total egg production, including eggs deposited and those left in the ovaries at death, and for the number of hatchlings. Although the pre-oviposition period was approximately 3 days shorter for females separated at 5 min than for those separated at 30 min and for undisturbed controls, no significant difference was found in adult life span either after mating or after the start of oviposition among the 3 groups (Table 1). Females separated at 5 or 30 min had more eggs in their body at death (Table 1) and the number of eggs deposited was lower than for undisturbed control females (Fig. 1b), indicating that artificial separation affected oviposition. Number and body weight of hatchlings were not significantly affected by artificial separation of mating pairs.

Experiment 2: effects of multiple mating

Total egg production, egg deposition, and number of hatchlings produced by each female increased slightly as the number of matings increased. However, the Kruskal–Wallis tests revealed that the effects of the number of matings were only marginally significant for total egg production and egg deposition and not significant for the number of hatchlings produced. No significant difference

between any of these data among the groups was detected with the Steel–Dwass multiple comparison test ($P > 0.05$) (Fig. 2). The number of males with which a female mated did not significantly affect the other data except for the pre-oviposition period, which was longer as females mated with more males (Table 2).

Whereas the BC of virgin females was empty ($N = 3$), that of singly mated females contained a large amount (mean ± SD, 9.3 ± 4.6 mg; $N = 6$) of colloidal substance. A second mating shortly after the first mating did not increase the BC contents significantly (10.3 ± 3.2 mg; $N = 7$) (Mann–Whitney's U test, $Z = 0.57$, $P = 0.57$, n.s.).

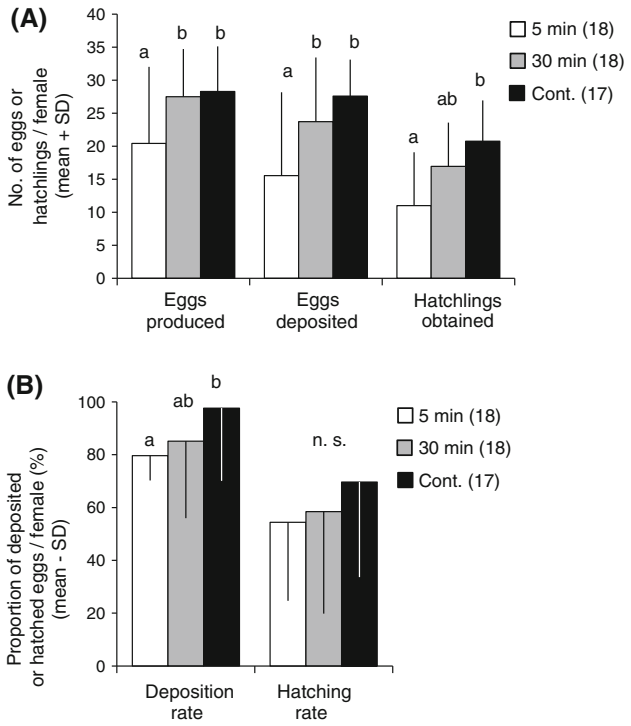

Fig. 1 Effect of interruption of mating after 5 or 30 min on total egg production, egg deposition, and the number of hatchlings (**A**) and the proportions of deposited eggs and hatched larvae (**B**) for a female *D. ishigakiensis*. See text for details. *Different letters on bars* denote a statistically significant difference at the 5 % level (Steel–Dwass test). *n.s.* non-significant, *Cont.* control. Sample sizes are shown in *parentheses*

Experiment 3: multiple mating in the field

Of 8 mating pairs found with extra male(s) on 7 February 2010, 5 were accompanied by one extra male and 3 by 2 extra males. Two females successively mated with the 2nd male but no female mated with the 3rd male on the same night (Table 3).

On 10 February 2010, 22 mating females with or without extra male(s) were observed in another sugarcane field. Thirteen mating pairs were with no extra male, 7 with 1 extra male, and 2 with 2 extra males. Table 3 shows the number of matings observed for each female. More than 25 % of females mated with 2 or 3 males. They had been accompanied by 1 or more extra males on their back. No multiple mating was observed for females without extra males. Of 22 females observed, only 1 female mated with 3 males and this individual was accompanied by 2 extra males.

Extra males were almost motionless on the back of other individuals until the first mating ended. Soon after the first mating male withdrew its copulatory organ from the genitalia of the female, the male on her back (2nd male) copulated with her. No struggle or aggressive behavior was observed among the males, including the male which had just finished mating. Although none of the females seemed to resist those males, as reported previously (Harano et al. 2010b), not all extra males succeeded in mating with the females. Some males left the mating pair by walking or dropping before mating with the first male ended; others moved down to the leaf and stayed there even after the female had disappeared. The 2-day observations indicated that mating success for the 2nd male, which mounted the back of a mating female ($N = 14$), was 42.9 % and that of the 3rd male, which mounted the back of the 2nd male ($N = 5$), was 20 %.

Table 1 Effects of separation of mating pairs on different fitness-related data for *D. ishigakiensis* females

	Control (mean ± SD)	30 min (mean ± SD)	5 min (mean ± SD)	$P^{\dagger}$
N	18	17	17	
Pre-oviposition period (days) (A)	53.0 ± 5.4^{a}	53.7 ± 8.0^{ab}	50.0 ± 18.8^{b}	0.03
Longevity after oviposition (days) (B)	18.5 ± 8.4	19.0 ± 11.0	17.1 ± 10.1	0.84
Longevity after mating (days) (A + B)	71.5 ± 9.8	72.7 ± 14.3	66.8 ± 14.8	0.21
No. of eggs found in dead females	0.7 ± 2.8^{a}	3.8 ± 7.2^{ab}	4.9 ± 7.3^{b}	0.02
Fresh weight of hatchlings (mg)	8.2 ± 0.5	8.3 ± 0.7	8.5 ± 0.7	0.49

Different letters adjacent to values indicate statistically significant differences at the 5 % level (Steel–Dwass test)

† P values calculated by use of the Kruskal–Wallis test

Experiment 4: the time course of changes in BC contents after mating

Mated females had 5.3 mg of colloidal substance in the BC 1 h after mating and the amount did not change significantly during the following 8 h (Fig. 3). The value decreased significantly by the 24th hour after mating.

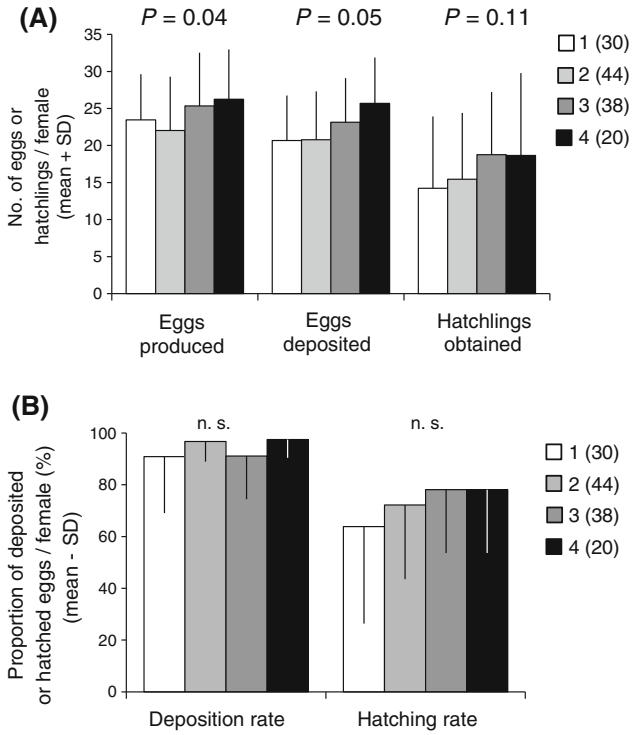

Fig. 2 The effect of the number of males mating with a focal female on total egg production, egg deposition, and the number of hatchlings (**A**) and on the proportion of deposited eggs and hatched larvae (**B**) for females of *D. ishigakiensis*. See text for details of calculation of results. *P* values are from the Kruskal–Wallis test. The Steel–Dwass post-hoc test failed to detect significant differences ($P > 0.05$) among female groups for the 3 values. *n.s.* non-significant. Sample sizes are shown in *parentheses*

Experiment 5: no expulsion of male substance by mated females

None of the 8 females observed during the 7 days after mating made any stain on the filter paper in the Petri dish hatchlings obtained, indicating that the reduction in the contents of the BC is not because of expulsion of male substance by the mated females.

Experiment 6: proteinase activity in virgin and mated females and virgin males

Proteinase activity was detected in samples from the spermathecal glands of virgin females with developed ovaries approximately 60 days after adult emergence, whereas no proteinase activity was detected in the spermathecal glands of females of similar age with undeveloped ovaries. For mated females, activity was detected not only in those glands but also in the BC (Fig. 4b). No temporal change in proteinase activity was apparent after mating. Weak proteinase activity was observed in the spermatheca 2 h after mating; this activity disappeared within 24 h, although for one replicate a weak signal was observed 48 h after mating. No evidence was obtained of proteinase activity in male accessory glands.

Of 6 proteinase inhibitors applied, leupeptin and AEBSF inhibited proteinase activity in samples from female spermathecal glands, and E-64, a specific cysteine proteinase inhibitor, did not affect gelatin degradation (Fig. 4c). Because leupeptin is an inhibitor of cysteine and serine proteinases and AEBSF is a specific inhibitor of serine proteinases, the proteinase activity found in the spermathecal glands was that of a serine proteinase. On the other hand, although trypsin, chymotrypsin, and cathepsin G are serine proteinases, specific inhibitors of these proteinases were not effective. Therefore, the proteinase found in spermathecal gland samples belongs to the serine proteinase family, but they were not likely to be trypsin,

Table 2 Effects of multiple mating on different fitness-related data for *D. ishigakiensis* females

	Number of matings				$P^{\dagger}$
	1 (mean ± SD)	2 (mean ± SD)	3 (mean ± SD)	4 (mean ± SD)	
N	30	44	38	20	
Pre-oviposition period (days) (A)	18.5 ± 8.8[a]	22.5 ± 5.4[b]	20.4 ± 6.5[ab]	24.9 ± 7.4[b]	<0.001
Longevity after oviposition (days) (B)	16.5 ± 9.8	15.1 ± 7.0	13.5 ± 6.8	11.2 ± 7.2	0.12
Longevity after mating (days) (A + B)	35.0 ± 8.4	37.5 ± 7.7	33.9 ± 8.1	36.0 ± 9.1	0.21
No. of eggs left in dead female	2.8 ± 6.9	1.2 ± 4.0	2.2 ± 4.5	0.6 ± 1.6	0.68
Fresh weight of hatchlings (mg)	8.4 ± 0.8	7.9 ± 1.5	8.5 ± 0.5	8.6 ± 0.7	0.08

Different letters adjacent to values indicate statistically significant differences at the 5 % level (Steel–Dwass test)

† *P* values calculated by use of Kruskal–Wallis test

Table 3 Frequency of multiple mating of *D. ishigakiensis* females in an 800 m^2 sugarcane field on Miyako Island on 7 and 10 February 2010

	Mated with			Total (%)
	1 Male (%)	2 Males (%)	3 Males (%)	
Number of females				
7 February	6[a]	2	0	8[a]
10 February	16 (72.7)	5 (22.7)	1 (4.6)	22 (100)

[a] The number of females which were not accompanied by extra males but mated with 1 male is not included in the data obtained on 7 February. See text for details

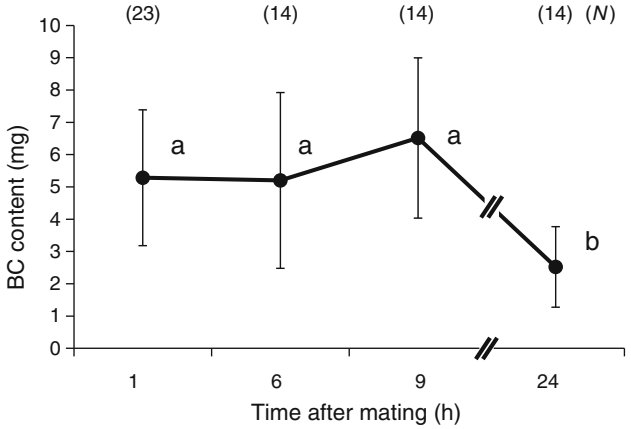

Fig. 3 Change in bursa copulatrix (BC) contents of female *D. ishigakiensis* after mating. *Different letters* denote statistically significant differences at the 5 % level (Steel–Dwass test). *Vertical bars* indicate SDs

chymotrypsin, or cathepsin G. This experiment was repeated several times and the same results were obtained in the replicates.

Discussion

The role of male substance

Females that were separated from the male 5 min after the start of mating produced significantly fewer eggs than did those that were left undisturbed until mating ended naturally. This difference is unlikely to be related to physical damage as a result of artificial separation of mating pairs because similarly separated females 30 min after the start of mating produced as many eggs as the undisturbed controls. The reduced egg production after early separation was probably because of the reduced amount of male substance transferred to the females (Harano et al. 2010b). However, the exact physiological mechanism underlying increased egg production as a result of prolonged copulation is unknown. In other insects, male-derived substances are incorporated into developing oocytes (Rooney and Lewis 1999; Hayashi and Kamimura 2002). Unlike many other insects, *D. ishigakiensis* do not eat any food as adults (Arakaki et al. 2004) and experience a long non-feeding period of as long as 9 months after cessation of feeding at the 3rd larval stadium (Tanaka et al. 2008). Mated females return to the soil for oviposition but they normally do not start laying eggs until a few weeks later. Under such circumstances, the male substance transferred to females may serve as a nuptial gift and is likely to be used as a precious nutrient for developing eggs.

No evidence was found for any significant effect of the male substance on the longevity of *D. ishigakiensis* females. Non-feeding females, for example *D. ishigakiensis*, may mainly allocate the male-derived nutrients to reproduction, whereas feeding females may allocate it to somatic reserves and maintenance (Rooney and Lewis 1999). The experiment with artificial separation and that with forced multiple mating did not provide clear evidence indicating a detrimental effect of the male substance on females in this beetle. Although the male substance had a delaying effect on the start of oviposition, this delay is slight and does not seem to be important to the females that stay underground until they die in the field. The predation risk there is probably low. It is also unlikely that oviposition delayed by a few days affects survival of their larvae, which have a developmental period as long as one and a half years.

Both early interruption of mating and forced multiple mating affected the pre-oviposition period in *D. ishigakiensis* females. This result might indicate the presence of some bioactive agent in the male substance that causes females to delay ovipositing. However, nothing is known about the identity of such a compound and the biological significance of this effect.

Possible mechanism of assimilating male substance in females

The fact that gradual reduction of male substance in the BC was not caused by expulsion by the female after mating may suggest another mechanism. Although male-derived material, for example spermatophore, is known to be disintegrated in the female body (Rooney and Lewis 1999),

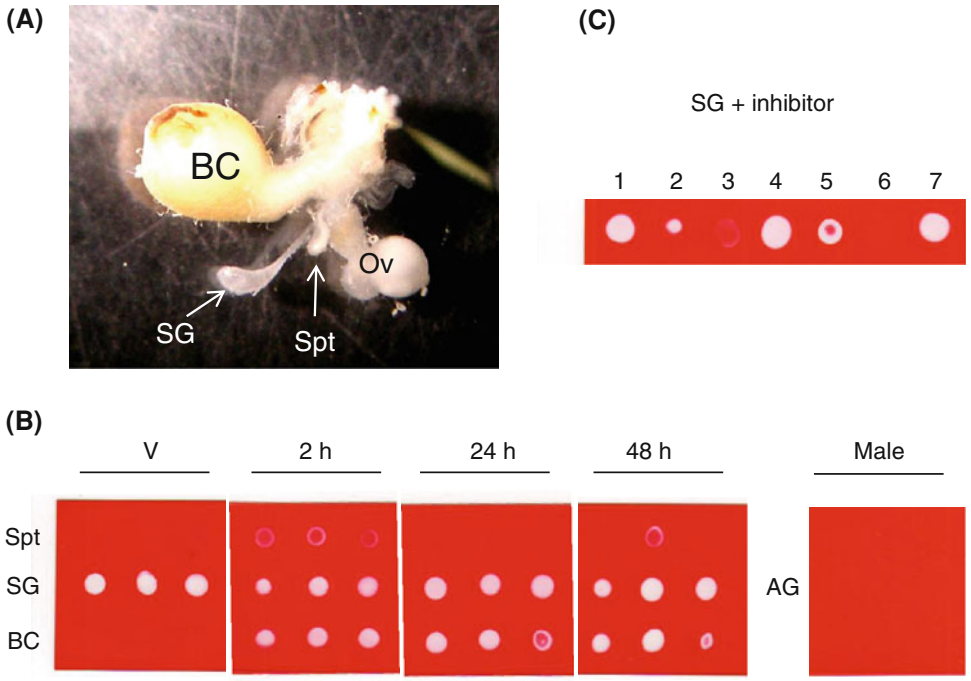

Fig. 4 Reproductive organs of female *D. ishigakiensis* (**A**), proteinase activity detected by film zymography in homogenates of reproductive organs of virgin and mated females 2–48 h after mating and of the reproductive accessory gland of virgin males (**B**), and the effect of 6 proteinase inhibitors on the proteinase activity found in homogenates of the female spermathecal gland (**C**). *White spots* indicate proteinase activity (**B**, **C**). The proteinase activity is shown in triplicate for different individuals (**B**). *BC* bursa copulatrix, *Ov* ovary, *Spt* spermatheca, *SG* spermathecal gland, *V* virgin female, *AG* male accessory gland. *1* DMSO (solvent control), *2* E-64 (cysteine proteinase inhibitor); *3* leupeptin (inhibitor for cysteine and serine proteinases), *4* chymostatin (chymotrypsin inhibitor), *5* trypsin inhibitor, *6* AEBSF (serine proteinase inhibitor), *7* cathepsin G inhibitor

the mechanism involved in the digestive process is not understood. Our FZ experiments suggested that a serine proteinase is present in BC samples from mated females. This proteinase is likely to be important in the degradation of the male substance because proteins are the major components of male secretion from the accessory gland in insects (Gillott 2003). No proteinase activity was found in the BC of virgin females and in the secretion of the male accessory gland, whereas activity was observed in the spermathecal glands as the ovaries developed, irrespective of mating status. Therefore, this organ is probably the source of the proteinase responsible for degradation of the male substance in this beetle, and the enzyme is likely to be produced in parallel with ovary development and transferred to the BC after mating. Another explanation is that the BC tissue is stimulated by mating to produce the proteinase. No data are available to conclude which explanation is more likely. In preliminary observations, we found that the male substance in the BC was lost within 10 days, leaving some darkened, sticky substance behind in the organ (Tanaka et al. unpublished observations). It is possible that females absorb and assimilate the digested liquid material, contributing to enhanced egg production.

Significance of multiple mating

The occurrence of multiple mating by *D. ishigakiensis* was confirmed by this study. However, no significant difference was detected between fecundity-related data for females that mated once and twice. This result may be because females had already obtained a sufficient amount of male substance (9.3 mg) in the BC after the first mating and the BC was fully expanded by the male substance. It is possible that the BC contents cannot be further increased by an additional mating because of the capacity of the BC. However, this result is not consistent with our previous study of this beetle (Harano et al. 2010b) in which a 2nd mating increased the BC content to 10 mg from approximately 6 mg after the first mating. The discrepancy between the two studies is hard to explain. One likely explanation is that in the previous study, males might have been exhausted because of air-transportation over 2000 km from Miyako Island to the Tsukuba laboratory and only small amounts of male substance were transferred to females. Relatively small amounts of BC content were also encountered in the present study with shipped males (Fig. 3). Another possibility is that variation in the amount of male substance among males depends on previous

mating experience, because males appear on more than one evening for mating in the field (Arakaki et al. 2004).

D. ishigakiensis females that have been forced to mate with additional males over several days tend to produce more eggs than those that mated only once. Although this result provides support for the nutritional function of the male substance in mated females, the enhanced egg production caused by multiple mating in this experiment has probably no ecological significance. In the field, after mating, females go underground until dawn (Harano et al. 2010b) and do not appear above the ground for mating again (Arakaki et al. 2004). Therefore, they normally have no chance of additional mating once they return to the soil. This study showed that the BC contents did not change during the first 9 h after mating but declined by half by the following day. Because females were allowed to mate with 3rd and 4th males a few days after the 2nd mating in the multiple mating experiment, their BC may have had some room to receive an additional amount of male substance. The additional deposition of male substance by the 3rd and 4th males probably resulted in enhanced egg production by females.

The possibility that *D. ishigakiensis* females enjoy some benefit from successive mating with 3 or 4 males during the same night cannot be excluded. However, multiple mating involving 3 or 4 males appeared to be rare in the field. Only 1 of 22 females (4.6 %) mated with 3 males on 10 February 2010 and no such female was found on 7 February. Although these observations are far from sufficient to generalize the behavior of this species, mating pairs accompanied by many extra males were sometimes encountered in the field (Fig. 5). While it is interesting to determine the factors affecting the mating success of these males, it is also important to examine how frequently males form such an aggregation. As already mentioned, mated females do not appear above ground again, whereas males may come out for mating during several evenings (Arakaki et al. 2004). This sexual difference in behavior implies that the ratio of males to females engaging in mating activity each day may increase gradually over the mating season, increasing the frequency of male aggregation around mating pairs.

D. ishigakiensis females did not appear to resist mating with extra males. This observation is consistent with other observations suggesting the absence of deleterious effects of multiple mating on female fitness. Although this study using virgin adults indicated that multiple mating does not affect the number and body size of their progeny, the situation may be changed if the first male to mate has only a small amount of male substance and sperm because he has already mated with other females. Another benefit might arise from the heterogeneity in progeny genetic constituents resulting from multiple mating. To examine this possibility and to investigate the evolution of multiple mating behavior in this beetle, the genetic contribution of different male parents to the progeny of each brood should be investigated with regard to their order of mating.

Fig. 5 A mating pair of *D. ishigakiensis* accompanied by 5 extra males in a sugarcane field on Miyako Island, Okinawa, Japan. *Black* and *white arrows* indicate a female and the first mating male, respectively. Other beetles are all extra males

Acknowledgments We would like to thank Mr Hiroaki Oroku of the MB-OPARC for his logistic support. We also thank Dr Koutaro Maeno for his kind help with experiments and Ms Ikuko Hashimoto for her assistance with rearing insects at NIASO.

References

Arakaki N, Sadoyama Y, Kishita M et al (2004) Mating behavior of the scarab beetles *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae). Appl Entomol Zool 39:669–674

Chapman T, Hutchings J, Partridge L (1993) No reduction in the cost of mating for *Drosophila melanogaster* females mating with spermless males. Proc R Soc B 253:211–217

Chapman T, Liddle LF, Kalb JM et al (1995) Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature 373:241–244

Fowler K, Partridge L (1989) A cost of mating in female fruit flies. Nature 338:760–761

Gillott C (2003) Male accessory gland secretions: modulators of female reproductive physiology and behavior. Ann Rev Entomol 48:163–184

Harano K, Tanaka S, Tokuda M et al (2010a) Factors influencing adult emergence from soil and the vertical distribution of burrowing scarab beetles *Dasylepida ishigakiensis*. Physiol Entomol 35:287–295

Harano K, Tanaka S, Yasui H et al (2010b) Multiple mating, prolonged copulation and male substance in a scarab beetle *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae). Int J Trop Insect Sci 30:119–126

Hayashi F, Kamimura Y (2002) The potential for incorporation of male derived proteins into developing eggs in the leafhopper *Bothrogonia ferruginea*. J Insect Physiol 48:153–159

Kotaki K (2005) Oosorption in the stink bug, *Plautia crossota stali*: follicle cells as the site of protein degradation. Inv Reprod Dev 47:147–153

Oyafuso A, Arakaki N, Sadoyama Y et al (2002) Life history of the white grub *Dasylepida* sp. (Coleoptera: Scarabaeidae), a new and severe pest on sugarcane on the Miyako Islands, Okinawa. Appl Entomol Zool 37:595–601

Partridge L, Farquhar M (1981) Sexual activity reduces lifespan of male fruitflies. Nature 294:580–582

Rooney J, Lewis SM (1999) Differential allocation of male-derived nutrients in two lampyrid beetles with contrasting life-history characteristics. Behav Ecol 10:97–104

Sadoyama Y, Oroku H, Nakamori H (2001) Injury of sugar cane roots by the white grub, *Dasylepida* sp. (Coleoptera: Scarabaeidae), in the Miyako Islands. Jpn J Appl Entomol Zool 45:89–91 (in Japanese with English abstract)

Tanaka S, Yukuhiro F, Yasui H et al (2008) Presence of larval and adult diapauses in a subtropical scarab beetle: graded thermal response for synchronised sexual maturation and reproduction. Physiol Entomol 33:334–345

Wakamura S, Yasui H, Akino T et al (2009a) Identification of (*R*)-2-butanol as a sex attractant pheromone of the while grub beetle, *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae), a serious sugarcane pest in the Miyako Islands of Japan. Appl Entomol Zool 44:231–239

Wakamura S, Yasui H, Mochizuki F et al (2009b) Formulation of highly volatile pheromone of the white grub beetle *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae) for development of monitoring trap. Appl Entomol Zool 44:579–586

Yamane T, Miyatake T (2010) Induction of oviposition by injection of male-derived extracts in two *Callosobruchus* species. J Insect Physiol 56:1783–1788

Yamane T, Miyatake T, Kimura Y (2008a) Female mating receptivity after injection of male-derived extracts in *Callosobruchus maculatus*. J Insect Physiol 54:1522–1527

Yamane T, Kimura Y, Katsuhara M, Miyatake T (2008b) Female mating receptivity inhibited by injection of male-derived extracts in *Callosobruchus chinensis*. J Insect Physiol 54:501–507