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VIDEO ARTICLE

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Sperm displacement behavior of the cuttlefish *Sepia esculenta* (Cephalopoda: Sepiidae)

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Abstract Sperm displacement behavior of cuttlefish (*Sepia esculenta*) was observed in a tank. Before ejaculation, male cuttlefish used their arms III to scrape out sperm masses attached to the buccal membranes of females. The removed sperm mass debris was directly visible and countable. Active sperm were present within the removed sperm debris, implying that the aim of this behavior is to remove competing male sperm. However, many sperm masses remained on the female buccal membrane even after the removal behavior, showing that sperm removal in *S. esculenta* is incomplete. The duration of sperm removal (an indicator of male investment in that process) was unaffected by the body sizes of mated pair, the duration of spermatangia placement at the current mating (for the hypothesis that the sperm removal serves to creat attachment space of spermatophores), or the estimated amount of sperm masses deposited from previous matings. Moreover, male *S. esculenta* performed sperm removal regardless of whether the last male to mate with the partner was himself, suggesting males remove not only the sperm of rivals but also their own. Although the number of removed sperm masses increased with the time spent on removal of sperm, male cuttlefish may shorten the duration of sperm removal to avoid the risk of mating interruption. We conclude that this time restriction would likely influence the degree of partial sperm removal in *S. esculenta*. A digital video image relating to the article is available at http://www.momo-p.com/showdetail-e.php?movieid=momo040729se01a.

Key words Cuttlefish · *Sepia esculenta* · Sperm competition · Sperm displacement · Sperm removal duration

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Introduction

Sperm competition, the competition between the ejaculates of two or more males for the fertilization of a given set of ova (Parker 1970), represents an important component of sexual selection and is a pervasive evolutionary force that has influenced a wide range of adaptations in many taxa (review in: Birkhead and Møller 1998). Most experimental studies of sperm competition, using sterile males or genetic markers, have demonstrated that the last male to mate with the female fathers the most offspring (e.g., Smith 1979; Siva-Jothy and Tsubaki 1989; Parker 1990; Hooper and Siva-Jothy 1996). One of the mechanisms of the last-male sperm precedence is "last in, first out" (Simmons and Siva-Jothy 1998). The last sperm to enter the female sperm-storage organ may push those from previous males to the back of the organ and are likely to be the first out when eggs need to be fertilized.

Another important mechanism that achieves last-male precedence is the removal of stored rival sperm from the female sperm-storage organ. Sperm displacement (i.e., removal of previous sperm and replacement with self's sperm; Parker 1970) increases the relative number of self's sperm within the female sperm-storage organ. Many previous studies on insects have revealed that a copulating male removes previously stored sperm either directly (e.g., removal by a specifically modified penis; Waage 1979) or indirectly (e.g., removal by the incoming ejaculate; Ono et al. 1989). These sperm displacement strategies should be highly effective for increasing fertilization success of the removing male (review in: Simmons and Siva-Jothy 1998), but are mostly unknown for taxa other than insects.

If possible, the male removes all stored rival sperm from the female sperm-storage organ prior to ejaculation, thereby completely avoiding subsequent sperm competition (Waage 1979; Simmons and Siva-Jothy 1998). In some sperm-removing species such as the damselfly *Mnais pruinosa pruinosa* (Siva-Jothy and Tsubaki 1989) and the earwig *Euborellia plebeja* (Kamimura 2000, 2004; Y. Kamimura,

personal communication), even partial sperm removal greatly increases the male reproductive success. Whether a species opts for complete or partial sperm removal will depend on biological and ecological variables, such as the morphology of the sperm-storage organ and the time between matings of the female (Simmons and Siva-Jothy 1998). However, sperm competition avoidance mechanism and adaptive significance of the partial removal of sperm are poorly understood.

In this study, we examine the sperm displacement behavior of cuttlefish *Sepia esculenta*. Most near-shore decapod cephalopods have a promiscuous mating system, and loliginid squids and cuttlefishes show particularly complex reproductive behavior (Hanlon and Messenger 1996). Although the mechanism underlying fertilization in cuttlefishes is far from clear, it likely occurs around the female buccal area (under the mouth), where males deposit their spermatophores (Hanlon and Messenger 1996). It is suggested that the water jetting of the male through its funnel may remove rival sperm in the common European cuttlefish, *S. officinalis* (Boal 1997; Hanlon et al. 1999), and giant Australian cuttlefish, *S. apama* (Hall and Hanlon 2002). To clarify several aspects of sperm competition, Hanlon et al. (1999) closely investigated the mating behavior of *S. officinalis* and reported the duration of sperm flushing and number of spermatophores transferred during a single mating. However, the behavioral strategy associated with sperm competition in other cuttlefishes has not been explored.

S. esculenta is widely distributed from central Japan to the Philippine Islands (Okutani 1995) and is one of the important resources for neritic fisheries in Japan. Members of the species grow to about 20 cm in mantle length during a short lifespan of 1 year or less (Natsukari and Tashiro 1991; Natsukari et al. 1991). Mature male and female *S. esculenta* migrate inshore to mate and spawn during the breeding period (from December to May in southern Japan; Watanuki and Kawamura 1999). Little is known about the reproductive behavior of *S. esculenta* (Arakawa 1960; Okutani 1979; Natsukari and Tashiro 1991). The female cuttlefish lays an egg capsule on the spawning substrate (the branches of plants or the nets of basket traps; Fujita et al. 1997) about every 5 min during an egg-laying session and produces 23–65 egg capsules during a single egg-laying period (Watanuki et al. 2000). Mating occurs in a head-to-head position so that the arms of the pair are intertwined throughout the event. The mating position lasts more than 2 min, and the mated pair remains almost motionless for the duration (Arakawa 1960). The male mating strategy involving sperm competition in *S. esculenta* has not previously been mentioned.

In the present study, we investigated the reproductive behavior of *S. esculenta* in a tank and found that visible sperm masses are removed by the male prior to ejaculation. We describe the process of direct sperm displacement and discuss the male behavioral mating strategy in regard to sperm competition in this species.

Materials and methods

Animals and husbandry conditions

This study was conducted at Marine World Uminonakamichi Aquarium in Fukuoka Prefecture, Kyushu, Japan. Between 14 March and 17 April 2000, we captured adult *Sepia esculenta* ($n = 54$) in basket traps off the coast of Shika-Island (33°44′N, 130°17′E) near the aquarium. All animals were temporarily kept in a rectangular sub tank ($200 \times 300 \times 75$ cm depth) to habituate to the housing conditions. A total of 19 cuttlefishes used for observations were transferred to an observation tank ($85 \times 135 \times 100$ cm depth) with an acrylic viewing window (90×110 cm). Between three and nine individuals (mean 7.6) were kept at any one time in the observation tank during the period 22 March to 30 May. The sex ratio was maintained at 1 : 1, except during the first 18 days (until 8 April), when there was a 3:1 male bias due to the sex ratio of the individuals captured. Discrimination of sex was made using the stripe patterns of the dorsal mantle and the animal's behavioral pattern. Each individual was identified by its external characteristics, especially by unique scars at the posterior end of the mantle. To avoid handling stress, we measured the mantle length after the death of the cuttlefish.

The observation tank was supplied constantly with natural seawater (132 l/h), and the water temperature ranged from 12.6 to 20.7°C (mean 15.7°C). Water quality was measured five times during the study period, and the values (mean ± SD; pH, 8.10 ± 0.04; NH_4–N, 0.07 ± 0.07 ppm; NO_2–N, 0.005 ± 0.004 ppm; NO_3–N, 1.3 ± 0.2 ppm) were maintained within previously reported recommended levels for culture systems for other cuttlefish (Forsythe et al. 1994) and squid (Walsh et al. 2002). Artificial light typically was provided from 0830 to 2100 hours, but the lighting period was sometimes extended until the end of observation. Some rocks were set on the sandy bottom of the tank, and branches were provided as a spawning substrate. The cuttlefishes were fed thawed fish and shrimp daily (1700–1800 hours) in sufficient quantities for the number of animals in the tank.

Observation and analysis of mating behavior

Behavioral observation and recording were made primarily between 1730 and 2100 hours through the window of the tank, but sometimes this period was extended. In addition, we often recorded the mating sequence and female egg-laying behavior during the daytime. The reproductive behavior was recorded using two digital video cameras, models DCR-VX1000 and TRV9-NTSC (Sony, Tokyo, Japan). The former was hand-held to record each phase of mating sequence in detail by using the zoom function, and the latter was mounted on a tripod to record a continuous series of reproductive behavior. Overall, we obtained 11.4 and 106.7 h of videotape recordings, respectively. The video recordings were analyzed on a high-resolution monitor (PVM-14M4J, Sony). The time data depended on the inter-

nal clocks of the video cameras. Mating behavior was analyzed at 30 frames/s. To count the number of sperm mass particles ($n = 30$) and the scratch behavior for sperm removal ($n = 21$), we used the close-up recordings from the hand-held camera. The sperm mass particles and scratch behavior were not always recorded at the same mating, resulting in the difference in the number of available data between them. In addition, the duration of spermatophore transfer was also measured from hand-held recordings. The number of spermatophores transferred per ejaculation could be readily counted from the digital video images on each frame ($n = 23$), because immediately after mating the outer sheaths either were ejected from buccal cavity of the female or were attached to the arms of the male. To avoid the effects of interruption by other individuals on the mating sequence, 54 of the 150 successful matings recorded (36%) were discarded from the behavioral data. In this study, we defined successful mating as the attachment of spermatophores to the paired female.

To confirm the presence of live sperm within the removed sperm debris, we used an inverted plastic conical container (diameter 13 cm) to collect the debris while the male was removing sperm. We collected four samples of sperm debris and, using microscopes, observed active sperm in each of them. The presence of live sperm was confirmed at 200 times magnification. Because the masses of removed sperm debris varied in size, we could not use their number to estimate the amount of removed sperm in this study.

Analysis of the factors affecting sperm removal duration

In the present study, we used the time allocated to sperm removal as an indicator of male investment in sperm removal. We evaluated the following six factors for their ability to affect the duration of sperm removal for each mating: the body sizes of the paired (1) male and (2) female; the duration of spermatangia placement (defined as that of post-spermatophore transfer behavior in this study; Hanlon et al. 1999) at the (3) current and (4) last matings; and the number of matings of the paired female during the previous (5) 3 and (6) 6 h. To assess the hypothesis that, to ensure the attachment space, the male cuttlefish determines the amount of sperm to be removed according to the amount of sperm subsequently transferred during the mating, parameter (3) was chosen in this analysis. If the hypothesis is correct, there is positive relationship between the durations of sperm removal and spermatangia placement. Moreover, we can hypothesize that the sperm removal duration depends on the quantity of sperm masses deposited on the buccal membrane of females. To demonstrate this hypothesis, parameters (4) to (6) were chosen as indicators of estimated amount of sperm deposited from previous matings, because there are no data about the number of sperm masses remained on the female's buccal membrane just before each mating. Parameter (3) (i.e., spermatangia placement duration at the current mating) was positively correlated with the number of spermatophores transferred to the paired female during a mating ($r = 0.59$, $p < 0.05$, $n = 14$). For these analyses, we used the 32 uninterrupted matings by 16 different pairs that occurred between 24 April and 25 May.

To compare the times spent for the sperm removal when the last male to mate with the partner was himself (i.e., the removing male) or not, we used a Mann–Whitney *U*-test. The statistical test procedures were performed with StatView 5.0 and the power analysis with GPOWER (Faul and Erdfelder 1992). For the power analysis, the effect size *d* was calculated by *t*-test for means (two-tailed), because some variants of power analyses for nonparametric tests can be conducted by adjusting the result obtained for the corresponding parametric test (cf. Singer et al. 1986). The calculated power value denotes the minimum sample size necessary for the statistical analysis.

Results

We observed 96 mating sequences (uninterrupted and successful) of 24 *S. esculenta* pairs between eight males and seven females (mean duration ± SD: 324 ± 133 s; range 89–857 s). The paired male usually stayed or swam beside his partner and began to mate with her mainly during the female egg laying (87% of 82 cases; the remaining 14 could not be discriminated). Both males and females mated with more than two partners during the breeding period, as expected for this species with a promiscuous mating system.

We divided the mating sequence into three phases as follows. The first phase was sperm removal (93 ± 41 s; 22–278 s; $n = 94$ by 24 pairs; Figs.1, 2a), which occurred while the pair was in the head-to-head mating position immediately after the start of the mating. With the sucker side of both arms III, the male scraped off sperm masses attached on the ventral portion of female buccal membrane during previous matings (mean number ± SD of scraping behaviors per mating: 8.4 ± 3.2; range 2–14; $n = 21$; Fig. 3); this behavior generated much debris in the water because of the

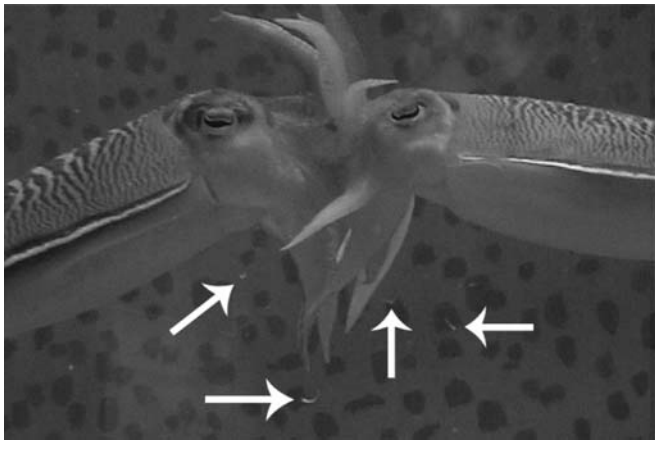

Fig. 1. Digital video image showing the sperm removal behavior of *S. esculenta* in a tank (http://www.momo-p.com/showdetail-e.php?movieid=momo040729se01a). The male (*left*) scraped out sperm masses attached on the ventral portion of the buccal membrane of the female by using his arms III. The *arrows* indicate the clumps of removed sperm

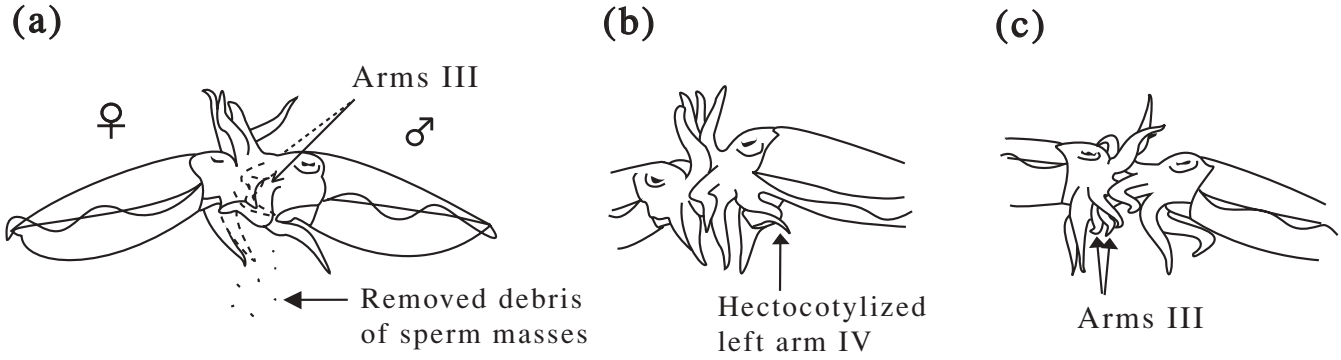

Fig. 2. Behavioral sequence of mating in *S. esculenta*: **a** sperm removal; **b** spermatophore transfer; **c** spermatangia placement. For details see text

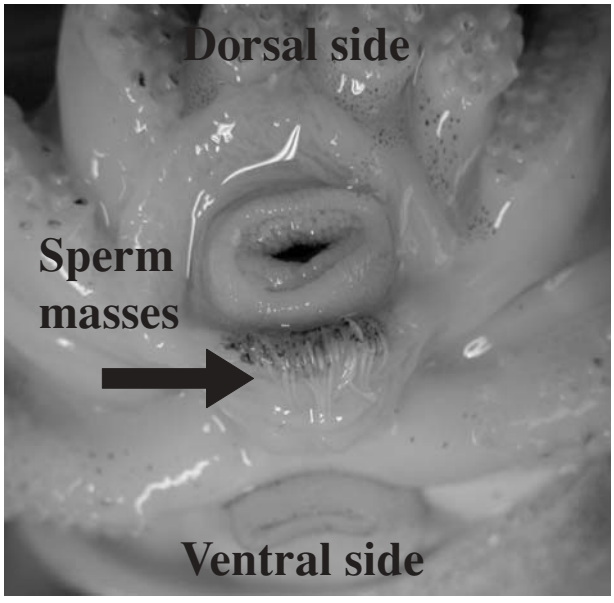

Fig. 3. Sperm masses (spermatangia) attached on the ventral portion of the female's buccal membrane

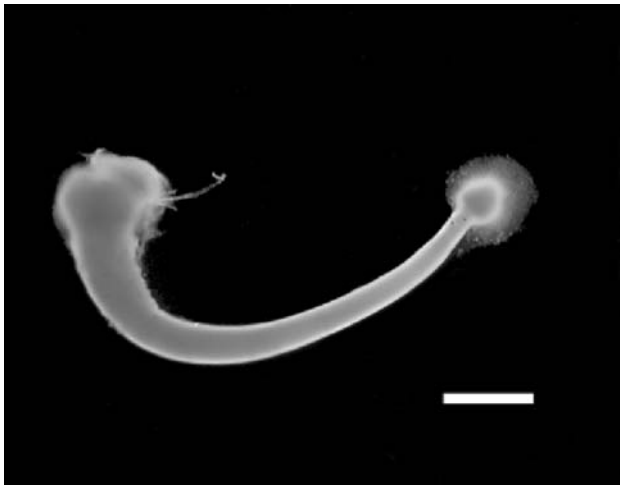

Fig. 4. One of the sperm mass debris removed from the buccal cavity of a female (*white bar*, 1 mm)

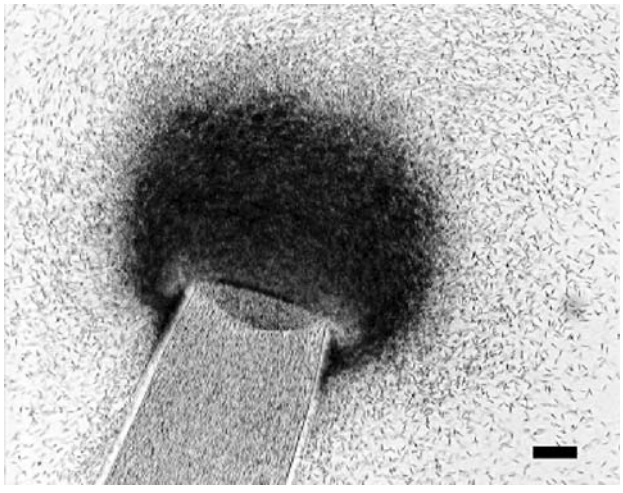

Fig. 5. Numerous sperm are within the removed sperm debris (*black bar*, 10 µm, 200×). Active sperm were observed leaving the tubular sac as long as 20 h after removal of the sperm masses

number of sperm mass particles that fell out of the female's buccal cavity (Fig. 1: counter no. 5–36 in the video image). We confirmed the presence of motile sperm within the sperm mass debris (Figs. 4, 5). Many sperm masses remained on the buccal membrane of the female even after the sperm removal behavior, showing that male cuttlefish do not completely remove all attached sperm masses. The number of sperm mass debris increased with the time spent on removal of sperm ($r = 0.74$, $p < 0.0001$, $n = 30$; Fig. 6).

Of the 61 unsuccessful matings during sperm removal, 54 (88%) were interrupted because of interference from other nearby individuals, especially males.The second phase of mating was that of spermatophore transfer (2.6 ± 0.6 s; 1.7–3.5 s; $n = 13$ by 5 pairs; Fig.2b). During this phase, the paired male swiftly wrapped his hectocotylized left arm IV around spermatophores that were ejected through his funnel and then pressed the coiled arm on the ventral portion of the female's buccal membrane. A mean of 8 ± 2.1 spermatophores (range 5–14; $n = 23$) were transferred to the female during a mating. The male typically transferred spermatophores once during each mating, except for one case in which they were transferred twice (1 of 96 matings, 1%).

Spermatangia placement (232 ± 120 s; 48–789 s; $n = 95$ by 24 pairs; Fig.2c) was the third phase of mating in our study.

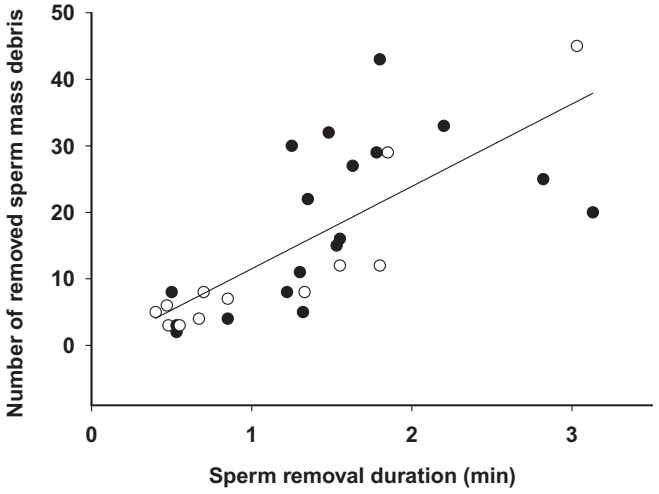

Fig. 6. Relationship between the number of clumps of removed sperm debris and sperm removal duration. *Solid circles* indicate successful matings (i.e., with spermatophore transfer), whereas *open circles* indicate unsuccessful matings (i.e., only sperm removal behavior)

During this phase, the male deposited the spermatophores on the female buccal area with his hectocotylized left arm IV and subsequently shifted the hectocotylus from this area to place his arms III there. Immediately after mating, the outer sheaths of the spermatophores were released from the female's buccal cavity into the water column, indicating that only spermatangia were attached on the ventral portion of the female's buccal membrane.

The male cuttlefish of a pair performed sperm removal in all observed matings, even when he had succeeded in attaching spermatophores to the partner during the last mating. Furthermore, the duration of sperm removal behavior did not differ significantly when the last male to mate with the partner was the removing male or a different male (Mann–Whitney *U*-test, $z = -1.74$, $n = 11$ by 5 males, 42 by 6 males, $p > 0.05$, power = 0.43, $d = 0.61$).

In analysis of the factors affecting sperm removal duration, the duration did not correlate with the body sizes of the paired male (mean mantle length ± SD = 19.3 ± 1.4 cm; $r = -0.05$, $p > 0.05$, $n = 32$) and female (15.8 ± 0.9 cm; $r = -0.21$, $p > 0.05$, $n = 32$). The sperm removal duration was also not affected by the duration of spermatangia placement at the current mating (Fig. 7a) that was not associated with the number of matings of the paired female during the previous 3 or 6 h ($r = 0.005$ and 0.02, respectively). Moreover, the other three indicators of the amount of sperm masses deposited from previous matings were not related to the variability in the duration of sperm removal (Fig.7b–d).

Discussion

Sperm displacement strategy

In all observed matings, male *S. esculenta* adopted sperm displacement strategy. Before ejaculation, the male removed sperm masses attached on the female's buccal membrane by using his arms III, not by flushing with water jetted from the funnel as reported for the other cuttlefishes *S. officinalis* (Boal 1997; Hanlon et al. 1999) and *S. apama* (Hall and Hanlon 2002). Hall and Hanlon (2002) have noted that movements of the male's arms II and III accompanied the flushing action during the mating, but have not mentioned the relationship between the arms' movements and sperm removal. We observed active sperm within the removed sperm debris, showing that the aim of this behavior is to remove competing male sperm. In particular, the removed debris of sperm masses was directly visible and countable, and this is the first report of this trait among animals even including the many insect species. This feature permits the accurate measurement of the time allocated to sperm removal behavior in *S. esculenta*.

The portion of the sperm masses removed in *S. esculenta* was part of the spermatangium attached on the ventral portion of female buccal membrane, where the seminal receptacle lies. A large number of sperm are stored in each spermatangium and the seminal receptacle (T. Wada, unpublished data), but the mechanisms underlying sperm travel between these structures and sperm fertilization currently are unknown. In the Japanese common squid, *Todarodes pacificus*, the spermatangium attached to the outer lip of the female may function as a sperm storage organ in addition to the seminal receptacle (Ikeda and Sakurai 2004). However, in *S. esculenta*, an unfertilized egg capsule transferred to the female's arms through its funnel would encounter the spermatangia attached there and would be fertilized by the sperm in and/or around the spermatangia. Therefore, the sperm displacement behavior of *S. esculenta* may have an immediate effect on fertilization success, that is, increased paternity in a subsequent spawning. This effect is probably supported by the timing of mating, which started during female egg laying, or the male guarding behavior against other males after mating (mean duration ± SD, 40.8 ± 46.4 min; $n = 89$; Wada 2002).

The hypothesis that the sperm removal serves creating attachment space was not supported in this study. If the male removes sperm masses attached by the previous matings to attach self's sperm, the time spent on the removal of sperm is expected to vary by the duration of spermatangia placement at the current mating (i.e., the number of transferred spermatophores during a mating; see Results). Moreover, the parameter may be positively correlated with the number of previous matings of the female if the hypothesis is correct. However, we did not find any significant relation for supporting the hypothesis in the present study. Therefore, the sperm removal of *S. esculenta* would not be done to ensure the attachment space of spermatophores.

Mating history and sperm removal

Generally, sperm removers should avoid removal of their own sperm when sperm competition occurs (Simmons and Siva-Jothy 1998). Therefore, if the male can recognize his own and/or his partner's mating history, he could reasonably be

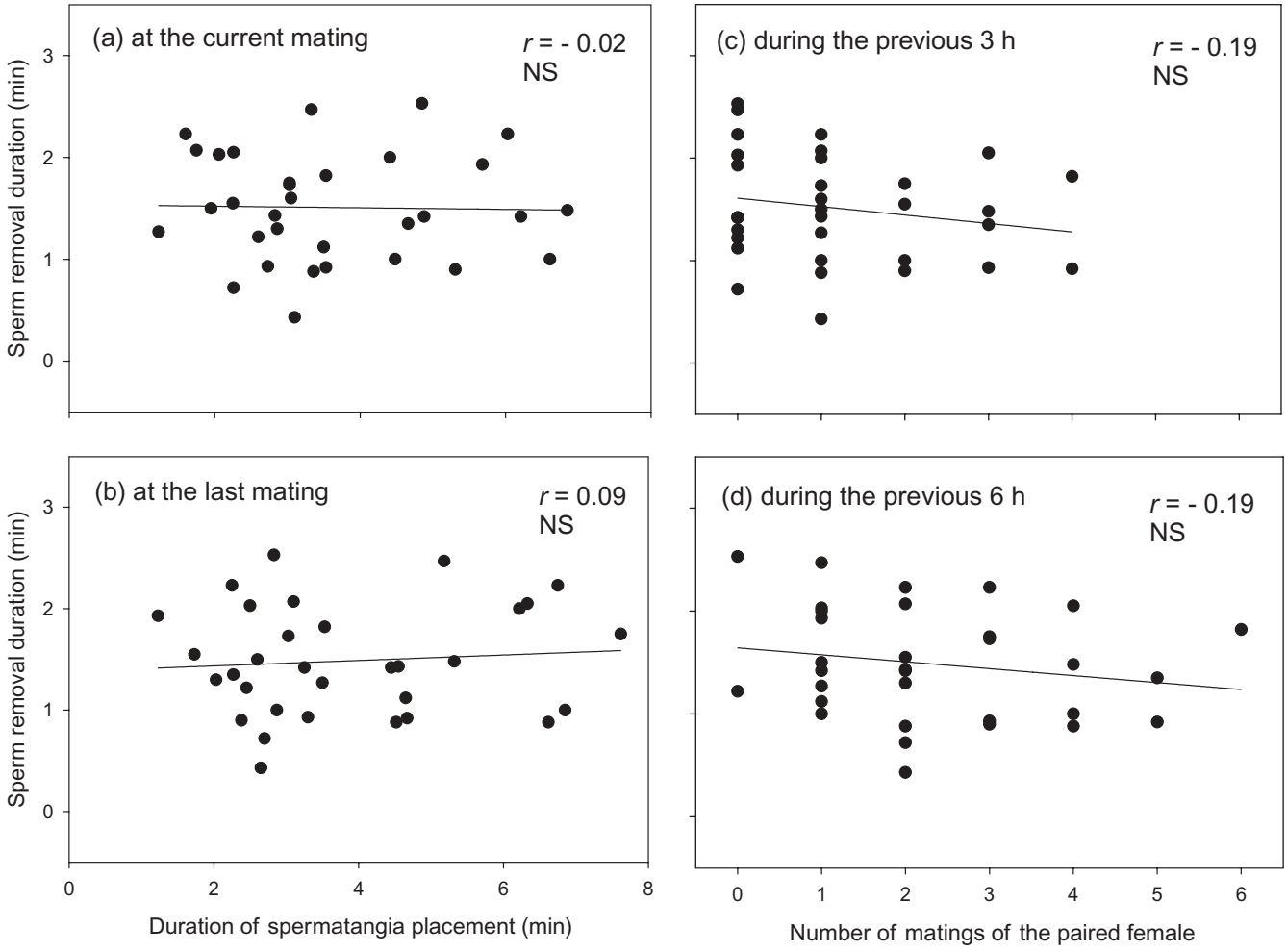

Fig. 7. Correlations between the sperm removal duration and four factors (except for the body sizes of the mated pair) for their ability to affect it; the duration of spermatangia placement at the **a** current and **b** last matings; and the number of matings of the paired female during the previous **c** 3 and **d** 6 h

expected to omit the sperm removal behavior or shorten the removal duration for repeat mating cases. However, male *S. esculenta* performed sperm removal prior to ejaculation regardless of whether the last male to mate with the partner was himself or not, and the duration of sperm removal behavior did not differ significantly between these cases. This result suggests that male *S. esculenta* remove not only rival sperm, but also their own: they are not likely to recognize their own or their partner's mating history. In *S. officinalis*, males can identify previously encountered opponents and then establish dominance rank (Adamo and Hanlon 1996), but the male cannot distinguish his mate from another female (Tinbergen 1939; Boal 1996). The ability to recognize a specific female as a mating partner may not be necessary for male *S. esculenta* if the immediate fertilization success of sperm transferred after sperm removal is sufficiently high. This theory holds even though the male removed only part of the previously attached sperm masses (see following).

Partial sperm removal

Sperm removal of male *S. esculenta* was performed at every mating, but even after that behavior many sperm masses remained on the surface of the buccal area of the female (Fig. 3). Moreover, three indicators of the amount of sperm masses deposited from previous matings (i.e., duration of spermatangia placement at the last mating, number of matings of paired female during the previous 3 and 6 h) did not significantly correlate with the duration of subsequent sperm removal (i.e., amount of removed sperm). These results show that sperm removal in *S. esculenta* is not complete. Because all males and females repeatedly mated with multiple partners in this study, sperm from multiple males would be attached on the female buccal membrane. Higher displacement rate of sperm should lead to increase the fertilization success of the displacer (review in: Simmons and Siva-Jothy 1998; Waage 1979, 1986). Therefore, why do male *S. esculenta* remove the attached sperm incompletely?

In some species of Odonata (Fincke 1984; McVey and Smittle 1984; Siva-Jothy and Tsubaki 1989), partial sperm removal is equivalent in sperm precedence to complete sperm removal, because the male displaces rival sperm from the particular region from which sperm are primarily used for fertilization immediately after copulation. Similarly in *S. esculenta*, there may be locational priority for fertilization among the spermatangia attached on the female's buccal membrane. However, we presume that the male cannot selectively remove particular spermatangia with his arms III because they are far larger than each spermatangium and because the attached spermatangia are densely clustered on the ventral portion of the buccal membrane.

Repeated matings often also compensate for partial sperm removal. In male earwigs *E. plebeja*, the displacement rate of sperm during a single mating is low (about 20%, Kamimura 2000), but repeated matings with the same female lead to high reproductive success for the male (Kamimura 2004; Y. Kamimura, personal communication). Although male *E. plebeja* may remove their own sperm during subsequent matings, the proportion of self-sperm to rival sperm within the spermatheca would increase gradually with subsequent matings. Although male *S. esculenta* sometimes remated consecutively with the previously mated female, multiple matings rarely occurred during a pairing.

Because the sperm removal duration by male *S. esculenta* was positively correlated with the number of removed debris of sperm masses (Fig. 6), males can remove more sperm if they spend more time in that activity. The females frequently rejected mating before the start of sperm removal behavior (T. Wada, unpublished data) but not during it. However, sperm removal behavior often was interrupted by another male, and most of those interrupted matings failed (no spermatophore transfer). In such situations, the paired male would shorten the duration of sperm removal to avoid the risk of mating interruption. This time restriction may be one of the most likely explanations for partial sperm removal in *S. esculenta*.

Conclusions and future study

The present study found that male *S. esculenta* removed spermatangia containing active sperm from previous matings, thereby reducing subsequent sperm competition. The removed sperm mass debris was easily visible, thereby permitting us to evaluate the time allocated for sperm removal. Although sperm displacement behavior is expected to increase the reproductive success of the removing male, we could not assess fertilization success in the present study. Future studies in *S. esculenta* will need to clarify the effect of its sperm displacement strategy by using genetic markers and field investigations, as reported for the giant Australian cuttlefish *S. apama* (Naud et al. 2004). Naud et al. (2004) indicated the negative relationship between the flushing time and the fertilization success, but their data was not enough to conclude the effect of sperm displacement behavior on fertilization success. Furthermore, we need to address female factors (i.e., female choice; Eberhard 1996) in the sperm displacement strategy.

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