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CHANGES IN THE NUMBER OF SPERMATOZOA IN THE FEMALE SPERM STORAGE ORGANS OF *ISCHNURA ASIATICA* (BRAUER) DURING COPULATION (ZYGOPTERA: COENAGRIONIDAE)

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Spermatozoan dynamics in the ♀ sperm storage organs of *I. asiatica* were examined with interrupted copulation experiments in the field. The copulation process was divided into 3 stages (I, II and III) according to the movements of the ♂ abdomen. ♀♀ interrupted just after the termination of stage I of copulation contained a much lower number of spermatozoa, both in the bursa copulatrix and in the spermatheca, than solitary ♀♀ captured before being attached by ♂♂. At the tip of the ♂'s secondary genitalia, there was a pair of horns which might be used to remove sperm from the bursa copulatrix and the spermatheca during copulation. The latter was joined to the base of the former by a spermathecal duct. Since each horn of the ♂ genitalia was significantly shorter than the spermathecal duct, the spermatheca might be inaccessible to ♂♂. The actual position of the horns in the ♀ sperm storage organs during stage I of copulation was observed by freezing copulating pairs using quick-freeze aerosol sprays. The horns were in the bursa copulatrix, but no horns had entered the spermatheca. Additional mechanisms of sperm removal from the spermatheca are proposed.

INTRODUCTION

Sperm competition has resulted in the evolution of many kinds of morphological, physiological and behavioral traits in males (PARKER, 1970). Since WAAGE (1979) demonstrated that *Calopteryx maculata* males use their specialized secondary genitalia to remove the sperm of rival males stored in the female repro-

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ductive organs before transferring their own sperm, mating behavior in Odonata has been repeatedly investigated and much evidence has been generated on sperm displacement mechanisms, such as sperm removal, sperm repositioning, inducing the female to eject stored sperm by sensory stimulation, and so on (CÓRDOBA-AGUILAR et al., 2003). Each mechanism has been considered to be an efficient way to gain high fertilization success for males in many species, resulting in high last-male sperm precedence immediately after copulation (e.g. FINCKE, 1984; SIVA-JOTHY & TSUBAKI, 1994; SAWADA, 1995).

Although sperm number is increasingly recognized as an important indicator of the fitness of males (e.g. SIMMONS, 2001), there have been few studies on the spermatozoan dynamics based on the number of spermatozoa in the female reproductive organs throughout copulation in Odonata (e.g. SIVA-JOTHY, 1987). Because odonate sperm were believed to be transferred into the female sperm storage organs as an interwoven mass with constant sperm density (WAAGE, 1979), many studies of sperm competition in Odonata have focused on sperm volume but not on the number of spermatozoa and have thus not provided any information on the relationship between sperm volume and spermatozoa number (WATANABE & ADACHI, 1987; REINHARDT, 2005).

Ischnura asiatica is a non-territorial zygopteran that widely inhabits open grasslands near water in Japan. The duration of copulation is more than three hours, and females oviposit alone, as in other *Ischnura* species (e.g. ROBERTSON, 1985; CORDERO, 1990; SAWADA, 1999). Although NARAOKA (1994) reported sperm removal in *I. asiatica* by examining the volume of both the bursa copulatrix and spermatheca of females in the field, the spermatozoan dynamics throughout copulation remain unknown. In the present study, we investigated the change in the number of spermatozoa in the female sperm storage organs in relation to the copulation process and examined the morphology of both the male and female genitalia.

MATERIAL AND METHODS

When a male *I. asiatica* encounters a female in the field, the mating attempt promptly starts, as described in TAJIMA & WATANABE (2009). That is, the male hovers over the female without any apparent courtship behavior, dashes toward the female to grasp her thorax, and then clasps her prothorax with his anal appendages. The female swings her abdomen forward and upward to the male when she is receptive. The male curves his abdomen and transfers sperm from his testis to the sperm vesicle. After sperm translocation, the male quickly curves the posterior part of his abdomen around so that the tip of the female abdomen is closer to his secondary genitalia, and they form a loop. The copulation process consists of three stages (Fig. 1). In stage I, the male depresses and stretches his first and second abdominal segments. This procedure is rhythmical, and the duration of stage I is variable. In stage II, the male thrusts his third abdominal segment at high frequency, but the frequency of the thrusts is gradually decreased. Stage III is a phase without any apparent movement of the abdomen in either sex. After stage III they separate.

In order to examine sperm removal, interrupted copulation experiments were conducted from

July to September 2006 and in June 2007. Eight solitary females and 23 copulating pairs were gently captured using a net in the morning (0500-1100), a time when many copulating pairs were found in the fields of Ibaraki Prefecture, the warm temperate zone of Japan. No pairs were disturbed by our netting procedure. Immediately after being captured, the pairs were moved into a small cylindrical cage (2mm mesh, φ 30cm, 20cm in height). Ten and seven copulating pairs were interrupted at the end of stage I and at the end of stage II, respectively. Six pairs were allowed to complete copulation. Immediately after the interrupted copulation, each female was placed in a plastic cup in a cool shade box and transported to the laboratory. The females were then decapitated, and their abdomens were dissected under a stereomicroscope to detach the sperm storage organs. Because the spermatheca is joined to the base of the bursa copulatrix by a long and narrow spermathecal duct (Fig. 2), we were easily able to separate the spermatheca from the bursa copulatrix. The bursa copulatrix and the spermatheca were put into separate tissue-homogenizers, each containing 0.5ml of saline, and ruptured. The number of spermatozoa was counted in a given volume more than five times in the same sample using a blood-haemocytometer, disregarding the volume of the bursa copulatrix and spermatheca due to their very small volume.

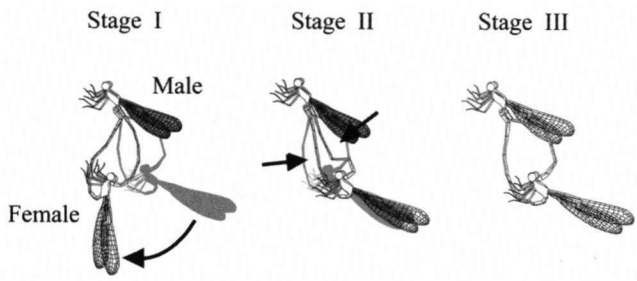

Fig. 1. The three stages in the copulatory sequence of *Ischnura asiatica*.

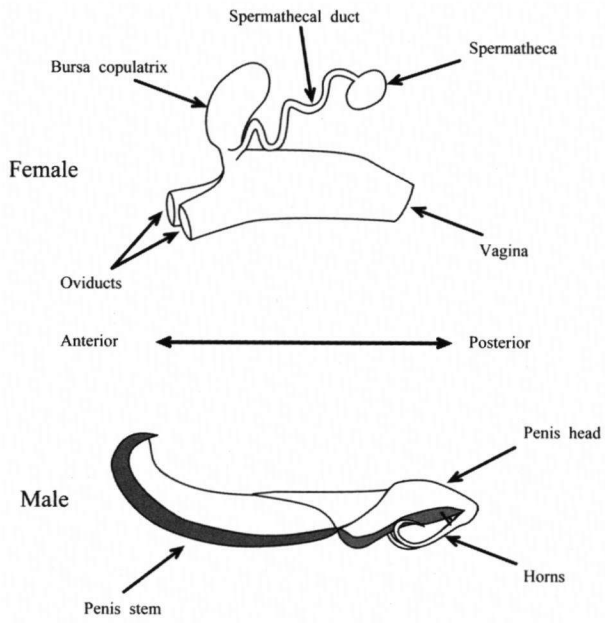

Fig. 2. The male secondary genitalia and the female genital organs.

The male's secondary genitalia consisted of two segments (Fig. 2). The penis stem is highly chitinized and its dorsal side bears a large and pliant membrane. The penis head is sclerotized and terminates in a small flap of cuticle, normally folded back along the ventral side, where paired horns are attached. The width and length of the paired horns and the spermathecal ducts were measured using a micrometer.

In order to confirm the actual position of the horns in the female genitalia, we obtained in-copula specimens during August and September 2007. Thir-

ty-two copulating pairs were gently netted in the fields during stage I, promptly frozen with quick-freeze aerosol sprays, and immediately transferred to a vial of absolute ethanol. The position of the male and female genitalia was examined by careful dissection under a stereomicroscope.

RESULTS

All captured solitary females contained sperm in the bursa copulatrix, indicating that they had already copulated, and the average number of spermatozoa was ca. 46,000 (Fig. 3). One out of the 10 females interrupted just after the termination of stage I contained no spermatozoa in the bursa copulatrix; the other 9 females had a few, with the average number of spermatozoa being ca. 3,900, which was significantly smaller than that in the solitary females (Mann-Whitney U-test, $U = 1$, $P = 0.001$). Therefore, more than 90% of bursal spermatozoa disappeared during stage I. The average number of spermatozoa contained just after the termination of stage II increased to ca. 40,000, which was significantly larger than that just after stage I (Mann-Whitney U-test, $U = 0$, $P = 0.0001$), suggesting that sperm were transferred during stage II. The number of spermatozoa contained just after the termination of copulation was also ca. 42,000, which was not significantly different from that just after stage II (Mann-Whitney U-test, $U = 18$, $P = 0.73$). The number of spermatozoa was not significantly different between solitary and post-copula females (Mann-Whitney U-test, $U = 23$, $P = 0.897$). Consequently, sperm previously stored in the bursa copulatrix seemed to be replaced by those from the current male mating.

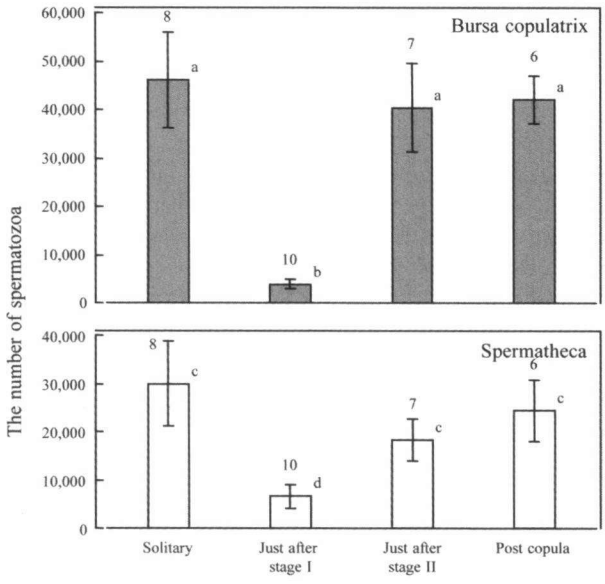

Fig. 3. Changes in the number of spermatozoa in the bursa copulatrix and the spermatheca of females at different copulatory stages (mean ± SE). The different letters indicate significant differences at a probability of less than 0.05 using the Mann-Whitney U-test. The numerals above the bar show the sample sizes.

There was also a change in the number of spermatozoa in the spermatheca (Fig. 3). Solitary females contained ca. 30,000 spermatozoa. No sperma-

tozoa were found in the spermatheca in three of the ten females interrupted just after the termination of stage I, the averaged number of spermatozoa (6,571) being significantly smaller than in solitary females (Mann-Whitney U-test, U = 10, P = 0.008). This indicates that about 80% of the spermathecal sperm disappeared during stage I, similar to the change in the bursal. The females interrupted just after the termination of stage II contained an average of ca. 18,000 spermatozoa, significantly more than just after stage I (Mann-Whitney U-test, U = 10.5, P = 0.013). This suggested that spermathecal sperm displacement might also occur as in the bursa copulatrix.

The number of spermatozoa in the spermatheca just after copulation (ca. 24,000) showed no significant difference from the number found just after stage II (Mann-Whitney U-test, U = 15, P = 0.44) and no significant increase was found in the number of spermatozoa contained in the spermatheca during stage III. Furthermore, there was no significant difference in the number of spermatozoa in the spermatheca between solitary and post-copula females (Mann-Whitney U-test, U = 23.5, P = 0.948).

The width of both horns of the male genitalia ranged from 27 to 53 μm (average of each horn 36 μm), while that of the spermathecal duct of the female ranged from 27 to 67 μm (average 49 μm) (Fig. 4). The horns were significantly narrower than the spermathecal duct (Mann-Whitney U-test: right, U = 20, P = 0.023; left, U = 20.5, P = 0.013). Therefore, any horn could easily be inserted into the spermathecal duct of the female.

However, the length of both horns ranged from 270 to 390 μm; thus, no horn was as long as any of the spermathecal ducts, whose lengths ranged from 480 to 820 μm (Fig. 4). The mean length of the spermathecal duct (610 μm) was significantly longer than that of either horn (Mann-Whitney U-test: right, U = 0, P = 0.00001; left,

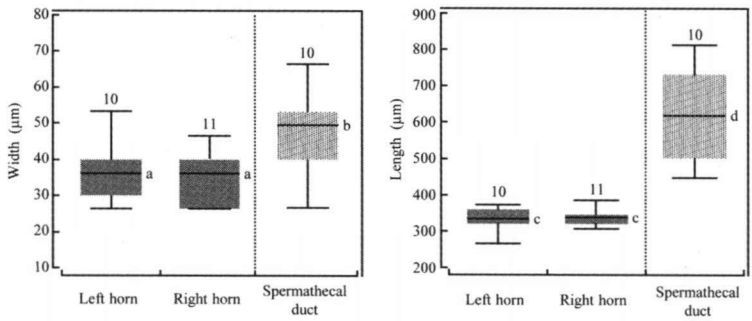

Fig. 4. Length and width of the horns of the male secondary genitalia and of the spermathecal duct of the female sperm storage organ. The box with the centre line represents interquartiles with the mean. The bars indicate the range of the maximum and minimum values. Different letters indicate significant differences at a probability of less than 0.05, using the Mann-Whitney U-test. The numerals above the bars show the sample sizes.

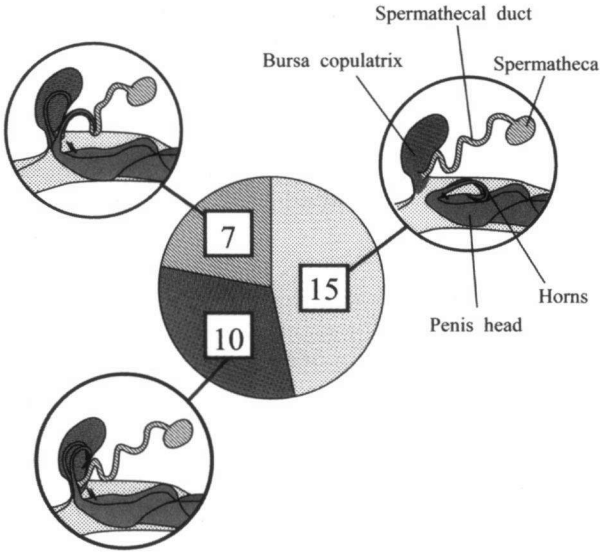

Fig. 5. The observed positions of the male secondary genitalia inserted into the female genital tract during stage I of copulation. The numerals indicate sample sizes.

U = 0, P = 0.000005). Thus, the spermatheca was inaccessible to every male.

In the in-copula specimens, the horns were found in three positions in the female reproductive organs (Fig. 5). In 15 out of 32 pairs examined, both horns were present in the vagina but not in the bursa copulatrix and also not in the spermathecal duct, indicating that the horns were totally withdrawn from the female sperm storage organs. In 10 pairs, both horns were found in the bursa copulatrix. Those males were probably accessing the bursal sperm, which suggests that males could remove sperm from the bursa copulatrix of females by using their horns. The horn might be pushed into, and pulled back out of, the bursa copulatrix by the male's abdominal movement during stage I. In each of the remaining seven copulating pairs, one horn was found in the spermathecal duct, indicating that the males' horns were able to penetrate into the spermathecal duct, though there were no pairs in which the horns reached the spermatheca.

DISCUSSION

Our results indicated that 90% and 80% of the spermatozoa were removed from the bursa copulatrix and the spermatheca, respectively, during stage I of copulation. Since most sperm stored in both sperm storage organs were removed and displaced by the current male, a high last-male sperm precedence immediately after copulation was confirmed in *I. asiatica*. NARAOKA (1994) has suggested that *I. asiatica* males remove 71% of bursal sperm volume and 61% of spermathecal sperm volume during stage I. In the case of *I. senegalensis*, SAWADA (1995) found decreases of 100% in the bursal sperm volume and 0% in the spermathecal sperm volume, while NAKAHARA & TSUBAKI (2007) reported decreases

of about 90% and 60% in the number of spermatozoa in the bursa copulatrix and spermatheca, respectively, in that species. In *I. elegans*, decreases of 100% in the bursal sperm volume and 0% in the spermathecal sperm volume were found (MILLER, 1987b).

Males of the *Ischnura* species have a pair of horns in their secondary genitalia, and the function of these horns seems to differ among the species (ROBINSON & NOVAK, 1997). MILLER (1987a) found that no horns entered the spermathecal duct in *I. elegans* during copulation. Because the spermathecal duct was significantly longer than the horns and there were no copulatory pairs in which the horns reached the spermatheca, *I. asiatica* males could not reach the spermatheca, as is the case in *I. elegans*, and so could not directly remove sperm from the spermatheca. In contrast, the horns of *I. graellsii* males can penetrate into the spermatheca and are probably able to directly remove sperm from the spermatheca (CORDERO & MILLER, 1992).

However, about 80% of spermathecal sperm were removed during stage I in *I. asiatica*. There are three plausible explanations for this:

- Depression of the bursa copulatrix – NARAOKA (1994) proposed that the depression of the bursa copulatrix during stage I by means of sperm removal led sperm to return from the spermatheca to the bursa copulatrix. Males then might directly remove them. Since males of *I. asiatica* were able to remove most sperm in the bursa copulatrix, there is a possibility that the decrease of spermathecal sperm is made possible by the depression of the bursa copulatrix.
- Stimulation of the female sensory system – MILLER (1987a) described the morphology of the female genital tract in *I. elegans* and stated that sensory stimulation by the male could lead to sperm removal. Because females have two cuticular plates with embedded mechano-receptive sensilla which communicate the presence of an egg to the muscles surrounding the sperm storage organs for fertilization, such muscles might have the function of inducing the release of sperm stored in the spermatheca (CÓRDOBA-AGUILAR, 2003). CÓRDOBA-AGUILAR (1999) reported that the appendages of male secondary genitalia are too wide to penetrate the spermatheca (in *Calopteryx haemorrhoidalis asturica*), but that the behavior of the shaft of the secondary genitalia mimics the movement of the egg, stimulating the mechano-receptive sensilla to induce spermathecal sperm ejection. In *Ceriagrion tenellum*, males also induce sperm ejection from the spermatheca (ANDRÉS & CORDERO, 2000). NAKAHARA & TSUBAKI (2007) also suggested that stimulation of the female genital tract during copulation could result in sperm ejection from the spermatheca in *I. senegalensis*. Because the morphology of the female sperm storage organs in *I. asiatica* is similar to that in *I. elegans* and *I. senegalensis*, females might have mechano-receptive sensilla on two cuticular plates of the vagina and may fertilize eggs in the same manner.
- Active sperm ejection – Sperm ejection from spermatheca by spermathecal

muscles during copulation is another possibility. Thus GONZÁLEZ-SORIANO & CÓRDOBA-AGUILAR (2003) found that, in *Paraphlebia quinta*, a copulating female ejected drops of sperm from its genital pore under repeatedly interrupted genital contact. CÓRDOBA-AGUILAR (2006) suggested that female sperm ejection was likely to be widespread among species lacking overt precopulatory courtship behavior. Although sperm ejection during copulation has not been clearly demonstrated, the active ejection of the sperm during copulation in *I. graellsii* females has been suggested (CORDERO & MILLER, 1992). Genital muscular movement might also bring about sperm ejection (CÓRDOBA-AGUILAR, 2003). Since the female genital tract in *I. asiatica* is covered in muscular tissue, females have the potential to expel sperm during copulation.

In conclusion, the mechanism involved in the disappearance of spermathecal sperm during copulation in *I. asiatica* was not direct sperm removal using horns but a different mechanism. The depression of the bursa copulatrix, the stimulation of the female sensory system, and active sperm ejection are all plausible explanations for the decrease in spermathecal spermatozoa. Therefore, males might achieve high last-male sperm precedence using one or more of these mechanisms. Further study is needed to clarify the mechanism of sperm disappearance from the spermatheca.

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