

## Seasonal variation of genital morphology and sperm removal in *Ischnura asiatica* (Odonata: Coenagrionidae)

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**Abstract.** During copulation, *Ischnura asiatica* (Brauer, 1865) males remove the sperm of the females' previous mates from the spermatheca by stimulating vaginal sensilla thereby inducing sperm ejection. Because a wider penis head stimulates the vaginal sensilla more intensely, larger males with wider penis heads can remove much more sperm from the spermatheca. There are two distinct body sizes for spring (large) and summer (small) generations of *I. asiatica*. In the present study we show that in spring, males have wider penis heads and females have a higher number of vaginal sensilla as compared to summer adults, suggesting that mating males remove more spermathecal sperm in spring than in summer. However, interrupted copulation experiments showed that females of the spring generation had a higher number of spermatozoa in both sperm storage organs than those of the summer generation. Solitary females of the spring generation also had higher numbers of spermatozoa stored than those of the summer generation, suggesting that spring females might have larger sperm storage organs than summer females. Although the removal rate of bursal sperm was almost the same between generations, the removal rate of spermathecal sperm in the summer generation was slightly higher than that in the spring generation. Consequently, spermatozoa derived from previously mated males have a higher probability of remaining in the sperm storage organs in spring- than in summer-generation females. The size of sperm storage organs in females might be critical to understanding sperm displacement. This aspect of female anatomy has not been previously considered in studies of sperm competition in odonates.

**Key words.** Dragonfly, damselfly, Zygoptera, bivoltine, interrupted copulation, male-female coevolution, penis head, spermatozoa number

### Introduction

Males of most odonate species displace the sperm of rivals stored in the female sperm storage organs (CÓRDOBA-AGUILAR et al. 2003). During copulation, the appendages on the secondary genitalia of the male enter the female sperm storage organs, and the rival's sperm is mechanically trapped

by the appendages. Sperm removal has been found to be associated with males' abdominal movements throughout copulation (TAJIMA & WATANABE 2010). Removal of the rival's sperm mass from the female's sperm storage organs has been reported for many species such as *Mnais pruinosa* Selys, 1853 (SIVA-JOTHY & TSUBAKI 1989), *Ischnura ramburii* (Selys, 1850) (WAAGE 1986) and *Enallagma cyathigerum* (Charpentier, 1840) (MILLER & MILLER 1981). In zygopteran species, sperm displacement mechanisms have been discussed from the viewpoint of sexual selection (CÓRDOBA-AGUILAR et al. 2003). Direct sperm removal, resulting in high last-male sperm precedence (e.g., FINCKE 1984), must be an efficient way for a copulating male to gain high fertilization success.

Males of *Ischnura* spp. have a pair of horns on their secondary genitalia, while the females have two types of sperm storage organ: a bursa copulatrix and a spermatheca that is connected to the bursa copulatrix by a long, narrow spermathecal duct (e.g., SAWADA 1995). There seem to be various mechanisms for spermathecal sperm displacement among *Ischnura* species (ROBINSON & NOVAK 1997). For example, the horns of *I. graellsii* (Rambur, 1842) males can enter the spermatheca so as to directly remove sperm from the spermatheca (CORDERO & MILLER 1992). However, the horns of *I. elegans* (Vander Linden, 1820) males cannot enter the spermathecal duct, hence these males cannot directly remove spermathecal sperm (MILLER 1987).

*Ischnura asiatica* is a non-territorial damselfly, inhabiting open and sunny grasslands around ponds. After emergence, both sexes perch on grass stems at the water's edge and exhibit mating behaviour. Although sexually mature females mate only once in a given day, they mate multiply throughout their life. The duration of copulation is more than three hours (NARAOKA 1994).

In *I. asiatica* an alternative mechanism of spermathecal sperm removal has been proposed. While direct sperm displacement from the bursa copulatrix occurs by the action of the male's secondary genitalia, the males cannot reach the spermatheca as in the case of *I. elegans* due to the females' spermathecal ducts being significantly longer than males' horns (TAJIMA & WATANABE 2010). Stimulation of mechano-receptive sensilla on the vaginal plates of females, which are used to communicate the presence of an egg to the muscles surrounding the sperm storage organs for fertilization during oviposition, may also result in sperm removal. When the males insert their

penis head into the vagina during copulation, the penis head approaches the sensilla. TAJIMA & WATANABE (2013) recently showed that the action of the penis head during copulatory movements mimics the movement of the eggs awaiting fertilization, stimulating the sensilla to induce spermathecal sperm ejection.

Wider penis heads stimulate the female sensory system more than smaller heads, resulting in a higher displacement rate of spermathecal sperm (TAJIMA & WATANABE 2013). Although body size does not always correlate with other morphological traits (NAVA-BOLAÑOS et al. 2012), larger males may have, in general, wider penis heads, and the ability of a male to displace spermathecal sperm might depend on body size. In *I. asiatica* on the mainland of Japan, there are two distinct body sizes for spring and summer generations (NARAOKA 1976). The adult flight season for the two generations does not overlap. In the spring generation, adults are larger than those in the summer generation, probably due to the prolonged larval period during overwintering (STOKS & CÓRDOBA-AGUILAR 2012). If the males in the spring generation should have a wider penis, then the efficiency of sperm removal by the males might differ from that of the summer generations. Another consideration is that the relationship between the female body size and the number of vaginal sensilla has not yet been clarified, though fewer sensilla means less stimulation by the penis head during copulation, resulting in less sperm being ejected from the spermatheca (CÓRDOBA-AGUILAR 2005). If the relative genital morphology of either males or females differs between generations, there should be a difference in the amount of sperm ejected from the spermatheca during copulation. Thus, we conducted interrupted copulation experiments using adult damselflies of *I. asiatica* of the spring and summer generations and examined the width of the penis head, the number of vaginal sensilla and the number of spermatozoa remaining in the female sperm storage organs. The effect of variation of genital size on sperm removal according to the generation is discussed.

## Materials and methods

### Study species' background information

The copulation process of *Ischnura asiatica* consists of three distinct stages (TAJIMA & WATANABE 2009). In stage I, the male rhythmically depresses

and stretches the first and the second abdominal segments. In stage II, the male thrusts with the third abdominal segment at high frequency, with the frequency of the thrusts gradually decreasing. Stage III is a phase without any apparent movement of the abdomen in both sexes. Sperm removal from female sperm storage organs occurs during stage I. The sperm of the present mate is transferred to the female during stage II and III (TAJIMA & WATANABE 2010).

#### **Field and laboratory work**

Samples were collected from several small ponds in Tsukuba city, Ibaraki Prefecture, located in the warm temperate zone of Japan, for spring (May) and summer (July to September) generations. Solitary individuals and copulating wheels in stage I were collected while walking slowly along the edge of the pond from 05:00 a.m. to 11:00 a.m. JST on sunny days. The hind wing lengths of solitary individuals were measured using digital callipers. Then, the individuals were put into a vial of absolute ethanol. Wheels were gently captured by net with minimal disturbance and immediately put into a small cylindrical cage with 2 mm mesh (diameter 30 cm, 20 cm in height). Stage I pairs that remained in the wheel position in the cage were used for the experiment.

Just after stage I ended, copulation was interrupted by separating the pair gently by hand and the hind wing length of each sex was measured by digital callipers (to the nearest 0.1 mm). Then, males were put into a vial of absolute ethanol, while females were placed in a plastic cup in a shaded box with an ice pack to reduce activity and were transported to the laboratory.

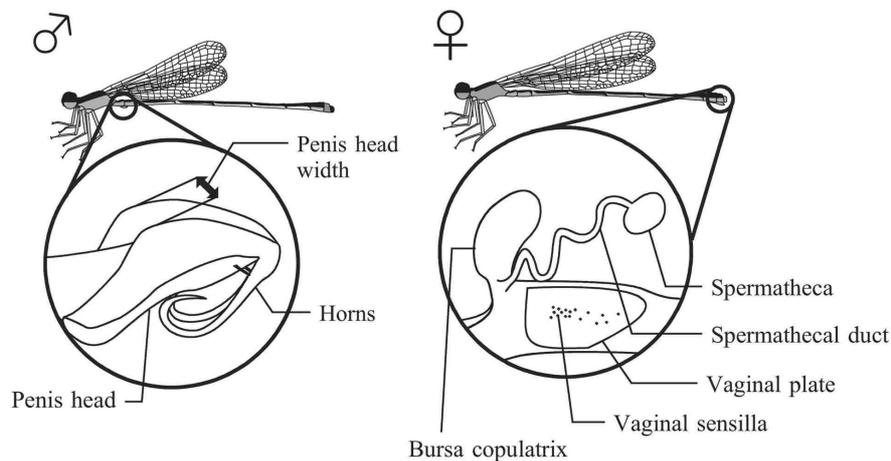
In ethanol-preserved males, the secondary genitalia were detached and measured using an ocular micrometer under a stereomicroscope ( $\times 75$ ). Fine forceps were used to fix the secondary genitalia horizontally to measure their size. Then, we identified the width of the parts of the penis head that would have been in physical contact with the vaginal plates (Fig. 1; TAJIMA & WATANABE 2009). We measured the widest part of penis head as the width of penis head.

The sperm count procedure followed that described by TAJIMA & WATANABE (2009). Each female was decapitated. The abdomen was cut with dissecting scissors and placed in insect Ringer's solution. Then, the internal

genitalia located in the eighth and ninth abdominal segments were detached using fine forceps. The spermatheca and the bursa copulatrix were each separated from the internal genitalia, and from each other, then put individually into tissue homogenizers containing a given volume (0.5 ml) of insect Ringer's solution and ruptured. The sperm samples were mixed by gentle pipetting. The number of spermatozoa in each organ sample was counted using a hemocytometer under a stereomicroscope ( $\times 75$ ). Five estimates were made for each samples and the mean was calculated. We did not measure the volume of bursa copulatrix and spermatheca, because each of them was too small in relation to the volume of insect Ringer's solution (0.5 ml).

The tissue around the vaginal plates was carefully removed using fine forceps. We counted the number of pore-embedded sensilla on both sides of vaginal plates as well as the number of vaginal sensilla under a stereomicroscope ( $\times 75$ ).

Data normality was tested using Shapiro-Wilk test. The data sets of the number of spermatozoa were not normally distributed, though other data sets were normally distributed. Then, differences in the number of spermatozoa stored in each sperm storage organ were analysed using a generalized liner model (GLM) with the number of spermatozoa as response variable,



**Figure 1.** Schematic representations of the tip of male penis and female internal genital organs of *Ischnura asiatica*.

and status (copulating solitary) and season (spring or summer) as factors assuming quasi-Poisson distribution. Differences in the hind wing length of males and females were analysed using a GLM with the hind wing lengths as response variable, and status (copulating solitary) and season (spring or summer) as factors assuming Gaussian distribution. Differences in the width of penis head and the number of vaginal sensilla between spring and summer generations were analyzed using the t-test. T-test and GLM were performed using SPSS version 12.0 and R version 2.13.2 (R DEVELOPMENT CORE TEAM 2011), respectively.

### Results

Six solitary males, nine solitary females and 17 copulating pairs in the stage-I wheel position were collected from the spring generation. Eight solitary males, 11 solitary females and 28 copulating pairs in stage I were collected from the summer generation. Based on body colour and their worn wing condition, all individuals were identified as sexually mature irrespective of copulation status.

The hind wing lengths of solitary and copulating males in the spring generation ranged from 14.1–16.0 mm and 14.1–17.0 mm (min.–max.), respectively, with a mean of around 15.5 mm (Tab. 1). On the other hand, mean hind wing length in the summer generation was around 13.5 mm, with ranges of 13.2–14.2 mm and 12.8–15.2 mm for solitary and copulating males respectively. Thus, the hind wing length of males in spring generation was significantly larger than that in summer generation ( $t = -11.01$ ,  $P < 0.01$ ). There was no significant difference between solitary and copulating males ( $t = -0.66$ ,  $P = 0.51$ ).

The hind wing lengths of solitary and copulating females in the spring generation ranged from 16.3–18.2 mm and 16.3–18.8 mm, respectively. Hind wing length in the summer generation ranged from 13.7–16.2 mm and 13.8–17.7 mm for solitary and copulating females, respectively (Tab. 1). The hind wing length was significantly different between solitary and copulating females of the summer generation ( $t = -2.58$ ,  $P = 0.01$ ). The females of the spring generation were significantly larger than those of the summer generation ( $t = -9.41$ ,  $P < 0.01$ ). Thus, two distinct body sizes were observed in both sexes.

**Table 1.** Hind wing lengths [mm] of the two sexes of *Ischnura asiatica* in each generation in Tsukuba, Ibaraki Prefecture, Japan (mean  $\pm$  SE).

	Spring (n)	Summer (n)
Solitary male	15.2 $\pm$ 0.3 (6)	13.7 $\pm$ 0.1 (8)
Copulating male	15.7 $\pm$ 0.2 (17)	13.5 $\pm$ 0.6 (28)
Solitary female	17.4 $\pm$ 0.2 (9)	14.8 $\pm$ 0.2 (11)
Copulating female	17.4 $\pm$ 0.2 (17)	15.8 $\pm$ 0.1 (28)

**Table 2.** Width of the penis head of males and the number of vaginal sensilla of females for copulating pairs of each generation of *Ischnura asiatica* in Tsukuba, Ibaraki Prefecture, Japan ( $\pm$ SE).

	Spring (n = 17)	Summer (n = 28)	t-test
Width of penis head [ $\mu$ m]	292.3 $\pm$ 3.7	276.3 $\pm$ 2.4	t = 3.80 P < 0.01
Number of vaginal sensilla	37.5 $\pm$ 1.1	32.5 $\pm$ 0.8	t = 3.80 P < 0.01

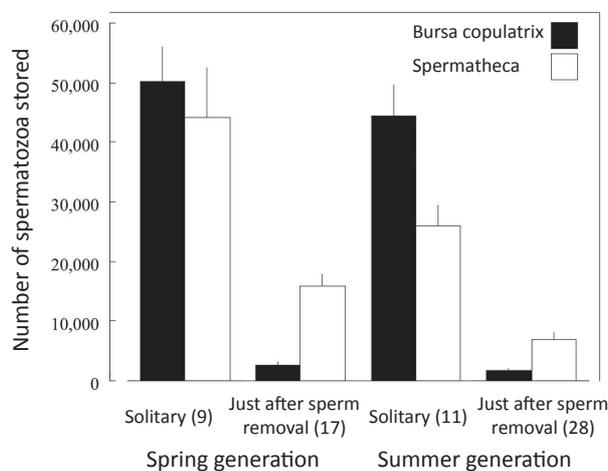
As shown in Table 2, the width of the penis head was about 292 and 276  $\mu$ m in the spring and summer generations, respectively; thus, the width of the penis head in the spring generation was significantly greater than that of the summer generation. Likewise, the number of vaginal sensilla in the spring generation was about 37.5, and significantly greater than that in the summer generation (32.5).

All solitary females stored sperm in both the bursa copulatrix and spermatheca. Every female from a tandem pair also contained sperm in the bursa copulatrix and/or in the spermatheca. As tandem pairs were interrupted before the male ejaculated, any sperm remaining in the sperm storage organs was derived from previously mated males, not the current male copulating.

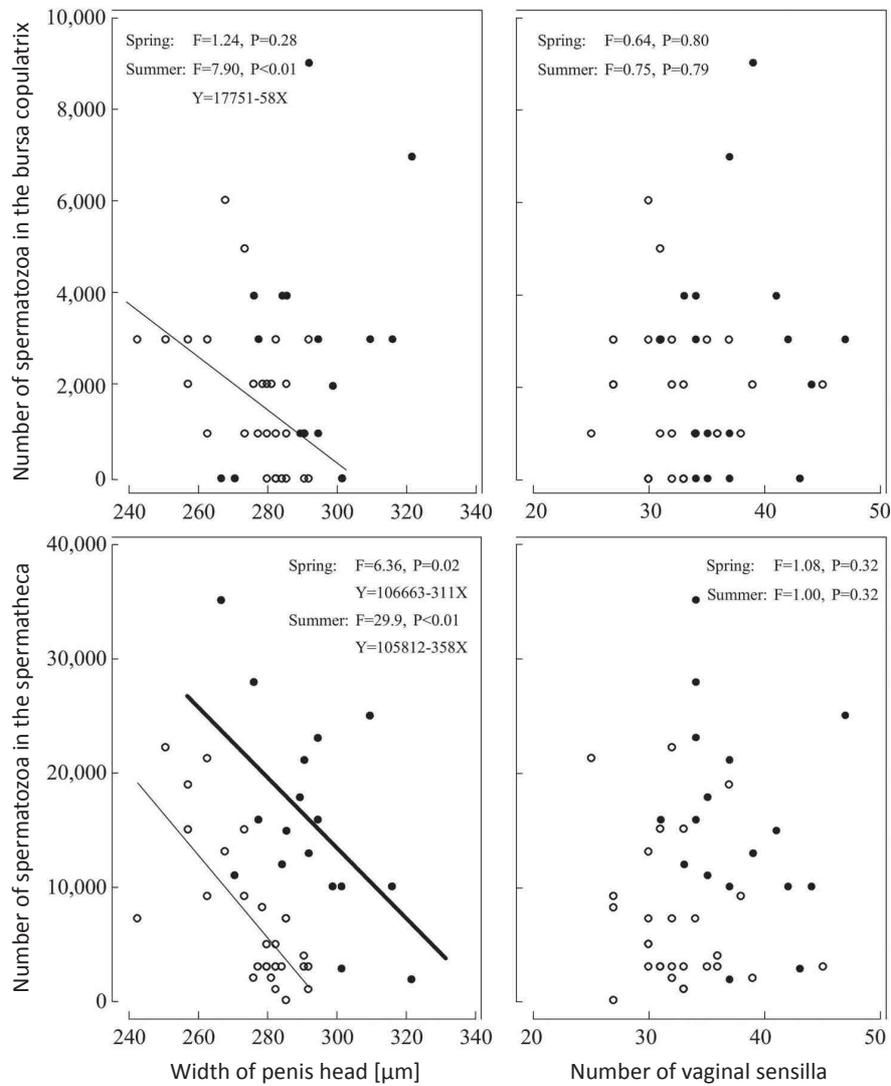
Solitary females in the spring generation had 50,222.2  $\pm$  5,816.1 ( $\pm$ SE) spermatozoa stored in the bursa copulatrix and 44,000.0  $\pm$  8,432.7 ( $\pm$ SE) spermatozoa stored in the spermatheca, (Fig. 2). On the other hand, solitary females in summer generation stored 44,363.6  $\pm$  5,284.7 ( $\pm$ SE) spermatozoa in the bursa copulatrix and 26,000.0  $\pm$  3,348.5 ( $\pm$ SE) spermatozoa in the spermatheca. Copulating females interrupted in the spring had 2,647.1  $\pm$  612.0

( $\pm$ SE) spermatozoa in the bursa copulatrix and  $15,764.7 \pm 2,089.1$  ( $\pm$ SE) spermatozoa in the spermatheca, while those in summer had  $1,714.3 \pm 290.3$  and  $7,000.0 \pm 1,180.8$  ( $\pm$ SE) spermatozoa in the bursa copulatrix and spermatheca respectively. The females in the spring generation had a significantly higher number of spermatozoa in the spermatheca though the differences in the bursa copulatrix were not significant (bursa copulatrix,  $t = -1.14$ ,  $P = 0.26$ ; spermatheca,  $t = -3.38$ ,  $P < 0.01$ ). Females that were interrupted while copulating had significantly fewer spermatozoa in the bursa copulatrix ( $t = 10.21$ ,  $P < 0.01$ ) and the spermatheca ( $t = 5.07$ ,  $P < 0.01$ ) than solitary females. On average, 94.7 and 64.2% of bursal and spermathecal sperm respectively were removed in the spring generation; in the summer generation those figures were 96.1 and 73.1%, respectively. Although the removal rate of bursal sperm was almost similar between generations, the removal rate of spermathecal sperm in the summer generation was slightly higher than that in spring generation.

Figure 3 shows the relationships between the genital traits for each sex and the number of spermatozoa remaining in each sperm storage organ in



**Figure 2.** The number of spermatozoa stored in solitary females and in females interrupted in copulation soon after sperm removal (Stage I) in the spring and summer generations of *Ischnura asiatica* in Tsukuba, Ibaraki Prefecture, Japan (mean  $\pm$  SE). Numerals in parentheses represent sample sizes.



**Figure 3.** Relationship between the genital traits of each sex and the number of spermatozoa retained in each sperm storage organ just after sperm removal (Stage I) in the spring and summer generations of *Ischnura asiatica* in Tsukuba, Ibaraki Prefecture, Japan. Closed circles and the thick line represent the spring generation. Open circles and the thin line represent the summer generation. F and P values were calculated by regression analysis.

the spring and summer generations. Although there was no significant relationship between the width of the penis head and the number of the spermatozoa stored in the bursa copulatrix in the summer generation, the wider penis head decreased the number of spermatozoa stored in spermatheca of females. On the other hand, the number of vaginal sensilla did not affect significantly the number of spermatozoa stored in the reproductive organs in either the spring or summer generations.

### Discussion

In *Ischnura asiatica* we found that males of the spring generation had a larger body size and wider penis head than those of summer generation. In this species, bursal sperm can be removed using the appendages of secondary genitalia, while spermathecal sperm is removed by stimulating vaginal sensilla using the penis head (TAJIMA & WATANABE 2009). Because a wider penis head stimulates the vaginal sensilla more, larger males can remove more sperm from the spermatheca. Therefore, the width of penis head indicates the male's capacity for spermathecal sperm removal in *I. asiatica*. According to our present results, sperm displacement ability of males in the spring generation might be higher than that in the summer generation.

Females in the spring generation had a significantly higher number of vaginal sensilla than those in summer generation. TAJIMA & WATANABE (2013) suggested that the number of vaginal sensilla was related to the number of spermathecal sperm removed during copulation. The muscles surrounding the spermatheca of females with higher number of vaginal sensilla in *Calopteryx haemorrhoidalis* showed more active contraction to eject sperm by artificial stimulation (CÓRDOBA-AGUILAR 2003). Thus, the spermathecal sperm might be more easily ejected in the females of the spring than summer generation. However, our data suggest that the number of sensilla was not related to the number of sperm remaining, suggesting that the sensitivity of sensilla might be different between the generations though the detailed morphological and physiological traits of sensilla have not yet been investigated.

The vaginal sensilla serve to control fertilization (MILLER 1987). During oviposition, as each egg passes from the oviduct between the vaginal plates, it stimulates the vaginal sensilla to release the sperm for fertiliza-

tion (MILLER 1987). The egg size must affect the intensity of the stimulation when the egg passes between the vaginal plates. The length of eggs in the spring and summer generation was measured as  $884.1 \pm 7.8 \mu\text{m}$  ( $\pm\text{SE}$ ,  $n=17$ ) and  $852.3 \pm 4.5 \mu\text{m}$  ( $\pm\text{SE}$ ,  $n=30$ ), respectively, with respective widths of  $182.6 \pm 1.8 \mu\text{m}$  ( $\pm\text{SE}$ ) and  $173.3 \pm 1.5 \mu\text{m}$  ( $\pm\text{SE}$ ) (YT & MW unpubl.). Thus, the eggs in the spring generation were significantly larger than those in the summer generation ( $t=3.80$ ,  $P<0.01$  for length;  $t=3.86$ ,  $P<0.01$  for width), and the intensity of the stimulation produced by the egg might also differ between generations. Because the number of sensilla was not related to the number of sperm stored in spermatheca, the differences in intensity of the sperm displacement between generations may be due only to differences in width of penis head. Spring males may be better at stimulating than summer males independently of the differences in the number of sensilla. Therefore, the differences in the number of sensilla can be explained as a natural selection factor involved in promoting fertilization according to egg size.

In a single copulation, males transfer sufficient spermatozoa to fertilize a female's lifetime egg production (e.g., SAKURAI 1998). If a female has fewer vaginal sensilla, she releases fewer spermatozoa for fertilization of each egg that passes through. The reduction of sperm release might cause failure of fertilization, resulting in the increase of unfertilized eggs (CÓRDOBA-AGUILAR 2005). On the other hand, if a female has too many sensilla, she might release excess spermatozoa for fertilization, resulting in a depletion of stored sperm. Presumably, the number of vaginal sensilla to control sperm release for each generation has evolved an optimal range. Because the intensity of the stimulation produced by the egg might differ between generations due to egg size, the optimal number of vaginal sensilla to control sperm release might differ between generations.

In the present study, solitary females of the spring generation had a higher number of spermatozoa in their sperm storage organs than those of the summer generation. TAJIMA & WATANABE (2009) reported that solitary females of the summer generation had about 46,000 and 30,000 sperm in the bursa copulatrix and spermatheca respectively while females just after copulation had about 42,000 and 24,000 sperm in the bursa copulatrix and spermatheca, respectively. There were no significant differences between the number of spermatozoa stored in the solitary females and females just after copula-

tion. This suggests that *I. asiatica* females may have a relatively high mating frequency though this requires verification. Thus, solitary females stored a number of spermatozoa similar to that found in females just after copulation.

In zygopteran species, the males often transfer a greater volume of sperm than the capacity of the female's sperm storage organs during a single copulation because the male seminal vesicle is of larger volume of that of the female sperm storage organs (e.g., WAAGE 1986). In this case a female's sperm storage organs would be fully filled with sperm, with excess sperm discharged after copulation (CÓRDOBA-AGUILAR 2006). Thus, the number of spermatozoa stored in solitary females might be a useful indicator of the capacity of their sperm storage organs.

In the spring generation, males with wide penis heads and females with high numbers of vaginal sensilla were found. The males with wide penis head might have higher sperm displacement ability. Consequently, males of the spring generation are expected to remove more spermathecal sperm than those of the summer generation. Accordingly, the interrupted females of the spring generation were expected to have emptied more spermathecal sperm than those of the summer generation. However, the interrupted females of the spring generation retained a higher number of spermatozoa in the spermatheca than those of the summer generation. This result suggests that the spermatheca size might affect the number of spermatozoa retained in the spermatheca just after sperm removal. CORDERO & MILLER (1992) reported that the volume of the spermatheca was correlated with the female body size in *I. graellsii*. The spring females might have larger sperm storage organs than summer females. The present study shows that the removal rate of spermathecal sperm in the spring generation was slightly lower than that in the summer generation though spring males could remove spermathecal sperm more easily due to their wide penis heads. Thus, the size of spermatheca in females might be critical for understanding sperm storage and sperm displacement. In addition, CÓRDOBA-AGUILAR (2009) hypothesized that a high sperm displacement rate caused a reduction of benefits of multiple mating for females. Because the rate of spermathecal sperm removal differed between generations, the benefits for the females of multiple mating might differ between generations. Consequently, the mating frequency of females might also differ between generations.

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