

Evolution of Sperm Displacement Mechanism  
and Genital Morphology in the Damselfly,  
*Ischnura asiatica*

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## FIGURE LIST

### GENERAL DISCUSSION

**Figure 1.** Relationship between the width of penis head and the number of vaginal sensilla after controlling for variation in body size. 28

## ABSTRACT

In most species of Odonata, males physically displace rivals' sperm stored in the female sperm storage organs. During copulation, appendages on the male secondary genitalia enter the female sperm storage organs, and trap and remove sperm masses from previous matings. Considerable variations of male and female genitalia among species has been clarified, indicating that males of some species are able to displace all sperm in female sperm storage organs, while males of other species displace not all sperm stored in female sperm storage organs. The *Ischnura asiatica* male has a pair of appendages at the tip of his genitalia that are shorter than the length of the narrow duct from the bursa copulatrix to the spermatheca in the female. Although the males remove bursal sperm during the copulation, the males have no appendages that can remove spermathecal sperm directly. However, reduction of the number of spermatozoa occurs both in the bursa copulatrix and in the spermatheca during copulation. An alternative mechanism for the reduction of the spermathecal sperm by the male was previously suggested. Females have mechano-receptive sensilla that communicate the presence of an egg to the muscles covering the sperm storage organs for the purpose of fertilization. Then, it is hypothesized that the penis head might mimic the movement of the egg, thus stimulating the sensilla to induce spermathecal sperm ejection by the female. In the present study, in order to clarify the mechanism of sperm removal by stimulating females' sensory system, an interrupted copulation experiment was

conducted in the fields in order to examine the relationship between sperm reduction and the morphology of the male's genitalia. The extent of sperm reduction in the spermatheca increased with the width of the penis head. Thus, the hypothesis of the indirect sperm removal mechanism by stimulating females' sensory system was supported. The females might suffer costs such as a loss of genetic variety of their offspring when the sperm displacement is complete. Thus, a counter-adaptation might have evolved in the females to prevent complete sperm displacement. In order to clarify females' adaptation for spermathecal sperm removal, the inter-population and seasonal variation of morphological factors affecting sperm removal, such as the width of the penis head and the number of vaginal sensilla, were examined, and counter-adaptations with which to prevent sperm ejection such as a decrease in the number of vaginal sensilla and an increase in the size of the spermatheca were found in females. Thus, the genital morphology of females and the indirect sperm removal mechanism that makes use of females' sensory system in the reproductive organs were shaped by sexual conflict. Proposing this co-evolutionary scenario for male-female genitalia in *I. asiatica* made it possible to reveal the role of females in genital coevolution.

Key words: bursa copulatrix; male secondary genitalia; Odonata; spermatheca; sperm competition; sperm removal; Zygoptera.

## GENERAL INTRODUCTION

Sperm competition has been conceptualized as a process of sexual selection defined as the competition between sperm of more than two males for the fertilization of eggs (Parker, 1970). In insects, once a male has transferred sperm to a female during copulation, the sperm is stored and maintained invariably in the female sperm storage organs, which have specialized tissue that provide both nutrition and a constant ionic environment so the sperm can survive throughout her life span (e.g. Villavaso, 1975). Therefore, insect sperm retain their fertilization capacity throughout the life span of the female, and the females fertilize eggs using the sperm at the time of oviposition. Typically, when a female oviposits, an egg passes down the oviduct to a special porch near the exit from the sperm storage organs. Females can control the position of the eggs in their internal genitalia and release exactly the number of spermatozoa needed to fertilize them in most insect species (Walker, 1980). Females of many insects can fertilize eggs without waste, as a single spermatozoon is generally used to fertilize a single egg (Thornhill & Alcock, 1983). If a female copulates with another male before the stored sperm is exhausted, temporal and spatial overlap of sperm from different males occurs in the female sperm storage organs, resulting in sperm competition. Therefore, sperm competition is expected to favor adaptations in males that prevent subsequent males from mating with a female or preempt the sperm stored by females from previous matings (Simmons, 2001). There is much evidence on

male morphological, physiological and behavioral traits that reduce the risk of sperm competition or prevent it, such as by guarding mates from rival males, depositing a mating plug on the female genitalia, transferring antiaphrodisiac substances, and removing rival sperm (Eberhard, 1996).

Parker (1970) stated that the postcopulatory guarding behavior of odonate males might function to protect mates from rival males and to avoid sperm competition with rival males, and concluded that sperm displacement and high last-male sperm precedence occurred. Waage (1979) demonstrated that the ebony jewelwing *Calopteryx maculata* males use their specialized secondary genitalia to remove the sperm of rival males that has been stored in the female sperm storage organs before transferring their own sperm in order to avoid sperm competition. Such morphological adaptations of the male's secondary genitalia are widespread in Odonata species (e.g. Waage, 1986).

Since Waage (1979) found the sperm removal mechanism in Odonata, the adaptive significance of males of the species in which the females are polyandrous has been investigated. Males could enhance the possibility that their own sperm is used for fertilization by removing the sperm previously stored in the sperm storage organs of females, resulting in an increase in the fertilization success of the males. Because sperm removal must be an efficient way to achieve high fertilization success for males, high last-male sperm precedence after copulation has been reported (e.g. Fincke, 1984; Siva-Jothy & Tsubaki, 1989). Thus, a sperm removal

mechanism might have evolved in the species in which the males could interfere with the sperm storage organs of the females. Consequently, the possibility of sperm removal behavior in animals with structures such as hooks or spines on their aedeagus, which is inserted directly into the sperm storage organs during copulation, has been proposed (Simmons, 2001). Sperm removal mechanisms similar to that in Odonata have been reported in many taxa, e.g. the longicorn beetle *Psacothoa hiralis* (Yokoi, 1990), the red flour beetle *Tribolium castaneum* (Haubruge et al., 1999). Furthermore, in taxa other than insects, the cuttlefish *Sepia esculenta* (Wada et al., 2005) and the nudibranch *Chromodoris reticulata* (Sekizawa et al., 2013) were reported to remove sperm or spermatophore stored in sperm storage organs.

It has been reported that the last-male sperm precedence occurs at a rate of nearly 100% just after copulation in Zygoptera (Córdoba-Aguilar et al., 2003). Zygoptera females usually have two types of sperm storage organs, the bursa copulatrix and spermatheca. The males can remove sperm stored in the bursa copulatrix in all species hitherto reported (e.g. Waage, 1986, Miller, 1987). On the other hand, the spermatheca is located at the back of the bursa copulatrix. Because the opening of the bursa copulatrix is located closer to the part connecting the oviduct and vagina where fertilization takes place, eggs are more easily fertilized by the sperm stored in the bursa copulatrix than in the spermatheca. Immediately after copulation, the sperm in the bursa copulatrix might be used for fertilization



more preferentially than the sperm in the spermatheca (Hooper & Siva-Jothy, 1996).

Although the sperm transferred by males that had previously mated with a female remain in the spermatheca after copulation if the last-mated male was not able to remove the sperm from the spermatheca, the bursa copulatrix is filled with the sperm transferred by the last-mated male just after copulation. Because the sperm in the bursa copulatrix is used for fertilization, the males might achieve high fertilization success if females start to oviposit immediately after copulation. The last-male sperm precedence would be high in that case.

In Zygoptera, there are many comparative studies of the morphology of the males' appendages and the length of the spermathecal duct and/or spermatheca in the females (Corbet, 1999). Males of some species are able to remove sperm from the spermatheca as well as from the bursa copulatrix using their appendages (e.g. Waage, 1979), while males of other species cannot remove sperm from the spermatheca (e.g. Siva-Jothy & Hooper, 1995). In *Ischnura* species, the males have two horn-like appendages on both sides of the tip of the secondary genitalia, whereas the females have a single spermatheca that is connected to the bursa copulatrix by the spermathecal duct. The appendages of *I. graellsii* males are able to reach inside of the spermatheca because the appendages are thinner and longer than the spermathecal duct; the males can thus directly remove the sperm stored in the spermatheca (Cordero & Miller, 1992). Contrarily, the males of *I. elegans*

could not directly remove the sperm stored in the spermatheca because their appendages are shorter than the spermathecal duct (Miller, 1987). In general, the sperm from previous matings that remain in the spermatheca are gradually mixed with the sperm transferred by subsequent males (Siva-Jothy & Tsubaki, 1989). The proportion of offspring sired by subsequent males tends to decrease with time after copulation. In *I. senegalensis*, the rate of last-male sperm precedence decreased to 50% at six days after copulation (Sawada, 1995). Sperm mixing causes a decrease in sperm precedence. Therefore, the males have to remove all sperm stored in the sperm storage organs of females in order to prevent sperm mixing so that the males can maintain a high rate of last-male sperm precedence for a long period.

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

Tajima & Watanabe (2009) clarified that a reduction in the number of spermatozoa occurred in both the bursa copulatrix and the spermatheca after the onset of copulation in *I. asiatica*, though each appendage of the male genitalia was significantly shorter than the spermathecal duct. No pairs in in-copula in which the appendages reached the spermatheca were collected from the fields. These results suggest that the decrease in spermathecal sperm is not caused by direct

sperm removal by the appendages of the male genitalia. In other words, there must be an indirect sperm removal mechanism in *I. asiatica* males.

*Ischnura asiatica* males might have evolved not only a direct sperm removal mechanism but also an indirect sperm removal mechanism (Tajima & Watanabe, 2009). Miller (1987) found that females of Odonata have a series of vaginal mechanoreceptive sensilla embedded in the pair of sclerotized vaginal plates that are located on both sides of the vagina. In *Orthetrum coerulescens*, females use the sensilla to communicate the presence of an egg to the muscles covering the sperm storage organs throughout the oviposition process in the vagina so that the egg can be fertilized (Miller, 1990). It has been suggested that males might use the fertilization mechanism of females to remove sperm from the spermatheca. Córdoba-Aguilar (1999) pointed out that indirect sperm displacement was mainly induced by stimulation of the female sensory systems.

During copulation, the aedeagus enters the female genital opening. When the males' appendages for direct sperm removal are restricted in the bursa copulatrix, the penis head, which is located in the front part of the aedeagus, comes into contact with the vaginal plates (Tajima & Watanabe, 2009). The male moves its abdomen in a piston motion, using the penis head to stimulate the vaginal sensilla. Thus, the penis head with abdominal movement might mimic the movement of the egg passing between the vaginal plates. The stimulation by the penis head might induce contraction of the muscle surrounding the spermatheca, and could thus

induce spermathecal sperm ejection (Córdoba-Aguilar, 2005).

The indirect sperm displacement via stimulation of the female sensory systems might have induced further evolution of the morphology of the female genitalia, as sperm removal has costs for the female, such as a loss of genetic diversity in their offspring (e.g. Siva-Jothy & Hooper, 1995). Therefore, in order to overcome these costs, a counter adaptation against the spermathecal sperm displacement by males is likely to have evolved. Consequently, it is likely that coevolution of the male and female genitalia has occurred in *I. asiatica* as a result of males removing spermathecal sperm via stimulation of the female sensory system.

Counter-adaptation against spermathecal sperm displacement by males might be evolved in females when the males increase genital size. Because the body size correlates with the genital size as morphological traits, large males have large genitalia. It is hypothesized that females develop the morphological traits to make sperm displacement difficult in the population including large males. The body size of adults is usually stable among local population because the body size might be determined by the trait of locality, such as the latitude, the area of the pond, the aquatic vegetation and so on (Stoks & Córdoba-Aguilar, 2012). Thus, the males must show different genital size among local populations. On the other hand, in *I. asiatica* in the mainland of Japan, there are two distinct body sizes for spring and summer generations (Naraoka, 1976). Therefore, the males also have different

size of genitalia between generations.

In my thesis, I confirmed the occurrence of sperm displacement via stimulation of the female sensory systems in *I. asiatica* (Chapter 1). In order to clarify the counter adaptation of the female genitalia, the variation among several local populations and the seasonal variation of morphological factors affecting sperm removal, such as the penis head and vaginal sensilla, were examined in the field populations and are reported in Chapters 2 and 3, respectively. I then discuss the evolution of the indirect sperm removal mechanism and the morphological factors affecting sperm removal in *I. asiatica*.

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## CHAPTER 1

Male Secondary Genitalia Mimic the Female Egg during Oviposition for Sperm Displacement in the Non-Territorial Damselfly *Ischnura asiatica* Brauer (Zygoptera: Coenagrionidae)\*

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## CHAPTER 2

Counter-Adaptation in Response to Sperm Removal by Stimulating the Sensory System in Female *Ischnura asiatica* (Odonata: Coenagrionidae)\*

\*Published in *Odonatologica*, **43**: 115-124 (2014)

## CHAPTER 3

Seasonal Variation of Genital Morphology and Sperm Removal in

*Ischnura asiatica* (Odonata: Coenagrionidae) \*

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## GENERAL DISCUSSION

There was a significant relationship between the penis head width and the vaginal sensilla number across the local populations in Chapter 2. Counter-adaptation by females to increased penis head size in males had occurred in *I. asiatica*. However, the variety in body size of both sexes among the local populations as well as the difference in generations has not been considered yet. Consequently, the fluctuation in spermathecal sperm during the copulation in Chapter 3 did not cooperate with the relationship resulting in as if the opposite case of Chapter 2. On the other hand, there were significant allometries between the mean body size and the mean width of penis head, and between the mean body size and the mean number of vaginal sensilla of each population and generations. Thus, these allometries were controlled by calculating appropriate residuals from regression lines (Fig. 1). Among local populations, there was a negative relationship between the residual width of penis head and residual number of sensilla.

The Tsukuba population of spring generation in 2008 for Chapter 2 and in 2009 for Chapter 3 showed a similar relationship between the width of penis head and the number of vaginal sensilla (Fig. 1). The summer generation showed that the females had a relatively lower number of vaginal sensilla than those in the spring generation. Although females in the summer generation might develop counter-adaptation among local populations as well as spring generation in Chapter 2, it is likely that the relative low number of sensilla in the females in

summer generation is not a counter-adaptation against the male sensory stimulation due to similar relative sperm displacement ability of males in the both generations. Therefore, the relative number of sensilla might be affected by another factor such as the difference of egg size as discussed in Chapter 3. The comparative work examining the genital traits of summer generation among local population may reveal the arms race between males and females in detail.

One central issue in the study of male-female genital coevolution is to clarify the benefits of the genital evolution for both males and females. The benefits of the genital evolution for males have been clarified in terms of the advantages conferred by morphological adaptation with regard to sperm competition (Simmons, 2001). The more sperm the males displace using their elaborated genitalia, the higher their fertilization success is (e.g. Yokoi, 1990). From the view point of the females, the morphology of the female genitalia might not be directly related to their reproductive success, because the number of progeny limits the reproductive success of females (Bateman, 1948). Thus, the benefits for females are less evident than those for males. On the other hand, it has been reported that the morphology of the female genitalia is related to the mating frequency of females (e.g. Arnqvist, 1998). Thornhill & Alcock (1983) suggested that females with an internal fertilization mechanism could mate multiple times as a result of their genital evolution since the females are able to store sperm under their own control. Consequently, the benefits for females of the evolution of their

genitalia may be related to the benefits of multiple mating (Córdoba-Aguilar et al., 2003).

The benefits of multiple mating for females can be divided into direct and indirect benefits (Simmons, 2001). The former are defined as material benefits such as those that affect the longevity of females and their number of progeny. Ridley (1988) noted that nuptial gifts, i.e., food resources that are transferred to females by males during courtship or copulation, are a direct benefit. In insects such as butterflies and crickets, the males transfer a spermatophore or sperm ampulla, which is a capsule or mass containing spermatozoa and nutrients, into the females during copulation (e.g. Kaitala & Wiklund, 1994). With multiple mating, females can obtain more nutrients via spermatophore or ampulla and can use them as a direct benefit to promote the maintenance and growth of female body (e.g. Wiklund et al., 1993), egg production (e.g. Simmons, 1990), egg maturation (e.g. Boggs & Gilbert, 1979). In addition, females can use sperm as an alternative source of nutrition by absorbing it from the sperm storage organs (Thornhill & Alcock, 1983). However, no evidence of nuptial gifts from odonate males has been reported (Córdoba-Aguilar et al., 2003). Odonate males transfer only sperm during copulation (Corbet, 1999). Furthermore, no absorption of sperm as nutrition has been demonstrated in odonate females because the tissue in the sperm storage organs has no sperm absorption function, as clarified by Siva-Jothy (1987).

The main indirect benefits that have been studied in females in Odonata are

the benefits of multiple mating (e.g. Fincke, 1996). Walker (1980) suggested three categories of indirect benefits: 1) ensuring genetic diversity in the offspring by using sperm from multiple males for fertilization; 2) ensuring fertilization by high-quality sperm by encouraging competition between males; and 3) increasing the chance of female mate choice. Siva-Jothy & Hooper (1995) examined how females ensure the genetic diversity of the sperm that remains in the spermatheca when it is not removed by subsequent males. When the sperm displacement rate is lower during copulation, females can store sperm derived from multiple males. Because the previous sperm remains in the spermatheca mixed with the sperm transferred by the subsequent male, the proportion of offspring sired by the subsequent male tends to decrease with time (e.g. Sawada, 1995). Consequently, females might oviposit genetically diverse eggs. In *I. asiatica*, none of the males can displace all of the sperm stored in the spermatheca, as shown in Chapter 1. Females can ensure the genetic diversity of the spermathecal sperm through multiple mating and by avoiding spermathecal sperm removal.

The relationship of fertilization to sperm quality is difficult to clarify in Odonata, because it is difficult to rear adults of most odonate species in the laboratory throughout successive generations (Córdoba-Aguilar et al., 2003). There is indirect evidence that *I. asiatica* females in the field may gain offspring of high quality when they mate and store the sperm of males of high quality, as shown in Chapters 2 and 3. In Chapter 2, it was clarified that the width of the

penis head varies among males within each population. Chapter 3 indicated that the males of both the spring and summer generations show variation in the width of the penis head and the extent of spermathecal sperm removal. Thus, males vary within the same population and within the same generation in their ability to induce spermathecal sperm ejection. Males with a low ability to induce spermathecal sperm ejection cannot transfer their sperm into the spermatheca because they did not remove the sperm of males that had previously mated with the female from the spermatheca. On the other hand, females would easily store sperm derived from males with a high ability to induce spermathecal sperm ejection because such males could remove almost all sperm in the spermatheca. As a result, females may choose males on the basis of a male's ability to induce sperm ejection by means of multiple mating and pursuing sperm ejection.

Increasing the chance of female mate choice by multiple mating of females has not been fully tested in non-territorial odonate species, because there is little information about qualities such as the body size, wing length, and so on, of the males before mating. For *Ischnura* spp. most males fly to search for females, and when they encounter one they promptly attack it (e.g., Takahashi & Watanabe, 2009). Females seem to have little chance to identify the qualities of the males. However, once in tandem with a male, a female must raise her abdomen to permit the male to engage the genitalia. Thus, males need some cooperation from females in order to copulate (Corbet, 1999), even though the tandem may have been



initiated by the male. On the other hand, females can show relatively strong mate refusal postures such as the opening of the wing and bending of the abdomen against approaches by males in *I. asiatica* (Arai, 1979). Such refusal postures might help females to reject unfavorable males, which would suggest that females can choose the males with which they mate.

Females' pursuit of sperm ejection by males might be related to the indirect benefits they receive from mating. The genetic diversity of the progeny might be ensured by pursuing sperm displacement. Córdoba-Aguilar et al. (2003) suggested that females choose males with which to mate on the basis of the male's ability to displace sperm, probably by pursuing sperm displacement. Therefore, females might gain indirect benefits not only from multiple mating but also by pursuing sperm removal. As shown in Chapter 1, the males of *I. asiatica* are not able to directly remove rival sperm stored in the spermatheca due to the long and narrow spermathecal duct as in other zygopteran species (e.g. Siva-Jothy & Hooper, 1995). On the other hand, from the viewpoint of males, indirect removal of the sperm stored in the spermatheca, which is connected to the bursa copulatrix by a long and narrow spermathecal duct, evolved over time. Thus, the evolution of an indirect sperm removal mechanism that stimulates the female's sensory system might be interpreted as an adaptation in response to the long and narrow spermathecal duct.

Females do not play a passive role in the indirect sperm removal by males, and might be expected to show counter-adaptations that make indirect sperm

removal more difficult for males. As shown in Chapter 2, a decrease in the number of vaginal sensilla developed as a counter-adaptation that functions to reduce the amount of sperm removed. A reduction in the number of sensilla makes females less sensitive to stimulation by the penis head during copulation (Córdoba-Aguilar, 2005), resulting in a reduction of the ejection of spermathecal sperm. Such a co-evolutionary response by females to increased penis head size in males may have occurred in *I. asiatica*.

Chapter 3 suggested that the capacity of the sperm storage organs was critical for sperm removal. Females do not need to store a large amount of sperm for fertilization because odonate females receive a sufficient amount of sperm for a lifetime of egg production in a single mating (Cordero, 1991). However, females might gain indirect benefits by storing large amount of sperm in their sperm storage organs. When the spermatheca is filled by sperm, it might be difficult for subsequent males to remove the sperm completely. Females with sperm storage organs with a large capacity can retain in the spermatheca more of the sperm transferred by males that had previously mated with the female after sperm removal. Thus, a high sperm-storage capacity in the spermatheca might be a counter-adaptation for sperm removal by males. On the other hand, having sperm storage organs with a high capacity might have a cost for the females (Michiels, 1989). The cost of the maintenance of stored sperm was clarified by Nakahara & Tsubaki (2007). Because females have to maintain sperm that is viable for

fertilization, they provide nutrition for the sperm from the inner walls of the sperm storage organs (Siva-Jothy, 1987). Thus, females must suffer the cost of maintaining the excess sperm. The trade-off between the benefit of a decrease in sperm removal during copulation and the cost of the maintenance of sperm might determine the capacity of the sperm storage organs.

The males of *I. asiatica* develop indirect mechanism for spermathecal sperm removal as counter-adaptation for long and narrow spermathecal duct. On the other hand, the counter-adaptation of females in order to reduce the amount of sperm removal is occurred by decrease of the number of vaginal sensilla. Thus, females' genital morphology and the indirect sperm removal mechanism using the sensory system in the females' reproductive organs were shaped by sexual conflict in *I. asiatica*. Although the sperm displacement mechanisms in Odonata such as the sperm displacement rate (e.g. Cordero & Miller, 1992), P2 value (e.g. Sawada, 1995), morphology of the male's appendages for sperm removal (e.g. Waage, 1979) and so on have been well-studied, there have been few reports on females' counter-adaptation in response to sperm displacement (Corbet, 1999, Córdoba-Aguilar et al., 2003). The co-evolutionary scenario of the male-female genitalia in *I. asiatica* suggests that females have a role in genital coevolution. This thesis might help elucidate the sperm removal mechanism in Odonata.

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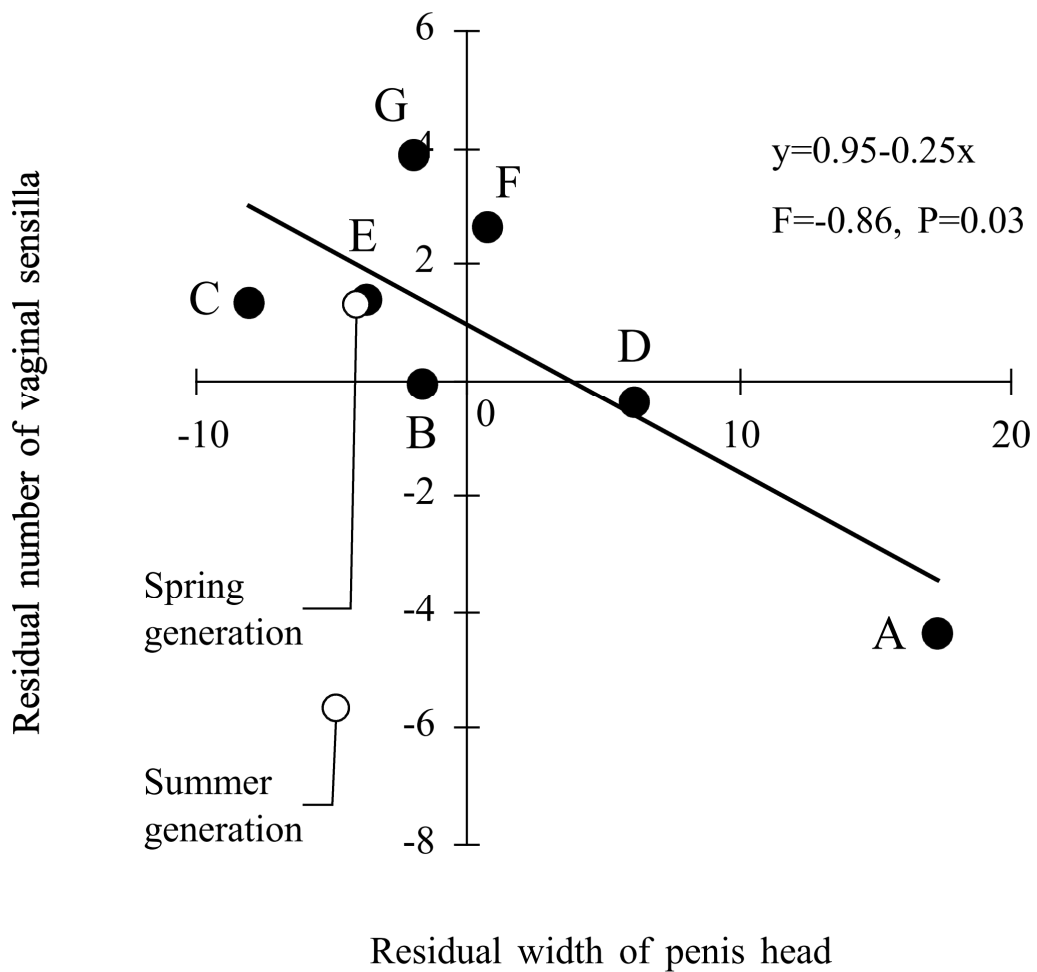


Fig. 1. Relationship between the width of penis head and the number of vaginal sensilla after controlling for variation in body size. Data set from Chapter 2 was shown as closed circle, and data set from Chapter 3 was shown as open circle. Solid line represents a regression line between the residual width of penis head and residual number of sensilla of the seven local populations (Chapter 2). Letters indicate each population (see Chapter 2).

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