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Abstract

In many insect species, males with larger body sizes are more successful at securing mates. Previous studies on the brachypterous grasshopper *Podisma sapporensis* have shown that male mating frequency varies greatly among populations. Here we test the hypothesis that differences in male mating success among populations are linked to differences in body size and shape. To do so, we collected males and females from three localities and conducted cross-breeding experiments. Overall, we found no link between mean male body size and mean number of matings across populations. However, stepwise regression indicates that within the populations, males with longer thoraxes and shorter hind femurs, mated more frequently, whereas females with longer thoraxes mated more frequently. These results suggest that in males, having short hind femurs, relative to body size, rather than body size itself, contributes positively to mating success — perhaps because of an advantage in holding onto females while avoiding females' resistance.

Key words

body size, morphological trait, stepwise regression, multiple choice experiment

Introduction

In many insect species, there is a general tendency for larger males to secure more matings (Thornhill & Alcock 1983, Simmons 1986, Forrest *et al.* 1991, Goulson *et al.* 1993, Kosal & Niedzlek-Feaver 1997). Three reasons are postulated for an advantage of larger males in mating. Firstly, females may actively choose mates of larger body sizes or of high quality through detecting the condition of specific male traits (Kotiaho *et al.* 1996, 1997). Secondly, where male-male competition is frequent, larger males are likely to dominate, so acquiring the opportunity to mate with females (Andersson 1994; Miyatake 1993, 1995). Thirdly, in species in which females are generally reluctant to mate, larger males are able to subdue reluctant females more efficiently, with the effect of more frequent matings (Allen & Simmons 1996).

In a previous study it was shown that females of the grasshopper *Podisma sapporensis* are generally reluctant to mate, females repelling approaching males (Sugano & Akimoto 2007). For successful mating, the males have to subdue females by grasping from behind with their legs. Thus, our first question about the mating system of *P. sapporensis* is whether being a larger male is advantageous in mating or not.

Another characteristic of mating in *P. sapporensis* is large differences in mating frequency among local populations in both sexes. In cross experiments (see below), using three local populations, we found that in one population the males were very vigorous and able to obtain frequent matings, whereas females of the same population were most reluctant to mate. In contrast, males of a second population were relatively inactive in mating, and females of the same population were most receptive.

We proposed that these results could be explained by hypothesizing an antagonistic coevolution between female ability to repel males and male ability to subdue reluctant females. If this hypothesis is correct, respective local populations are at different equilibria, depending on the local conditions, in which female ability to repel males is balanced with male ability to overcome female resistance. In this respect, the critical issue is whether female ability to repel or male ability to subdue are attributed to general body size or to specific body shape (some combination of morphological traits). If male and female morphological traits have been affected by such sexual conflict, the strength of which varied from population to population, morphological divergence may have been promoted among local populations, perhaps leading to geographic variation and speciation.

The present study aims to clarify these questions using three populations of *P. sapporensis*. We conducted cross pairings among the three populations in the laboratory and examined the relationships between the number of matings for each adult and its morphological traits. The specific questions to be answered were 1) whether differences in mean mating frequency among the populations reflected differences in mean body size or not, and 2) which morphological trait or trait-set is responsible for the variation in mating frequency, irrespective of the differences among local populations.

Methods

The grasshopper and cross-experiments.—*P. sapporensis* (Figs 2,3), a univoltine grasshopper, is distributed on islands of northern Japan and the Russian Far East (Bugrov *et al.* 2000a). This species ranges from lowland to alpine areas and is mainly found along the edges of forests (Miyatake & Kanou 1992). On a microgeographic scale, it is patchily distributed on the host plant *Petasites japonicus giganteus*. It is characterized by conspicuous geographic variation in body coloration (Miyatake & Kanou 1992), morphological traits (Akimoto *et al.* 1993; Tatsuta & Akimoto 1994, 1997, 1998, 2000), and karyotypes (Walchałowska–Śliwa *et al.* 2008a, 2008b).

We conducted cross-breeding experiments, using local populations in Hokkaido in 2000. Based on Bugrov *et al.* (2000b), we selected three localities with high grasshopper density: Teine (lat 43°06'N, long 141°13'E; 150 m above sea level), Shimokawa (lat 44°17'N, long 142°40'E; 150 m above sea level) and Akan (lat 43°26'N, long 144°08'E; 400 m above sea level). The three localities were 150 to 240 km from one another. In mid June, we collected 200 to 250

Table 1. Mean number of matings per individual over seven-days of observation and mean length of thorax for males and females of each population. Means and s$_{\overline{x}}$ are shown. Different letters show significant differences between means based on the Tukey-Kramer method.

Locality	No. matings		Thorax length, mm	
	male	female	male	female
Akan	2.9± 1.79b	5.9± 2.26a	4.9± 0.24a	5.7± 0.34a
Shimokawa	3.5± 2.05ab	3.9± 2.94b	4.3± 0.28c	5.2± 0.35b
Teine	4.1± 2.09a	0.7± 0.80c	4.5± 0.29b	5.3± 0.47b

second to fifth instars from each locality in order to obtain virgin adults. In each locality, grasshoppers were collected from within a circular area with radius of 100 m.

The nymphs were placed in plastic containers (40×21×27cm, length × width × height) and reared at 21°C under a photoperiod of 14L:10D in an environmental chamber. Approximately 100 nymphs from the same locality were maintained in one container. The position of the containers on the rearing shelves was displaced every day in order to avoid position effects in the chamber. The nymphs and adults were fed on leaves of *Petasites japonicus giganteus*.

We conducted the crossing of any two populations using a multiple choice method. This method enables any male and any female to choose a mate from their own population or from a different population, so we can easily evaluate mate preference for other populations relative to their own population. We prepared all cross-combinations of three local populations, *i.e.*, three cross-combinations, Teine *vs* Shimokawa, Shimokawa *vs* Akan, Akan *vs* Teine. Each cross-combination was replicated three times, using separate containers. Ten males and ten females from one population were simultaneously placed in a container (70 × 30 × 40 cm,

length × width × height) along with ten males and ten females from a different population, such that 40 adults were present in the container. Since the adults did not mate for several days after final molting, only adults more than three days old with a complete set of appendages were used in the crossings. Virgin grasshoppers were used in all crossings.

The grasshoppers were individually distinguished by marking with white paint. Each grasshopper's behavior was monitored for eight hours daily, from 9:00 to 17:00 h, and all matings established in this interval were recorded. (Except that observations were stopped if 10 matings were observed before 17:00 h.)

The adults in each container were monitored consecutively for seven days; this seven-day observation period for one container is referred to as a trial. In each trial, adults were not replaced. Each adult was used only in a single trial. Observations were always made by the same researcher. Each day, after observation, the females from each container were transferred into another container in order to prevent copulation until the next observation period. During observation, the temperature was raised to 23°C in order to enhance the activity of the grasshoppers. Thus, the number of matings was recorded for each adult in each trial.

After the observation in a trial, all the grasshoppers were fixed in 80% ethanol and used for measurements of eight morphological traits: head length (HL), thorax (pronotum) length (TL), fore femur length (FFL), fore femur width (FFW), midfemur length (MFL), mid femur width (MFW), hind femur length (HFL), and hind tibia length (HTL) (Fig. 1). The measurements were made using an electronic digital caliper and a stereomicroscope with an ocular micrometer.

Statistics.—For each locality, the mean number of matings per adult in a trial was calculated, and mean body size, represented by thorax length as well. The effect of each morphological trait on

Fig. 1. Measurements of eight morphological traits of *P. sapporensis*.
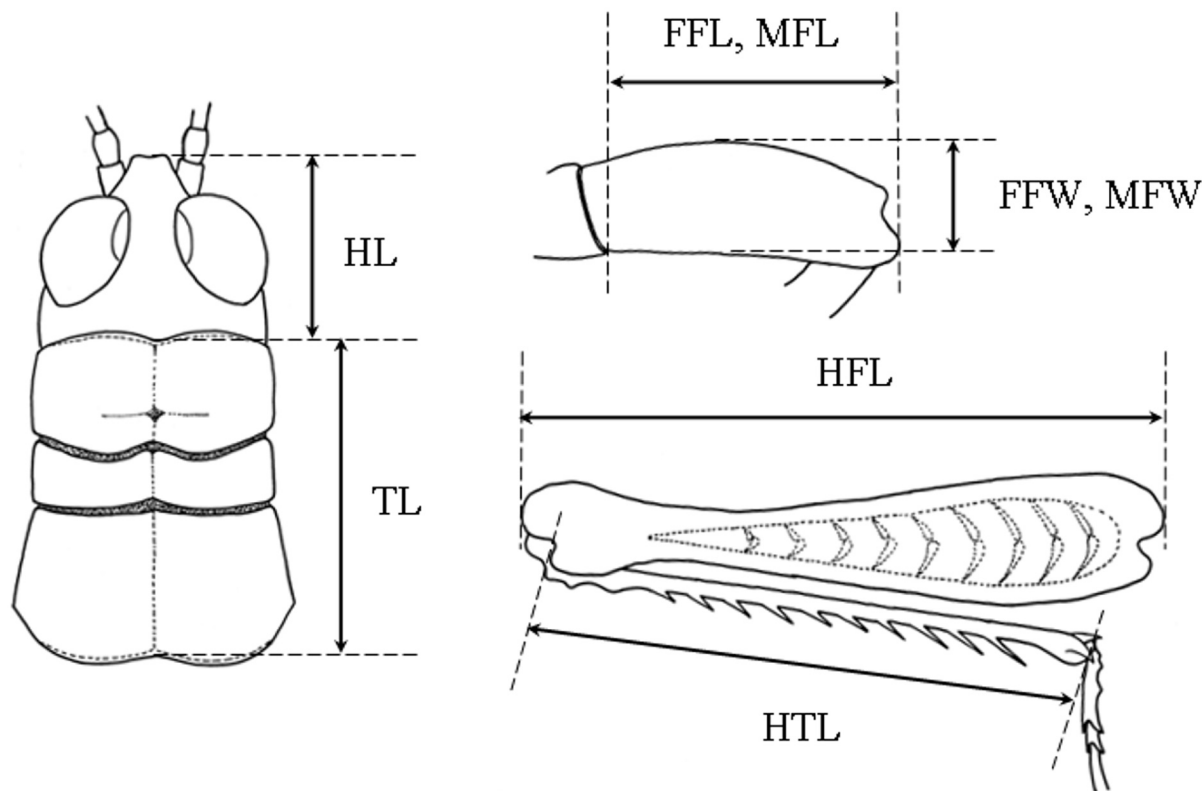
the number of matings was evaluated for both sexes with multiple regression. The distribution of the dependent variable, the number of matings per adult, was highly skewed from a normal distribution, in particular for females. Thus, in the analysis, the number of matings was transformed with the Box-Cox method, to render the distribution closer to a normal distribution.

Before analysis of the effects of morphological traits, we tested the effects of cross combination (three types), replications within each cross combination (three replicates), and localities (three localities) — separately for males and females, using nested ANOVA. In the ANOVA the replications were nested within cross combinations and specified as a random effect. Thus, the effect of cross combination on mating was tested relative to the effect of replications. When any variable was statistically significant in this analysis, that variable was included in the model of the next analysis, together with eight morphological traits. The effects of the morphological traits were analyzed using stepwise multiple regression (backwards model), deleting any traits that were not significant sequentially from the model. The reason for the use of stepwise multiple regression is that the inclusion of the highly correlated morphological traits into the model leads to multicollinearity.

In order to assess the extent to which variation in mating frequency was explained by the factors of cross combinations, localities and morphological traits, we calculated the variance components of these factors for both sexes using the residual maximum likelihood method (REML) (Shaw 1992). In this calculation, cross combinations and localities were specified as random effects. All the statistical analyses were conducted with the software JMP version 5 (SAS Institute 2002).

Results

The mean number of matings per individual in a trial differed significantly among the localities in both males and females (Table 1). Teine males mated most frequently, followed by Shimokawa males, and Akan males mated least frequently. However, the rank order of mating frequency was opposite in females (Akan>Shimokawa>Teine). These results were the same as those in a different experiment using the same three populations (Sugano & Akimoto 2007). Across all nine 7-d trials, individuals varied widely in propensity to mate. Eleven of 180 males and 35 of 180 females did not mate, whereas one male mated nine times, and two females mated 11 times each.

However, these population propensities for mating were not linked to population mean body size. Although the rearing of nymphs was conducted under the same laboratory conditions with host leaves of the same quality, body size varied significantly among localities both in males and in females (Table 1, Fig. 3). For males, although the Akan population had the largest body size, they mated least often. For females, the Teine population was not the biggest, but females repelled approaching males most intensively, showing the strongest reluctance for mating.

Although the small number of populations used makes statistical analysis impractical, these results indicate that male propensity for mating and female reluctance are not linked to body size. Thus, we examined which morphological traits were associated with mating frequencies in males and females across populations, after the effects, of the propensity to mating intrinsic to the population, were controlled statistically.

When we evaluated the effects of cross combinations, replications within cross combinations, and localities, on mating frequency, there was no significant effect in replications for either males (df = 6, F = 0.04, p = 0.99) or females (df = 6, F = 0.50, p = 0.81), suggesting that replications could be pooled in the later analyses. However, the effects of cross combinations and localities were significant both in males (for cross combinations, df = 2, F = 8.32, p < 0.001; for localities, df = 2, F = 7.33, p = 0.001) and in females (for cross combinations, df = 2, F = 56.37, p < 0.001; for localities, df = 2, F = 169.24, p < 0.001). Thus, these variables were incorporated into the multiple regression model, together with eight morphological traits. In the stepwise regression, localities and cross combinations were always held as variables in the model, and the morphological traits were subject to backward selection.

Stepwise regression indicated that in males, thorax length and hind femur length had significantly positive and negative effects, respectively, on mating frequency (Table 2). This result suggests that differences in body shape rather than general body size are important for securing mating in males. However in females, of the eight morphological traits, only thorax length affected the mating frequency significantly. Therefore females with longer thoraxes mated more frequently.

To determine the extent to which the cross combinations affect the results of the morphological analysis, we used a general linear model including cross combinations, localities, the morphological traits that were significant, the interactions between cross combinations and, as the independent variables, these morphological traits.

Table 2. The results of stepwise regression (backward selection) for the effect of each morphological trait on the number of matings. The eight morphological traits were included in the initial model as well as localities and cross combinations. The traits shown as "Not included" were not included in the final regression model. Localities and cross combinations were always held in the model of the stepwise regression. Par is the parameter estimated.

	Male				Female			
	Par	df	F	p	Par	df	F	p
Head length	Not included				Not included			
Thorax length	1.037	1	4.86	0.029	0.563	1	6.07	0.015
Ffem length	Not included				Not included			
Mfem length	Not included				Not included			
Hfem length	-0.783	1	9.52	0.002	Not included			
Htib length	Not included				Not included			
Ffem width	Not included				Not included			
Mfem width	Not included				Not included			
Locality		2	4.46	0.0013		2	120.27	<0.001
Cross combination		2	2.35	0.098		2	23.82	<0.001

In males, the analysis showed that neither of the two interactions was a significant predictor of mating frequency (for the cross combination with thorax length, df = 2, F = 0.22, p = 0.80; for the cross combination with hind femur length, df = 2, F = 2.90, p = 0.058). In females as well, interaction between the cross combination and thorax length was not significant (df = 2, F = 1.70 p = 0.19). These results suggest that the effects of thorax length and hind femur length in males and thorax length in females on mating frequency are invariant across populations.

In males, the variance components of cross combinations and localities were relatively small, accounting for 0.8 % and 5.8 %, respectively, of the total variance in mating frequency. The remaining variance was explained by variation in the morphological traits and the residuals. In contrast, in females, cross combinations and localities accounted for 13.8% and 66.9% respectively, of the total variance. This result suggests that mating propensity native to localities can explain the majority of the variation in female mating frequency.

Discussion

A notable characteristic of mating behavior of *P. sapporensis* is a strong female resistance against males' attempts to copulate. In *P. sapporensis*, the mating process commences with the male jumping on the back of the female then trying to prevent her from rejecting him. Females repel any males approaching them by jumping away or kicking, and they twist their abdomen away when males on their back try to push their genitalia closer to female genitalia. Females occasionally dislodge the mounted males by kicking them upward. As a male is refused, he often nibbles the females' back or vibrates his hind legs rhythmically. Although the function of this behavior is currently unknown, we suggest it might help promote copulation by females.

The brachypterous species *P. sapporensis* uses no acoustic signals in mating, and mating success depends on whether males can hold onto females or not (Sugano & Akimoto 2007). This observation corroborates the results of the present analysis, which indicate that mating success in males is affected by body shape, not by body size. It is likely that larger males with relatively short legs have an advantage in holding onto females (*e.g.*, by avoiding females' kicking). This link between shorter hind femurs in males and higher mating frequency is found across the localities.

In this respect, it is interesting to point out that the relationship between thorax length and hind femur length differs significantly among the localities: the Teine males with the highest mating frequency, and the Shimokawa males with the medium mating frequency, both had the shortest hind femurs relative to their body (least square mean, 10.8 mm). In contrast, the Akan males with the lowest mating frequency were characterized by having relatively long legs.

Although the relationship between shorter hind femurs and higher mating frequency is not specific to any local population, males of the Teine population may have been subject to more intense selective pressures for relatively shorter femurs. A previous study on geographic variation in *P. sapporensis* showed that the slope of male hind femur to body size, differed significantly among local populations, whereas that of other appendage traits did not differ (Akimoto *et al.* 1993). The high geographic variation in the length of male hind femurs shown in the present and previous studies may imply that strong sexual selection is operating specifically on this male trait in some populations.

The analysis of mating frequency in females showed that the majority of variation in female mating frequency was due to locality-specific propensities. About 81% of the variance was explained by the differences among female localities, plus cross combinations (6.6% in the case of males). As a result, only a small variance was left for morphological analysis in females, leading to unstable results in the multiple regression. For example, the morphological trait that showed significance changed, depending upon whether the dependent variable was transformed or not, or on whether the variables other than morphological traits were included in backwards selection. In contrast, in males, the two morphological traits that exhibited significance were robust, irrespective of the presence or absence of transformation of the dependent variable.

As the present experiments were conducted under artificial conditions, one has to be careful in generalizing these results. In the field, *P. sapporensis* is subject to predation and parasitism. These pressures may result in a reduction in mating frequency, and furthermore the operation of predation and parasitism may be biased toward adults with specific morphology. For example, relatively long legs may be adaptive for escaping from predators, but unsuitable for holding onto females. Thus, it is possible that the length of male hind femurs is determined by a balance between predation pressure and sexual selection within local populations. On the other hand, in the limited space of the cage, it may be difficult for the females to escape completely from males approaching them. If so, limited space would moderate sexual selection acting on the males. Thus, it is necessary to examine whether this result can be applied to wild situations or not.

Another biasing factor could be interpopulation crosses, in which males of a population were forced to mate with females of a different, as well as their own, population. Combinations between local populations might have had a significant effect on the trait that contributed positively to mating frequency. However, the analysis based on the GLM shows that the interactions of the cross combinations with characters that exhibited significant effects were not significant, suggesting that the effects of such characters did not change significantly depending on the cross combinations used. Thus, the hypothesis that male body shape or female body size are critical for mating is maintained, even if the differences between cross combinations are taken into account.

It should be noted that females probably do not gain direct fitness benefits from frequent matings. The theory of sexual conflict predicts that frequent matings result in larger costs to females through predation, infection of diseases and physical damage from the males (Arnqvist & Rowe 2005). Thus, evidence that females of larger body size mated more frequently suggests that larger body size is not advantageous to females if costs of mating are considered. Therefore, it is also necessary to examine whether or not smaller body size is advantageous to females in escaping superfluous matings in the wild.

In conclusion, laboratory experiments indicated that males with long thoraxes (*i.e.*, large body size) and short hind femurs are likely to secure matings, although the mechanical function of hind femurs during mating is unclear. This result, coupled with large geographic variation in the allometry of hind femurs relative to body size, suggests that sexual selection is acting on male hind femur length in some populations. In future studies, it is necessary to clarify how males use their hind femurs in mating and the extent to which male traits are subject to sexual selection in the wild. On the other hand, in females, it is necessary to assess whether or not they have been selected toward smaller body size in the wild.

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Fig. 2. *Podisma sapporensis* mating pair, Teine population, Hokkaido Japan. See Plate II.

Fig. 3. *Podisma saporensis* females from the three populations illustrating size differences; left to right from Akan, from Shimokawa and from Teine. See Plate II.