Repeatability of sperm number across multiple matings in three cricket species, *Gryllodes sigillatus*, *Gryllus veletis*, and *Gryllus texensis* (Orthoptera: Gryllidae)

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Abstract: Although studies of various taxa have shown that males can alter the number of sperm in their ejaculates according to the risk of sperm competition, few studies have examined the extent to which the number of sperm transferred by males across multiple matings is repeatable. We assess the within-male and between-male components of variation in sperm number by counting the sperm in multiple ejaculates of males of three cricket species and determining the repeatability of sperm number. Sperm number was highly repeatable across multiple matings in all three species, leaving open the possibility that variation in sperm number is based, in part, on heritable genetic variation.

Résumé : Bien que des études de différents taxons aient démontré que les mâles peuvent modifier le nombre de spermatozoïdes dans leurs éjaculats en fonction des risques de compétition entre les spermatozoïdes, peu d'études se sont attardées à déterminer à quel point le nombre de spermatozoïdes transmis par les mâles au cours d'accouplements multiples est reproductible. Nous évaluons ici les composantes de la variation, chez un même mâle et d'un mâle à l'autre, par dénombrement des spermatozoïdes dans les éjaculats multiples des mâles chez trois espèces de grillons et par détermination de la répétabilité du nombre obtenu. Le nombre des spermatozoïdes s'est avéré très reproductible au cours des multiples accouplements des mâles des trois espèces, laissant entrevoir la possibilité que le nombre de spermatozoïdes soit en partie régi par la variation génétique héréditaire.

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Introduction

In many species of insects, females often mate with more than one male, resulting in competition between the sperm of rival males for the fertilization of a single female's eggs. Such competition represents a potent selective force, and a wide array of male secondary traits in these insects has been attributed to selection on males to prevent females from remating or to incapacitate the sperm of their rivals (Parker 1970, 1984). Evolutionary biologists have begun to consider the possibility that male sperm-allocation strategies may also

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be tied to the risk of sperm competition. Under most circumstances, an increased risk of sperm competition favours an increase in the number of sperm transferred, but this increased gametic investment comes at the cost of a male's ability to invest in future matings (Parker 1998).

Comparative studies across a wide range of taxa have broadly supported the prediction that ejaculate expenditures should increase with an increase in the risk of sperm competition (Harcourt et al. 1981; Svärd and Wiklund 1989; Møller 1991; Stockley et al. 1997). In addition, a number of studies have shown that, within species, males exhibit considerable plasticity in ejaculate expenditure in response to varying sperm competition risk (e.g., Gage 1991; Gage and Baker 1991; Baker and Bellis 1993; Gage 1998). Notwithstanding the ability of males to customize their ejaculates, the average ejaculate expenditure of males of a given population should be subject to stabilizing selection imposed by both the social environment and the costs of producing sperm (see also Morrow and Gage 2001). Consequently, we might expect that variation between males in the number of sperm transferred at mating should account for less of the overall variance in sperm number than variation in the number of sperm transferred across multiple matings within males.

Here we assess the within-male and between-male components of variation in sperm number by counting the sperm in multiple ejaculates of males of three cricket species, *Gryllodes*

sigillatus, *Gryllus veletis*, and *Gryllus texensis* (Orthoptera: Gryllidae) and determining the repeatability of sperm number. Repeatability is a statistic derived from quantitative genetics theory, and measures the extent to which differences between individuals contribute to the overall variation in a trait (Boake 1989). Repeatability (r) is formally defined as $(V_G + V_{Eg})/V_p$, where V_G is genetic variance, V_{Eg} is variance arising from permanent but nongenetic factors (e.g., maternal effects), and V_p is total phenotypic variance. Repeatability provides an upper estimate of the heritability of a trait and some indication of its likely response to selection (Falconer 1981), and is a technique that is especially appropriate to behavioural studies (Boake 1994). Repeatability partitions the phenotypic variance of a trait into a between-individual component and a within-individual component. The within-individual component is entirely environmental in origin, caused by temporary differences in environment between successive measurements; in the present study, this component incorporates any effect due to social environment (i.e., sperm competition risk). The between-individual component is both genetic and environmental in origin, with the environmental component arising from factors that affect individuals permanently.

Crickets are useful model systems with which to address sperm allocation patterns, because they are typically subject to high levels of sperm competition (Zuk and Simmons 1997) and there is evidence from some species that males are capable of customizing their ejaculates in accordance with the risk of sperm competition (Gage and Barnard 1996; Schaus and Sakaluk 2001). Males transfer their sperm in the form of a spermatophore, a discrete vessel that remains attached outside the female's body after mating. The spermatophore is easily removed, simplifying the recovery and enumeration of sperm. Males typically manufacture their spermatophore well in advance of copulation, and copulate only if a fully formed spermatophore is present in their spermatophoric pouch (Loher and Dambach 1989).

Materials and methods

Experimental crickets were obtained from stock colonies and maintained according to standard procedures (Sakaluk 1991; Burpee and Sakaluk 1993). *Gryllodes sigillatus* originated from approximately 200 subadult and adult crickets collected at Tucson, Ariz., U.S.A., in October 1995; *G. texensis* originated from crickets collected at Austin, Tex., U.S.A., in summer 1995; and *G. veletis* were F_1 offspring of crickets collected locally. Late-instar nymphs were checked daily for adult eclosion, and adults were held individually to ensure their virginity.

The number of sperm contained in the spermatophore was determined for each of three successive spermatophores produced by a male after his first spermatophore had been discarded. These sperm counts were made as part of a larger study in which the number of rival males with which a male was held prior to mating (0, 1, or 6) was varied from one mating to the next (Schaus and Sakaluk 2001). The order in which males experienced the three treatment densities was randomized from one replicate to the next. Because any effect of social environment on sperm allocation would increase the within-male component of the overall variance in

sperm number while leaving the between-male component unaffected, our estimate of the repeatability of sperm number can be regarded as conservative.

Experimental males were housed in 3.5-L plastic jars with the prescribed number of rivals for a period of 24 h prior to mating and provided with cricket chow and water ad libitum. Experimental males were distinguished from nonexperimental males by a drop of model paint applied to the pronotum or hind femur. Following the 24-h period, the male was removed and placed in another jar with a virgin female of known mass and age and allowed to mate. If the pair failed to mate within 1 h, the male was reassigned to his current treatment density and given an opportunity to mate with a different virgin female 24 h later. Upon mating, the spermatophore was immediately removed from the female with forceps, and the male was assigned to his next treatment density. This protocol was repeated until each male had mated three times, once in each social environment ($n = 24$ males for each species).

In *G. veletis* and *G. texensis*, the spermatophore consists of a simple sperm-containing ampulla, whereas in *G. sigillatus*, the ampulla is accompanied by a gelatinous non-sperm-containing mass, the spermatophylax, which functions to keeps the female preoccupied during the time it takes the ampulla to be emptied of sperm (Sakaluk 1984, 1985). After matings in all three species, the ampulla was immediately placed in 4 mL of distilled water, except that, in the case of *G. sigillatus*, this first necessitated the separation of the ampulla from the spermatophylax. The ampulla was subsequently cut into several pieces using microscissors, after which the mixture was forced repeatedly through a fine-gauge 1-cc syringe, until the ampulla had been sheared into smaller pieces. To prevent sperm agglutination, the solution was stirred vigorously for 1 min using a Fisher Vortex Genie 2. In the case of *G. sigillatus*, the solution was further diluted by half, because of the higher density of sperm in this species. Five 10-μL samples were pipetted onto a uniquely labeled microscope slide equipped with a grid, which subsequently was set aside to dry. The mean number of sperm per 10-μL sample was determined at ×100 magnification. Sperm counts were made blind with respect to the male from which the spermatophore had been obtained.

Results

Repeatability was determined as the intraclass correlation in sperm number, which was derived from a one-way ANOVA using the GLM (general linear model) module of SAS (SAS Institute Inc. 1988). The intraclass correlation coefficient (r_i) was calculated as (groups MS – error MS)/[groups MS + ($n – 1$) error MS], where MS is mean square and n is the number of repeated measures per individual (Zar 1984).

Variation in sperm number was due largely to variation among males rather than to variation among ampullae of individual males. The repeatability of sperm number varied from 0.36 to 0.79 across species, and was highly significant statistically in all cases (all $P < 0.002$; Table 1).

Discussion

Differences between males explained a significant and substantial portion of the variance in sperm number in all three species. This result is somewhat surprising for two reasons:

Table 1. Repeatability of sperm number, measured as the intraclass correlation (r_i) of the number of sperm contained in each of three spermatophores produced by individual males, in three cricket species.

	r_i	$F_{[23,48]}$	P
Gryllus veletis	0.790	12.26	0.0001
Gryllus texensis	0.544	4.58	0.0001
Gryllodes sigillatus	0.361	2.70	0.0019

(1) males of some cricket species are capable of adjusting the number of sperm allocated from one ejaculate to the next (Gage and Barnard 1996; Schaus and Sakaluk 2001), which should increase the within-male component of the variance in sperm number; and (2) the range over which males can vary the number of sperm in their ejaculates should be subject to stabilizing selection and, hence, most of the genetic variation in average ejaculate expenditure should be exhausted. Morrow and Gage (2001) recently reported significant differences between males in sperm length in 10 different taxa, including field crickets (*Gryllus bimaculatus*), a result they found equally puzzling for similar reasons.

Of the three cricket species, *G. sigillatus* exhibited the lowest repeatability, accounting for less than half of the total variance in sperm number. However, *G. sigillatus* is unusual in that the male's spermatophore includes a large gelatinous mass, the spermatophylax, which the female detaches and feeds on after mating. The spermatophylax serves as a protective device, ensuring that the majority of a male's sperm are evacuated from the sperm ampulla before it too is removed and eaten by the female (Sakaluk 1984). In most cricket species, however, the ampulla is transferred to the female unprotected by a spermatophylax, giving females the freedom to terminate sperm transfer at any time after mating by prematurely removing the ampulla (Sakaluk 2000). The ability of male *G. sigillatus* to entice females into relinquishing at least some of their control of the insemination process by providing a nuptial food gift may afford males greater latitude in varying the number of sperm allocated to the sperm ampulla relative to males of non-gift-giving species. This in turn could account for the lower repeatability of sperm number exhibited by *G. sigillatus*.

Because the repeatability sets an upper limit to the heritability of a trait (Falconer 1981), the results of the present study leave open the possibility that variation in sperm number is predicated, to some extent, on heritable genetic variation (Boake 1994). While repeatabilities should be interpreted with some caution (Boake 1989; Aragaki and Meffert 1998), studies in which the actual heritability of sperm number has been measured have reported significant additive genetic variation. For example, in various species of domestic livestock, several parameters of ejaculates, including sperm concentration, have been found to be significantly heritable (e.g., Parlevliet et al. 1994; Ducrocq and Humblot 1995).

We are not aware of any studies that have determined the heritability of sperm number for any insect species, although previous studies have shown that relative ejaculate size (the proportion of male body mass devoted to the ejaculate) is significantly heritable in crickets (Sakaluk and Smith 1988) and seed beetles (Savalli et al. 2000). Radwan (1998) re-

cently demonstrated significant additive genetic variation in male sperm competition success in mites (but see Gilchrist and Partridge 1997). If this result proves replicable across other species, then genetic variation in sperm allocation would be an obvious factor underlying variation in sperm competition ability. Perhaps of even greater interest would be studies designed to investigate the genetic underpinnings of plasticity in sperm allocation, given the increasing number of studies suggesting that such plasticity is ubiquitous across species subject to varying sperm competition risk and intensity (e.g., Gage and Barnard 1996; Simmons and Kvarnemo 1997; Wedell and Cook 1999).

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