Current Biology, Vol. 15, 271–275, February 8, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.01.032

Sperm Viability Matters in Insect Sperm Competition

Francisco García-González*
and Leigh W. Simmons
Evolutionary Biology Research Group
School of Animal Biology (M092)
The University of Western Australia
Nedlands, Western Australia 6009
Australia

Summary

Experimental studies in insects have shown how sperm competition can be a potent selective force acting on an array of male reproductive traits [1–4]. However, the role of sperm quality in determining paternity in insects has been neglected, despite the fact that sperm quality has been shown to influence the outcome of sperm competition in vertebrates [5–8]. A recent comparative analysis found that males of polyandrous insect species show a higher proportion of live sperm in their stores [9]. Here, we test the hypothesis that sperm viability influences paternity at the within-species level. We use the cricket *Teleogryllus oceanicus* to conduct sperm competition trials involving prescreened males that differ in the viability of their sperm. We find that paternity success is determined by the proportion of live sperm in a male's ejaculate. Furthermore, we were able to predict the paternity patterns observed on the basis of the males' relative representation of viable sperm in the female's sperm-storage organ. Our findings provide the first experimental evidence for the theory that sperm competition selects for higher sperm quality in insects. Between-male variation in sperm quality needs to be considered in theoretical and experimental studies of insect sperm competition.

Results and Discussion

We used the Australian field cricket, *Teleogryllus oceanicus*, a species in which sperm are transferred from an externally attached spermatophore, to test the hypothesis that sperm viability, measured as the proportion of live sperm in an ejaculate, determines paternity success when males compete to fertilize a female's ova. The fact that the ejaculate in this species is contained in a spermatophore allowed us to determine the repeatability of sperm viability across ejaculates of individual insects. Furthermore, previous work in this species found that there is no variation among males in the number of sperm transferred to the female's spermatheca for any given duration of spermatophore attachment [10]; thus, we were able to control for sperm numbers when analyzing the influence of sperm viability on competitive fertilization success.

The proportion of offspring sired by the second male to mate with a female (P_2) was explained by the viability of his sperm. Factors affecting P_2 were analyzed with generalized linear models with binomial error structure and logit link function. Second-male sperm viability positively influenced P_2 (change in deviance $\chi^2_1 = 7.09$, $p = 0.0077$, $n = 45$; Cohen's standardized effect size estimated according to Rosenthal [11], $d = 0.81$) (see Figure 1). The mean percentage of viable sperm from two spermatophores collected across 135 males was 66% (standard error = 1.0, range 25%–87%). Previous studies of this insect have found that neither sperm numbers nor sperm size influences paternity [10]. Our experimental design controlled for the number of sperm transferred from each male. We found no significant effects for mating interval, the order of matings, male age, male size, or male weight. We did find that very young males had a greater proportion of nonviable sperm than middle-aged and older males (change in deviance $\chi^2_1 = 9.05$, $p = 0.003$, $n = 137$; Cohen's standardized effect size $d = 0.52$) (see Figure 2). However, we minimized the effects of age in our experiment by placing only males that were in their middle age in competition (see Experimental Procedures), so that male age did not contribute to the effect of sperm viability on fertilization success, as confirmed by the nonsignificance of this term in the generalized linear model.

We observed complete first or last male sperm precedence (i.e., $P_2 = 0$ or $P_2 = 1$, respectively) in 29% of the sperm-competition trials. Insemination failures are likely to be responsible for at least some of these extreme P_2 values; the proportion of mated males that did not sire offspring in this study was around 15%, whereas previous studies of this species have found that around 5% of matings do not involve sperm transfer [12]. Importantly, our conclusion that second-male sperm viability influences P_2 was unaffected when the data were reanalyzed excluding those cases in which there was complete first- or last-male sperm precedence (change in deviance $\chi^2_1 = 4.80$, $p = 0.0028$, $n = 32$; Cohen's standardized effect size $d = 0.80$).

Our results provide direct experimental evidence that sperm quality in the cricket *T. oceanicus* plays an important role in determining which male has the advantage when males compete for fertilization, and they support the hypothesis, implicit in sperm-competition theory, that selection should maximize sperm quality. Results from a recent study implied a role for sperm viability in sperm competition in the stalk-eyed fly *Cyrtodiopsis whitei*. In this insect, the patterns of sperm precedence are influenced by the presence of sex chromosome meiotic drive [13]. Fry and Wilkinson [14] have shown that drive-carrying males suffer reduced progeny production when their sperm are exposed to the seminal fluid of another male and that sperm from drive males are incapacitated within the female's reproductive tract by seminal fluid from standard nondriving males. Although the effect of seminal fluid on sperm viability of driving males could not account fully for the degree of sperm precedence [14], Fry and Wilkinson's results nonetheless pro-

*Correspondence: pgarcia@cyllene.uwa.edu.au

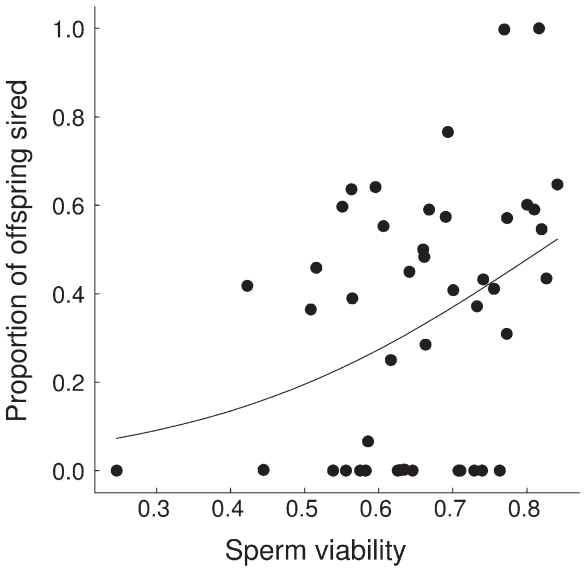

Figure 1. Relationship between the Proportion of Viable Sperm of the Last Male to Mate a Female and the Proportion of Offspring Sired by Him

The line represents the fit of the generalized linear model.

vide indirect evidence that the viability of stored sperm can influence paternity in competitive contexts in *C. whitei*.

Our results also show that there is considerable between-male variation in sperm viability and that this variation is significantly greater than the variation within males. Sperm viability (the proportion of live sperm) assessed in two different spermatophores across 135 males showed highly significant repeatability ($R = 0.47$, $p << 0.0001$). The repeatability of sperm viability for the subset of males used in the sperm-competition trials ($n = 92$; see Experimental Procedures) was $R = 0.57$ ($p << 0.0001$). In vertebrates, differences in fertilizing capacity among males are well known, and a number of studies have shown repeatability in ejaculate quality (see for instance [6–8, 15]). For insects, the results of Hunter and Birkhead [9] pointed to the existence of high intraspecific variation in sperm viability. However, to our knowledge, the repeatability of sperm viability has not previously been assessed in insects. This is surprising given that sperm viability is a trait that is being increasingly assayed in sperm-competition studies [14, 16–18]. All these previous studies have looked at the viability or survival of sperm stored in the female's sperm-storage organ, and the difficulties of recovering successive ejaculates from the same male might explain the paucity of measures of the repeatability of sperm quality within males.

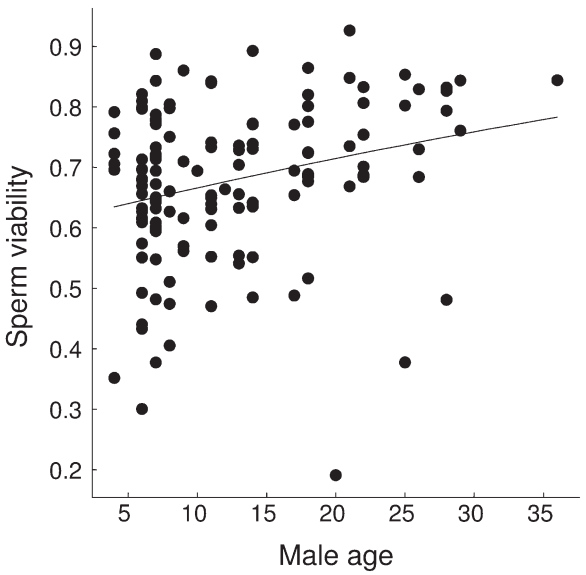

Figure 2. Relationship between Male Age (Days) and Sperm Viability (Proportion of Live Sperm in the Ejaculate)

The line represents the fit of the generalized linear model.

How can we explain the maintenance of intraspecific variation in a trait that plays such an important role in male fitness? Differences among males in their genetic constitution and in their ability to buffer production errors in sperm and/or in their ability to maintain live sperm are all likely candidates. It is also known that fitness traits show high variation because of their greater mutational variability [19]. In addition, variation in sperm quality could be the result of trade-offs between reproductive effort and other biological demands, such as immune function [20, 21]. Finally, if sperm quality were maternally inherited, as it seems to be in domestic fowl *Gallus domesticus*, directional selection acting on males under sperm competition might not result in a loss of genetic variation [22, 23].

Our study highlights the importance of considering variation in sperm quality in studies of sperm-competition mechanisms. Theoretical models for predicting mechanisms have been developed on the assumption that all sperm in an ejaculate are fertilization competent. Thus, the predicted distributions of paternity are calculated on the basis of empirical measures of the numbers of sperm in the fertilization set, and these distributions are compared to those derived empirically in sperm-competition trials [1, 12, 24–29]. This combined theoretical and empirical approach has proved useful in exploring mechanisms of sperm utilization, although some anomalous results have also been obtained [1]. For example, P_2 distributions in *T. oceanicus* are neither bimodal nor skewed, and mean P_2 values are around 0.5 [10, 30], suggesting a mechanism of random sperm mixing—the "fair raffle" principle, in which the fertilization success of each male is related to the relative numbers of sperm each male has in the fertilization set [26]. However, despite the absence of order effects on paternity, the apparent lack of influence of sperm numbers on paternity has led to the conclusion that random sperm mixing cannot be the mechanism operating in *T. oceanicus* [10]. But given that males vary in the proportion of dead and live sperm in their ejaculates, the random mixing model should be redefined in terms of numbers of viable sperm rather than absolute numbers of sperm. We can use the proportion of viable sperm of each of the two males mated to a female to predict the expected paternity of the second male (P_2). Expected values of P_2 can be calculated from the number of viable sperm

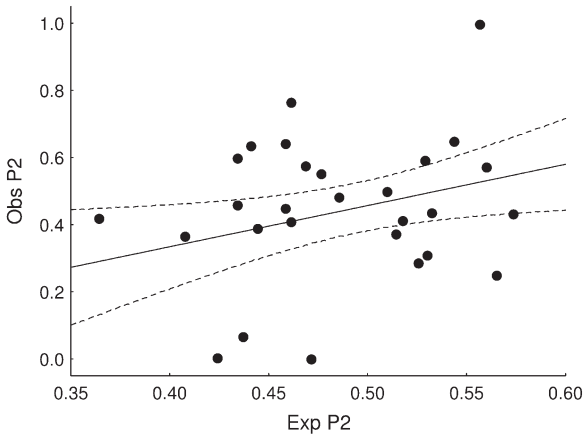

Figure 3. Correlation between the Observed P_2 Values and the P_2 Values Predicted under a Sperm-Mixing Mechanism in which Sperm Viability Corrects for Sperm Numbers at the Site of Fertilization

Dashed lines are 95% confidence limits for the regression line.

of the second male in relation to the total number of viable sperm from both males; that is, $r_2/(r_1 + r_2)$, where r_2 is the proportion of viable sperm from the second male to mate with the female and r_1 the proportion of viable sperm from the first male. The calculation rests on previous evidence that males transfer similar amounts of ejaculate for any given duration of spermatophore attachment [10] and the fact that sperm numbers were controlled in our sperm-competition trials. When we conservatively excluded those cases in which P_2 values were either 0 or 1, assuming that these values arose from insemination failures, the correlation between the observed and predicted P_2 values was significant ($F_{1,30} = 5.35$, $r = 0.39$, $p < 0.028$; Cohen's standardized effect size $d = 0.84$). Importantly, the intercept did not differ from zero ($p = 0.56$), and the slope did not differ from 1.0 ($B = 1.22 \pm 0.53$, $p = 0.67$; Figure 3). Including cases in which P_2 values were either 0 or 1 yielded a weaker relationship ($F_{1,43} = 4.0$, $r = 0.29$, $p < 0.052$; Cohen's standardized effect size $d = 0.61$), but again, the intercept did not differ from zero ($p = 0.47$) and the slope did not differ from 1.0 ($B = 1.10 \pm 0.55$, $p = 0.85$). However, as outlined above, there is good reason to believe that at least some of those cases arise because of insemination failure.

These results support the idea that the mechanism of sperm competition is similar to a fair raffle, in which the male with higher representation of viable sperm in the female's sperm storage organ wins the majority of fertilizations. Sperm viability is so far the only known factor that accounts for paternity in sperm-competition contexts in this species. Other factors that might also explain the patterns of paternity in this species include cryptic female choice, random effects such as insemination failure or sperm clumping, or loading of the raffle because of differences in the relative qualities of viable sperm. More generally, these results suggest that sperm viability should be taken into account in all future studies that attempt to deduce mechanism from relative numbers of sperm in the fertilization set.

Finally, sperm-competition theory suggests that males may respond to current information on sperm-competition risk (the probability that a given male will be in direct competition for fertilizations) by increasing ejaculate expenditure [2, 31, 32]. Such strategies of ejaculate allocation evolve because the costs of ejaculate production are not trivial, and males are expected to partition their ejaculates optimally. Numerous experimental studies have provided evidence in support of this prediction by showing that males increase the numbers of sperm ejaculated [1, 2, 31]. However, males do not react as predicted in all species [33]. In the light of our results, it would be worth examining whether males increase their investment in producing and maintaining ejaculates with a greater representation of viable sperm as a response to an increase in the risk of sperm competition. Recent studies of fish with alternative mating tactics suggest that increased sperm quality, rather than quantity, may be an alternative route to fertilization success under sperm-competition risk [34, 35].

In conclusion, we show that sperm viability alone influences paternity under sperm competition in an insect. Intrinsic differences in sperm viability between males translate into differences in competitive-fertilization success; the latter lead to the patterns of paternity observed. These findings provide experimental support at the within-species level for the hypothesis that sperm viability is a male adaptation to sperm competition in insects.

Experimental Procedures

Materials

Animals used in this study were obtained from an outbred laboratory stock derived from 120 adult females collected in Carnarvon, North Western Australia. Crickets were reared in plastic containers in a constant-temperature room (25°C), maintained on a 12:12 hr light: dark cycle, fed with cat chow ad libitum, and supplied with a Petri dish containing a pad of moist cotton wool for oviposition. Sexes were separated before the penultimate instar.

In order to assign paternity, we used a morphological marker, white eye (*we*). Phenotypic expression of the marker is homozygous recessive, following mendelian inheritance. Importantly, previous studies indicate that the mutation does not affect the fertilization capacity of the sperm or embryo viability [10], making the white eye character a suitable neutral marker for paternity-determination purposes. Homozygous *we* crickets were kept and maintained as their black-eyed (*be*) counterparts. Emerging adult males were sought twice a week, collected, and kept in groups of about 20 individuals with access to about 10 females to allow for a continuous production of fresh spermatophores. Males were marked individually with a numbered tag secured to the pronotum with cyanoacrylic glue.

Sperm-Viability Assays and Sperm-Competition Trials

Fresh spermatophores were removed from the subgenital pouch of mature males and ruptured in 20 μl of Beadle saline (128.3 mM NaCl, 4.7 mM KCl, and 23 mM $CaCl_2$) to assess sperm viability. After the optimal amounts of live/dead stain reagents (Live/Dead sperm-viability assay, Molecular Probes) required [16, 36] were established, 5 μl of sperm were mixed with an equal volume of 1:50 diluted 1 mM SYBR-14 and left in the dark for 10 min before 2 μl of 2.4 mM propidium iodide was added. The sample was incubated in the dark for 10 min and then observed under a fluorescence microscope (blue excitation filter at $\lambda = 490$ nm). The assay stains live sperm green with the SYBR-14, a membrane-permeant nucleic acid stain, and stains dead sperm, with damaged membranes, red with propidium iodide. Five hundred sperm per sample were scored to obtain proportions of live and dead sperm; in a series of preliminary tests,

this number provided repeatable proportions of live and dead sperm within a sample.

Sperm viability was assessed in two different spermatophores for each of 135 males. Sperm viability (the proportion of live sperm) showed highly significant repeatability (see text), which was calculated after Becker [37]. Sperm viability did not differ between eye-color morphs ($t_{133} = 0.78$, $p = 0.43$; percentage of viable sperm in *be* males = 66.8% ± 1.3, n = 70; percentage of viable sperm in *we* males = 65.2% ± 1.6, n = 65). Middle-aged males (n = 92) were used in sperm-competition trials to minimize the effects of age on sperm viability (see results) (mean ± the standard error age of the first male = 20.4 ± 0.99 days, range 10–36 days old; age of the second male = 20.7 ± 1.07 days, range 10–40 days old; difference in age between first and second males = 0.29 ± 1.07). In brief, the sperm-competition protocol involved 46 virgin *we* females mated sequentially in random order to two prescreened males, one *be* and the other *we*. Pairs were observed closely to ensure matings occurred. After mating, the males were left to guard the females for 40 min, thus preventing females from removing the spermatophore. Spermatophores were experimentally removed after 40 min to standardize the numbers of sperm received by each female. For any given duration of spermatophore attachment, there is no significant among-male variation in the number of sperm transferred [10]. After their matings, females were provided with a Petri dish containing damp cotton and allowed to oviposit for 21 days. Forty-five females (21 mated in order *we-be*, 24 mated in order *be-we*; 23 mated first to the male with higher sperm viability, 22 mated first to the male with lower sperm viability) laid eggs. Eggs were incubated at 25°C until hatching. Upon hatching, nymphs were scored as sired by the first or second male depending upon eye color. A total of 21,452 nymphs were scored (mean ± the standard error number of nymphs per female = 476.7 ± 30.5, range 64–909).

Statistical Analyses

The variables affecting the proportion of offspring sired by the second male to mate with a female (P_2) were analyzed with generalized linear models with binomial error structure and logit link function [38] in Genstat 7.2.0.208 (VSN International). The number of offspring sired by the second male was fitted as the response variable, and the total number of offspring produced by each female as the binomial denominator. We included all likely, biologically relevant explanatory variables that could potentially explain paternity in the maximal model (sperm viability, pronotum width, weight, and age of the two males, plus mating interval and order of the matings, either *we-be* or *be-we*). We then dropped terms sequentially until the model included only terms whose elimination would significantly decrease the explanatory power of the model. We corrected for overdispersion with Williams's procedure [39]. None of the terms, apart from the viability of the second male (see text), showed explanatory power; the terms' significance, inferred from changes in the deviance—distributed as a χ^2 with degrees of freedom equal to the difference in degrees of freedom between the models compared—of the model caused by each variable, returned probabilities higher than 0.2, and the proportion of deviance explained was lower than 3%. Therefore, in the text, we only show the minimal model, which included just the viability of the second male.

A generalized linear model with binomial error structure and logit link function was also utilized to analyze the relationship between sperm quality and male age in 137 males for which at least one ejaculate was recovered. The number of viable sperm was fitted as the dependent variable, and the total number of sperm screened per male as the binomial denominator.

Acknowledgments

We thank Maxine Beveridge for her help collecting nymphs. The study was supported by grants from the Secretaria de Estado de Educación y Universidades and the European Social Fund to F.G.-G. and from the Australian Research Council to L.W.S.

Received: November 5, 2004
Revised: November 25, 2004
Accepted: November 25, 2004
Published: February 8, 2005

References

1. Simmons, L.W. (2001). Sperm Competition and Its Evolutionary Consequences in the Insects (Princeton, New Jersey: Princeton University Press).
2. Wedell, N., Gage, M.J.G., and Parker, G.A. (2002). Sperm competition, male prudence and sperm-limited females. Trends Ecol. Evol. *17*, 313–320.
3. Birkhead, T.R., and Møller, A.P. (1998). Sperm Competition and Sexual Selection (San Diego, California: Academic Press).
4. Smith, R.L., ed. (1984). Sperm Competition and the Evolution of Animal Mating Systems (New York: Academic Press).
5. Dziuk, P.J. (1996). Factors that influence the proportion of offspring sired by a male following heterospermic insemination. Anim. Reprod. Sci. *43*, 65–88.
6. Birkhead, T.R., Martinez, J.G., Burke, T., and Froman, D.P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc. R. Soc. Lond. B. Biol. Sci. *266*, 1759–1764.
7. Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B., and Parker, G.A. (2004). Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. *14*, 44–47.
8. Donoghue, A.M., Sonstegard, T.S., King, L.M., Smith, E.J., and Burt, D.W. (1999). Turkey sperm mobility influences paternity in the context of competitive fertilization. Biol. Reprod. *61*, 422–427.
9. Hunter, F.M., and Birkhead, T.R. (2002). Sperm viability and sperm competition in insects. Curr. Biol. *12*, 121–123.
10. Simmons, L.W., Wernham, J., García-González, F., and Kamien, D. (2003). Variation in paternity in the field cricket *Teleogryllus oceanicus*: No detectable influence of sperm numbers or sperm length. Behav. Ecol. *14*, 539–545.
11. Rosenthal, R. (1991). Meta-Analytic Procedures for Social Research, Volume 6, Revised Edition (London: Sage Publications).
12. García-González, F. (2004). Infertile matings and sperm competition: The effect of "Nonsperm Representation" on intraspecific variation in sperm precedence patterns. Am. Nat. *164*, 457–472.
13. Wilkinson, G.S., and Fry, C.L. (2001). Meiotic drive alters sperm competitive ability in stalk-eyed flies. Proc. R. Soc. Lond. B. Biol. Sci. *268*, 2559–2564.
14. Fry, C.L., and Wilkinson, G.S. (2004). Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. Evolution Int. J. Org. Evolution *58*, 1622–1626.
15. Froman, D.P., and Feltmann, A.J. (1998). Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). Biol. Reprod. *58*, 379–384.
16. Bernasconi, G., Hellriegel, B., Heyland, A., and Ward, P.I. (2002). Sperm survival in the female reproductive tract in the fly *Scathophaga stercoraria* (L.). J. Insect Physiol. *48*, 197–203.
17. Snook, R.R., and Hosken, D.J. (2004). Sperm death and dumping in *Drosophila*. Nature *428*, 939–941.
18. Fedina, T.Y., and Lewis, S.M. (2004). Female influence over offspring paternity in the red flour beetle *Tribolium castaneum*. Proc. R. Soc. Lond. B. Biol. Sci. *271*, 1393–1399.
19. Houle, D., Morikawa, B., and Lynch, M. (1996). Comparing mutational variabilities. Genetics *143*, 1467–1483.
20. Sheldon, B.C., and Verhulst, S. (1996). Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. *11*, 317–321.
21. Skau, P.A., and Folstad, I. (2003). Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. Behav. Ecol. *14*, 40–47.
22. Froman, D.P., Pizzari, T., Feltmann, A.J., Castillo-Juarez, H., and Birkhead, T.R. (2002). Sperm mobility: Mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus*. Proc. R. Soc. Lond. B. Biol. Sci. *269*, 607–612.
23. Simmons, L.W. (2003). The evolution of polyandry: Patterns of genotypic variation in female mating frequency, male fertilization success and a test of the sexy-sperm hypothesis. J. Evol. Biol. *16*, 624–634.
24. Cook, P.A., Harvey, I.F., and Parker, G.A. (1997). Predicting vari-

ation in sperm precedence. Philos. Trans. R. Soc. Lond. B Biol. Sci. *352*, 771–780.
25. Harvey, I.F., and Parker, G.A. (2000). "Sloppy" sperm mixing and intraspecific variation in sperm precedence (P_2) patterns. Proc. R. Soc. Lond. B. Biol. Sci. *267*, 2537–2542.
26. Parker, G.A., Simmons, L.W., and Kirk, H. (1990). Analysing sperm competition data: Simple models for predicting mechanisms. Behav. Ecol. Sociobiol. *27*, 55–65.
27. Lessells, C.M., and Birkhead, T.R. (1990). Mechanisms of sperm competition in birds: Mathematical models. Behav. Ecol. Sociobiol. *27*, 325–337.
28. Eggert, A.K., Reinhardt, K., and Sakaluk, S.K. (2003). Linear models for assessing mechanisms of sperm competition: The trouble with transformations. Evolution Int. J. Org. Evolution *57*, 173–176.
29. Neff, B.D., and Wahl, L.M. (2004). Mechanisms of sperm competition: Testing the fair raffle. Evolution Int. J. Org. Evolution *58*, 1846–1851.
30. Simmons, L.W. (2001). The evolution of polyandry: An examination of the genetic incompatibility and good-sperm hypotheses. J. Evol. Biol. *14*, 585–594.
31. Parker, G.A., Ball, M.A., Stockley, P., and Gage, M.J. (1997). Sperm competition games: A prospective analysis of risk assessment. Proc. R. Soc. Lond. B. Biol. Sci. *264*, 1793–1802.
32. Ball, M.A., and Parker, G.A. (1998). Sperm competition games: A general approach to risk assessment. J. Theor. Biol. *194*, 251–262.
33. Schaus, J.M., and Sakaluk, S.K. (2001). Ejaculate expenditures of male crickets in response to varying risk and intensity of sperm competition: Not all species play games. Behav. Ecol. *12*, 740–745.
34. Vladic, T.V., and Jarvi, T. (2001). Sperm quality in the alternative reproductive tactics of Atlantic salmon: The importance of the loaded raffle mechanism. Proc. R. Soc. Lond. B. Biol. Sci. *268*, 2375–2381.
35. Burness, G., Casselman, S.J., Schulte-Hostedde, A.I., Moyes, C.D., and Montgomerie, R. (2004). Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav. Ecol. Sociobiol. *56*, 65–70.
36. Damiens, D., Bressac, C., Brillard, J.-P., and Chevrier, C. (2002). Qualitative aspects of sperm stock in males and females from *Eupelmus orientalis* and *Dinarmus basalis* (Hymenoptera: Chalcidoidea) as revealed by dual fluorescence. Physiol. Entomol. *27*, 97–102.
37. Becker, W.A. (1984). Manual of Quantitative Genetics (Washington: Pullman).
38. Wilson, K., and Hardy, I.C.W. (2002). Statistical analysis of sex ratios: An introduction. In Sex Ratios: Concepts and Research Methods, I.C.W. Hardy, ed. (Cambridge: Cambridge University Press), pp. 48–92.
39. Williams, D.A. (1982). Extra-binomial variation in logistic linear models. Appl. Stat. *31*, 144–148.