Seminal Fluid Affects Sperm Viability in a Cricket

Leigh W. Simmons*, Maxine Beveridge

School of Animal Biology, Centre for Evolutionary Biology, The University of Western Australia, Crawley, Australia

Abstract

Recent studies have suggested that males may vary the quality of their ejaculates in response to sperm competition, although the mechanisms by which they do so remain unclear. The viability of sperm is an important aspect of ejaculate quality that determines competitive fertilization success in the field cricket *Teleogryllus oceanicus*. Using *in vitro* mixtures of sperm and seminal fluid from pairs of male crickets, we show that seminal fluid can affect the viability of sperm in this species. We found that males who invest greatly in the viability of their own sperm can enhance the viability of rival sperm, providing the opportunity for males to exploit the investments in sperm competition made by their rivals. Transitive effects of seminal fluids across the ejaculates of different males are expected to have important implications for the dynamics of male investments in sperm competition.

Introduction

Sperm competition theory predicts that multiple mating by females should favour the evolution of increased male expenditure on the ejaculate as males compete to fertilize available ova [1]. Moreover, because ejaculates are costly to produce, selection is predicted to favour phenotypic plasticity in expenditure such that males invest more in their ejaculate when they encounter females who are more likely to mate with additional males [1]. Consistent with theory, among species, positive evolutionary associations have been found between testes mass and estimates of the strength of selection generated by sperm competition [2,3], and within species, exposing males to an elevated risk of sperm competition can result in them transferring more sperm at copulation [4].

Empirical studies have focussed primarily on the effects of sperm competition on the numbers of sperm ejaculated, with little attention being paid to variation in ejaculate quality [5]. However, there is now some evidence to suggest that males may also be able to make strategic adjustments to the quality of their ejaculates. Thus, in fish [6,7], fowl [8], and humans [9], males have been shown to be capable of making rapid adjustments to the swimming velocity of sperm contained within their ejaculates, in response to social status, the attractiveness of their mating partner, or the perceived risk that they will face sperm competition. Male crickets, *Teleogryllus oceanicus*, are capable of making adjustments to the quality of their sperm in response to both the risk and intensity of sperm competition; when males perceive the presence of a potential rival they produce ejaculates with a greater proportion of viable sperm than when isolated from other males [10]. Moreover, the males of this species also appear to recognise individually distinct cuticular hydrocarbon residues left on a female after mating, making fine-grained assessments of the actual number of males in competition and reducing their expenditure on sperm viability accordingly [11,12]. The mechanism by which males make strategic adjustments in sperm quality is unknown, but

recent work on fowl suggests that adjustments in seminal fluid quality may be an important modulator of sperm performance [13].

Seminal fluid is known to contain important biologically active compounds that affect male fertility [14]. The role of seminal fluid proteins in influencing female fecundity is also well documented [2], particularly in *Drosophila melanogaster* where seminal fluid proteins reduce female receptivity to further matings, and increase rates of oviposition at a cost to female lifespan [15,16]. However, with the notable exception of *D. melanogaster* [17,18,19] the function of seminal fluid in the context of competitive fertilization success is less well studied. Cameron et al.'s [20] theoretical analysis predicts that selection should favour phenotypic plasticity in male expenditure on both sperm and non-sperm components of the ejaculate that affect its competitive weight, but the predictions from their models depend critically on which component of the ejaculate, sperm or seminal fluid, influences its competitive weight. These findings call for empirical studies that examine how sperm and non-sperm components of the ejaculate influence its competitiveness. For *T. oceanicus* we know that sperm viability, rather than sperm numbers per se, is the principal determinant of competitive fertilization success [21]. Here, by separating sperm from seminal fluid components of the ejaculate, and conducting reciprocal recombination within and among males, we show how seminal fluid affects the viability of self and rival sperm.

Methods

Male crickets were derived from an outbred laboratory culture, and isolated as final instar nymphs in individual boxes supplied with cat chow and a cotton plugged tube containing water. Crickets were maintained in a constant temperature room (25°C with a 12:12 hour light:dark cycle), and monitored daily. Fourteen days after they moulted to adulthood, 42 unrelated

crickets were paired at random to provide 21 experimental pairs. A spermatophore was removed from the genital pouch of each male and placed onto a separate cavity slide in 20 µl of Beadle saline (128.3 mM NaCl, 4.7 mM KCl, and 23 mM CaCl$_2$), the evacuating tube was cut from the neck of the spermatophore with fine scissors, and evacuation observed under a binocular microscope. Under these conditions sperm were discharged from the spermatophore over a period of ~20 min, following which there was a brief period (10–20 sec), before the seminal fluid was discharged (see Fig. 1). After sperm ceased to be discharged, they were immediately removed along with 10 µl of the Beadle saline, and placed into a clean 0.5 ml eppendorf tube. Once the seminal fluid had discharged, it was likewise placed into a clean eppendorf tube. Reciprocal combinations of sperm and seminal fluid were then made for each pair of males. Thus 2.5 µl of sperm from each male in a pair was mixed with either 2.5 µl of his own seminal fluid or 2.5 µl of the other male's seminal fluid. The viability of sperm in each of the four samples was then assessed using a live-dead assay (Molecular Probes). Each 5 µl aliquot of sperm and seminal fluid was mixed with 5 µl 1:50 diluted 1 mM SYBER-14 and the sample incubated in the dark for 10 min, before adding 2 µl propidium iodide and incubating for a further 10 min. Samples were viewed under a florescence microscope and the number of live (stained green by SYBR-14) and dead (stained red by propidium iodide) sperm in the first 500 sperm counted was used to calculate the proportion of live sperm in the sample.
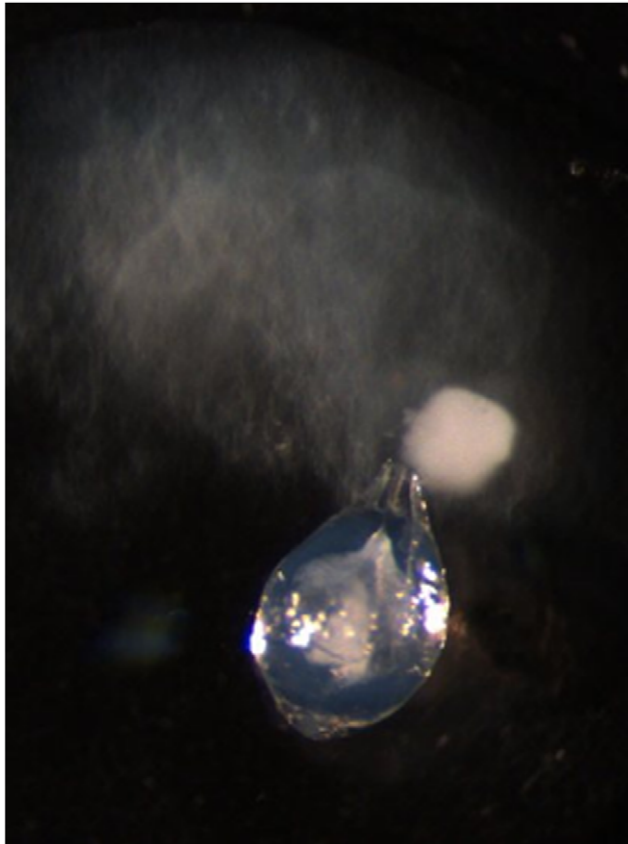

Figure 1. An evacuated spermatophore. The seminal fluid (white) discharges only after all sperm (grey) have left the spermatophore. doi:10.1371/journal.pone.0017975.g001

Results

Across all males, when mixed in the male's own seminal fluid the mean ±SE viability of sperm was 0.78±0.02, and ranged from 0.48–0.93 (Fig. 2). For each male pair we were able to identify the male with the higher (H) and lower (L) sperm viability when his sperm were mixed with his own seminal fluid; on average males within pairs differed significantly in their sperm viability (higher males 0.83±0.01, lower males 0.73±0.02; matched-pairs t = 5.23, df 20, P<0.0001). The difference in the viabilities of sperm between males of a pair when in their own seminal fluid ranged from as little as 0.6% to as much as 31%. Thus some males were closely matched while others differed greatly. We contrasted the viability of sperm in own versus rival seminal fluid using a regression analysis approach. If the seminal fluid of rivals had no impact on a male's sperm viability, the viability of his sperm should be the same whether mixed with his own or rival seminal fluid. Thus, under the null hypothesis regressing sperm viability in own seminal fluid on sperm viability in rival seminal fluid is expected to yield a slope of 1.0 and an intercept of zero. When sperm from the male with a high viability ejaculate were combined with the seminal fluid from his rival with a relatively low viability ejaculate, the viability of his sperm was unaffected; the slope did not differ from 1.0 (1.14±0.32, t = 0.45, df 19, P = 0.658) and the intercept did not differ from zero (−0.19±0.26, t = 0.71, P = 0.484) (closed symbols in Fig. 3). In contrast, for the relatively low viability male of a pair, the viability of his sperm was enhanced when mixed with seminal fluid from his rival with high sperm viability; the slope was significantly less than 1.0 (0.34±0.19, t = 3.39, P = 0.003) and the intercept was significantly greater than zero (0.49±0.14, t = 3.38, P = 0.003) (open symbols in Fig. 3). The data in Figure 3 show that the males who benefited from rival seminal fluid were those whose sperm had very low viability when in their own seminal fluid.

Discussion

We show that seminal fluid influences the viability of sperm in the cricket *T. oceanicus*. Sperm viability in this species is a key factor determining competitive fertilization success [21], so that seminal fluid is likely to be under significant postcopulatory sexual
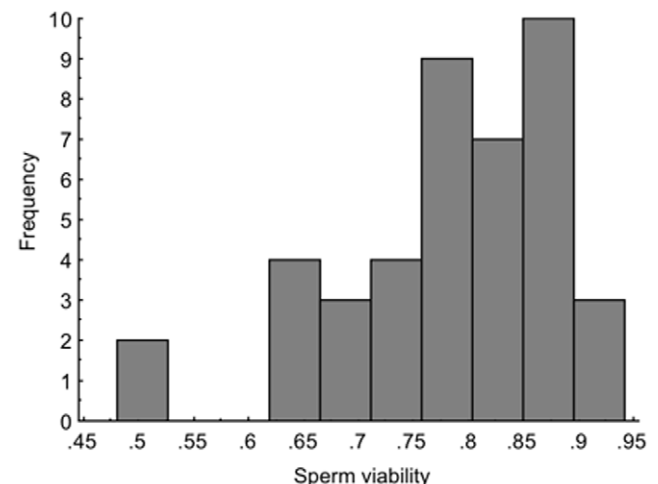
Figure 2. Frequency distribution of sperm viability for the males used in this study. The data are for sperm mixed with the male's own seminal fluid. doi:10.1371/journal.pone.0017975.g002
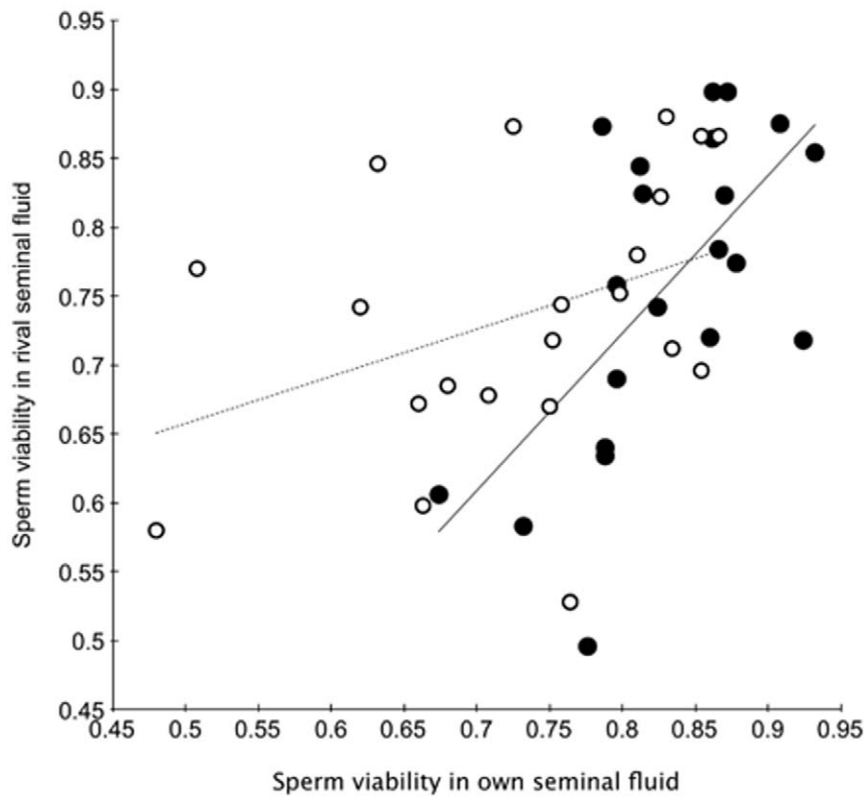
Figure 3. Relationship between viability of sperm in own versus rival seminal fluid. Closed (open) symbols show the effects of the seminal fluid from males of a pair with relatively high (low) sperm viability on their lower (higher) viability rival.
doi:10.1371/journal.pone.0017975.g003

selection. Recent molecular studies of cricket accessory gland genes have identified 30 genes predicted to encode seminal fluid proteins, a significant proportion of which appear to be under positive selection [22,23]. Assuming that the patterns we have observed *in vitro* extend to *in vivo* interactions among ejaculates, our study suggests that one important function of seminal fluid proteins may lie in sperm competition.

Sperm incapacitation has long been suggested as a potential mechanism of sperm competition, but empirical evidence for incapacitation has generally been equivocal [24,25,26]. Two recent studies have reported opposite effects of seminal fluid on rival sperm viability. In *D. melanogaster*, seminal fluid was found to increase the viability of sperm, irrespective of its source [26]. Thus, the viability of sperm was increased after the addition of own and rival seminal fluid. In contrast, in multiple mating social bees and ants, own seminal fluid was found to increase sperm viability, while rival seminal fluid was found to decrease sperm viability [27]. Our study shows that the effects of seminal fluid on sperm viability are likely to depend strongly on ejaculate-by-ejaculate interactions [28,29]. Consistent with the work on *D. melanogaster*, we show that the viability, and thus competitive weight of a male's sperm, can be increased by the seminal fluid of his rival. Thus, seminal fluid produced by males with relatively high sperm viability was found to increase the viability of sperm in the ejaculate of an inferior competitor. However, males who produced ejaculates with high sperm viability were unaffected by the seminal fluid of their rivals. Thus, different effects can be observed, dependent on the potency of donor and recipient seminal fluids.

The considerable variation in seminal fluid potency that we observe could provide an avenue by which males can exploit the

seminal fluid investments of their rivals [30]. For example, if males mating to unmated females invest heavily in seminal fluid to enhance the viability of their sperm, subsequent males could reap the benefits of their rivals past investment while saving their own seminal fluids for future matings. Although male *T. oceanicus* exhibit intrinsic genetically based differences in sperm viability [31], they also show phenotypic plasticity in this trait, reducing their investment in sperm viability when mating with females that have already received sperm and seminal fluids from previous males [10,12]. Strategic adjustments in seminal fluid investments are predicted by recent theoretical models that incorporate both sperm and non-sperm components of ejaculate investment [20], and our data suggest that adjustments in seminal fluid potency could underlie the strategic adjustments in sperm viability observed in this species [10,12]. Indeed, recent work with *D. melanogaster* has revealed that males can adjust the protein composition of their seminal fluids in response to perceived risk and intensity of sperm competition [32].

The effects of seminal fluid on sperm viability that we document here are remarkably similar to the effects of seminal fluid on the viability of own and rival embryos previously documented in this species. Male *T. oceanicus* that impart high viability to their own embryos also impart high viability to embryos sired by their rivals [33]. Paternal effects on embryo viability have been found to be genetically correlated with a male's investment into his accessory glands [34]. It may be that different seminal fluid compounds act independently on sperm and embryos to impart viability. Alternatively, seminal fluid compounds may affect qualitative differences in sperm, other than their viability, that impact developing embryos. The DNA integrity of sperm can be

protected by seminal fluid proteins [35,36], which in turn can determine the viability of embryos sired [37,38,39]. Identifying the seminal fluid compounds responsible for effects on sperm and embryo viability in *T. oceanicus* will allow us to explore more fully the evolutionary significance of this biologically important fluid.

Acknowledgments

References

1. Parker GA, Pizzari T (2010) Sperm competition and ejaculate economics. Biological Reviews 85: 897–934.
2. Simmons LW (2001) Sperm Competition and its Evolutionary Consequences in the Insects. Princeton: Princeton University Press.
3. Birkhead TR, Møller AP (1998) Sperm Competition and Sexual Selection. London: Academic Press.
4. Wedell N, Gage MJG, Parker GA (2002) Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol 17: 313–320.
5. Snook RR (2005) Sperm in competition: not playing by the numbers. Trends Ecol Evol 20: 46–53.
6. Rudolfsen G, Figenschou L, Folstad I, Tveiten H, Figenschou M (2006) Rapid adjustments of sperm characteristics in relation to social status. Proceedings of the Royal Society of London B 273: 325–332.
7. Gasparini C, Peretti AV, Pilastro A (2009) Female presence influences sperm velocity in the guppy. Biol Lett 5: 792–794.
8. Cornwallis CK, Birkhead TR (2007) Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. Am Nat 170: 758–770.
9. Kilgallon SJ, Simmons LW (2005) Image content influences mens semen quality. Biol Lett 1: 235–255.
10. Simmons LW, Denholm A, Jackson C, Levy E, Madon E (2007) Male crickets adjust ejaculate quality with both risk and intensity of sperm competition. Biol Lett 3: 520–522.
11. Thomas ML, Simmons LW (2007) Male crickets adjust the viability of their sperm in response to female mating status. Am Nat 170: 190–195.
12. Thomas ML, Simmons LW (2008) Male-derived cuticular hydrocarbons signal sperm competition intensity and affect ejaculate expenditure in crickets. Proceedings of the Royal Society of London B 276: 383–388.
13. Cornwallis CK, O'Conner EA (2009) Sperm: seminal fluid interactions and the adjustment of sperm quality in relation to female attractiveness. Proceedings of the Royal Society of London B 276: 3467–3475.
14. Poiani A (2006) Complexity of seminal fluid: a review. Behavioral Ecology and Sociobiology 60: 289–310.
15. Wolfner MF (1997) Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. Insect Biochemistry & Molecular Biology 27: 179–192.
16. Chapman T (2008) The soup in my fly: evolution, form and function of seminal fluid proteins. PLoS Biol 6: e179.
17. Clark AG, Aguade M, Prout T, Harshman LG, Langley CH (1995) Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. Genetics 139: 189–201.
18. Fiumera AC, Dumont BL, Clark AG (2007) Associations between sperm competition and natural variation in male reproductive genes on the third chromosome of *Drosophila melanogaster*. Genetics 176: 1245–1260.
19. Fiumura AC, Dumont BL, Clark AG (2005) Sperm competitive ability in *Drosophila melanogaster* associated with variation in male reproductive proteins. Genetics 169: 243–257.
20. Cameron E, Day T, Rowe L (2007) Sperm competition and the evolution of ejaculate composition. Am Nat 169: E158–E172.
21. García-González F, Simmons LW (2005) Sperm viability matters in insect sperm competition. Current Biology 15: 271–275.
22. Andrés JA, Maroja LS, Bogdanowicz SM, Swanson WJ, Harrison RG (2006) Molecular evolution of seminal proteins in field crickets. Molecular Biology and Evolution 23: 1574–1584.
23. Braswell WE, Andres JA, Maroja LS, Harrison RG, Howard DJ, et al. (2006) Identification and comparative analysis of accessory gland proteins in Orthoptera. Genome 49: 1069–1080.
24. Price CSC, Dyer KA, Coyne JA (1999) Sperm competition between *Drosophila* males involves both displacement and incapacitation. Nature 400: 449–452.
25. Snook RR, Hosken DJ (2004) Sperm death and dumping in *Drosophila*. Nature 428: 939–941.
26. Holman L (2009) *Drosophila melanogaster* seminal fluid can protect the sperm of other males. Functional Ecology 23: 180–186.
27. den Boer SPA, Baer B, Boomsma JJ (2010) Seminal fluid mediates ejaculate competition in social insects. Science 327: 1506–1509.
28. Bjork A, Starmer WT, Higginson DM, Rhodes CJ, Pitnick S (2007) Complex interactions with females and rival males limit the evolution of sperm offence and defence. Proceedings of the Royal Society of London B 274: 1779–1788.
29. García-González F (2008) The relative nature of fertilization success: implications for the study of post-copulatory sexual selection. BMC Evol Biol 8: 140.
30. Hodgson DJ, Hosken DJ (2006) Sperm competition promotes the exploitation of rival ejaculates. Journal of Theoretical Biology 243: 230–234.
31. Simmons LW, Roberts B (2005) Bacterial immunity traded for sperm viability in male crickets. Science 309: 2031.
32. Wigby S, Sirot LK, Linklater JR, Buehner N, Calboli FCF, et al. (2009) Seminal fluid protein allocation and male reproductive success. Cur Biol 19: 751–757.
33. García-González F, Simmons LW (2007) Paternal indirect genetic effects on offspring viability and the benefits of polyandry. Current Biology 17: 32–36.
34. García-González F, Simmons LW (2005) The evolution of polyandry: intrinsic sire effects contribute to emryo viability. Journal of Evolutionary Biology 18: 1097–1103.
35. Aitken RJ, Baker MA (2006) Oxidative stress, sperm survival and fertility control. Molecular and Cellular Endocrinology 250: 66–69.
36. Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, et al. (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformation index. Fertility and Sterility 81: 349–354.
37. O WS, Chen HQ, Chow PH (1988) Effects of male accessory sex gland secretions on early embryonic development in the golden hamster. Journal of Reproduction and Fertility 84: 341–344.
38. O WS, Chen H, Chow PH (2006) Male genital tract antioxidant enzymes - Their ability to preserve sperm DNA integrity. Molecular and Cellular Endocrinology 250: 80–83.
39. Dowling DK, Simmons LW (2009) Reactive oxygen species as universal constraints in life-history evolution. Proceedings of the Royal Society of London B 276: 1737–1746.

Author Contributions

Conceived and designed the experiments: LWS. Performed the experiments: MB. Analyzed the data: LWS. Contributed reagents/materials/analysis tools: LWS. Wrote the paper: LWS.