Sperm competition experiments between lines of crickets producing different sperm lengths

Edward H. Morrow^{1*} and Matthew J. Gage²

¹Department of Ecology and Environmental Science, University of Umeå, SE-901 87 Umeå, Sweden

²Centre for Ecology, Evolution and Conservation, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

Sperm numbers can be important determinants of fertilization success in sperm competition. However, the importance of variation in sperm size is less well understood. Sperm size varies significantly both between and within species and comparative studies have suggested that some of this variance can be explained by sperm competition. In this study we examine whether variation in sperm length has consequences for fertilization precedence using controlled sperm competition experiments in the field cricket *Gryllus bimaculatus*. This species is an ideal model for such investigations because the mechanism of sperm competition generates complete mixing of different males' spermatozoa in the female (thereby allowing individual sperm to express their own competitive abilities). We successfully bred lines of crickets, the males of which produced short, medium and long sperm types with narrow and non-overlapping distributions. Males of different lines were then sequentially mated with control females in order to create two-male sperm competitions. The paternity outcomes of these competitions were measured after matings using an irradiated male technique (with a full reciprocal design that controls for natural fertility and any irradiation effects on gamete competitiveness) over a 12 day oviposition period. However, having successfully bred diverging sperm length lines and competing males that differed in sperm length, we found no evidence that a male's sperm size explained any of the variation in their relative fertilization success. Males from lines producing longer sperm showed no fertilization advantage over males producing shorter sperm across 97 double matings. There was also no advantage for males producing a sperm length close to the population mean over those competitors whose sperm length had been selectively diverged across 63 matings.

Keywords: sperm precedence; P_2 ; fertilization; gamete; flagellum

1. INTRODUCTION

Sperm competition (Parker 1970) is a powerfully selective force that is responsible for a broad range of physiological, behavioural and anatomical adaptations (Smith 1984; Birkhead & Møller 1998). Biologists are currently attempting to understand the precise mechanisms of sperm competition at the level of the gamete. The importance of sperm numbers in sperm competition is well known (Parker 1982; Letsinger & Gromko 1985; Simmons 1987) and may be the force that generates the evolution of profligate sperm numbers (Parker 1982). However, the role that sperm size plays when ejaculates compete for the fertilization of a female's ova is much less well understood, despite significant variation in sperm form both across and within species (e.g. Gage 1998; Ward 1998; Morrow & Gage 2000, 2001a).

Variation in sperm size may influence sperm power (Katz & Drobnis 1990) and swimming speed (Gomendio & Roldan 1991) as well as longevity (Stockley *et al.* 1997; Parker 1998) due to changes in the energetic demands of longer or shorter flagella. For example, in broadcast spawning echinoids there is evidence that sperm velocity and longevity covary between and within species (Levitan 1993). Levitan (2000) has recently shown that, in the sea urchin *Lytechinus variegatus*, sperm velocity and longevity are traded off against each other and, furthermore, that sperm velocity is positively related to the fertilization rate, which may translate into reproductive

success when ejaculates compete. Sperm size as well as numbers may therefore have direct effects on the outcome of sperm competition. However, the empirical progress in addressing such questions lags behind theoretical advances, partly because of the practical difficulties of working with sperm experimentally *in vivo*. Two empirical studies have explored the role of sperm size in sperm competition: fertilization success is greater for males producing larger sperm in bulb mites (Radwan 1996) and nematode worms (LaMunyon & Ward 1998, 1999). However, males in both these groups produce aflagellate amoeboid-type sperm that move with pseudopodia and may therefore not represent a general phenomenon for the majority of males that produce flagellate spermatozoa.

Significant positive relationships have been found across species between the intensity of sperm competition (which is measured through behavioural classifications, paternity analyses and residual testes size) and sperm length in a number of internally fertilizing taxa, for example birds (Briskie *et al.* 1997), mammals (Gomendio & Roldan 1991; but see Harcourt (1991), butterflies (Gage 1994) and moths (Morrow & Gage 2000) for moths). Accordingly, it appears that higher levels of sperm competition select for males in order to partition resources towards longer and perhaps faster and/or more powerful spermatozoa. However, the exact mechanism of sperm competition will dictate sperm size evolution. In contrast, for example, externally fertilizing fishes have shown a negative relationship between sperm size and mating pattern (Stockley *et al.* 1997). If the mechanism of sperm

*Author for correspondence (ted.morrow@eg.umu.se).

competition in external fertilizers approaches that of a lottery where sperm numbers are more important than sperm size, speed or power, selection may act on males that maximize sperm number at the expense of individual gamete size.

In this study we experimentally explore the significance of variation in sperm size by running controlled sperm competitions between male crickets (*Gryllus bimaculatus*) that have been bred for divergence in sperm length. *Gryllus bimaculatus* is an ideal model for very important reasons. First, it has been possible to produce selected lines of males that differ genetically in their sperm size phenotype (Morrow & Gage 2001b). Sperm length screening and the selection of short and long sperm producing lines enabled the production of males that diverged significantly and continuously in sperm length across four generations (Morrow & Gage 2001b). Morrow & Gage (2001b) found significant heritability ($h^2 = 0.52 \pm 0.06$ s.e. and $p = 0.01$) and sex linkage of sperm length genes. After four generations, the mean sperm length of the short line was $958 \pm 4.15 \mu\text{m}$ ($n = 50$ males) and that of the long line was $1001 \pm 5.15 \mu\text{m}$ ($n = 50$ males). In addition, we preserved a medium length line that had been subjected to the same sample size criteria (in order to control for any inbreeding effects), but in which the sperm length was not selected for and, therefore, remained neutral at the population mean. Accordingly, we are able to compete short versus long sperm-length males and/or medium versus divergent (long/short) sperm-length males and examine whether sperm size influences sperm competition success.

Gryllus bimaculatus achieves insemination by an externally attached spermatophore. Accordingly, we were able to control the length of time that a spermatophore is attached to a female (ideally to the maximum necessary for complete emptying of the spermatophore) so that we were able to eliminate the confounding influences of variation in sperm number entering the competition as a result of premature spermatophore removal by the female. Finally, unlike most polyandrous species, the mechanism of sperm competition (which will select for optimal sperm form and function) is understood in *G. bimaculatus*. This cricket shows a complete sperm mixing mechanism of sperm competition (Parker *et al.* 1990). In other words, sperm compete on a directly numerical basis for the proportions of fertilizations obtained by each ejaculate. A homogenous mixing mechanism means that the phenotype of an individual spermatozoon has the opportunity of expressing itself within the female's reproductive tract under circumstances of sperm competition. In species where sperm competition between males occurs at the level of the entire ejaculate, factors such as the order (e.g. in many odonates) (Waage 1984) or timing (e.g. in domestic fowl) (Birkhead *et al.* 1995) of matings have an overriding influence on the patterns of paternity. However, when spermatozoa of different males are mixed homogeneously within the female reproductive tract, this allows individual sperm to express their individual abilities in achieving fertilization success (Parker *et al.* 1990).

Accordingly, this experiment is designed to investigate the significance of variation in sperm length for sperm competition success empirically in a carefully selected

model, namely *G. bimaculatus*. We examine the paternity of competition between two males where the only systematic difference between the competitors is the length of the sperm they have been bred to produce. We explore whether there are differences in fertilization precedence between longer and shorter sperm-length lines and also whether the population median sperm length is at a fertilization advantage over the diverged long and short male lines.

2. METHODS

(a) Sperm length selection

The male and female *G. bimaculatus* used in this experiment were taken from the fifth generation of three lines, which were successfully selected for short, long and medium sperm length as described in Morrow & Gage (2001b). The selection criteria were based on a within-family protocol using original parents all derived from the same laboratory population; this design avoided the requirement of replicating lines since we used 10 independent families in each line for maintaining random selection on any trait independent of sperm length. Ten families within each sperm length line were screened at each generation (five males per family) and the most divergent/convergent male in each family was selected for siring the next generation with a female from a different family (but the same line). The final generation that sired the males used in these competitions had diverged mean sperm lengths of between $957.6 \pm 4.15 \mu\text{m}$ ($n = 50$ males) and $1001.03 \pm 5.15 \mu\text{m}$ ($n = 50$ males) for the short and long lines, respectively. The medium line underwent no selection for sperm length but was cultured using identical population sizes in order to control for potential inbreeding influences. The realized heritability (h^2) across our four long and short line generations was 0.52 ± 0.06 ($p = 0.01$) (Morrow & Gage 2001b). In addition to selected divergence in sperm length, there is also direct evidence that sperm length is male specific in *G. bimaculatus* and is repeatable between subsequent spermatophores collected on different days (Morrow & Gage 2001a).

(b) Sperm competition experiments

The crickets were maintained at $28 \pm 2^\circ\text{C}$ under a 14 L:10 D photoperiod. Final instar males and females from each of the families within the selected lines were isolated in 5 l plastic cages in single sex groups in order to ensure that no mating occurred prior to experimentation. Males and females were not used until at least 10 days after adult eclosion in order to ensure sexual maturity.

Sperm competition experiments were carried out using the 'irradiated male' technique (see Boorman & Parker 1976). By exposing males to a non-pathological but sterilizing source of gamma radiation high levels of dominant and lethal chromosome mutations occur within the spermatozoa. These irradiated sperm are still able to 'fertilize' ova, but a high proportion of the zygotes die at an early stage in development due to the induced mutations. This male sterilization is permanent and over the short term (2 or 3 weeks) has no other effects on male mating behaviour (Simmons 1987). Females were therefore mated sequentially to a non-irradiated and irradiated male and, by controlling for natural infertility and the effectiveness of the sterilization procedure, it is possible to determine the proportion of eggs fertilized by sperm from either ejaculate, depending on which ova develop. The potential influences of irradiation on sperm competitiveness are controlled for by employing a fully reciprocated experimental design.

Half of the males from each of the short (S), medium (M) and long (L) lines selected were irradiated with 7 krad gamma radiation (dose rate of 385 rad min^{-1} and total duration 18.2 min) using a ^{134}Cs gamma radiation source at the Department of Immunology, Liverpool University. In order to avoid confusion in identifying irradiated and non-irradiated males (which are usually referred to in the literature as R and N, respectively) in males belonging to the three selected lines, irradiated males are identified in the following text with an asterisk, i.e. S*, M* and L* and non-irradiated males without an asterisk. The following competitive crosses were then executed.

- (i) Group 1. Females were mated twice, once with a male from the short line and once with a male from the medium line. Reciprocal matings were carried out giving mating patterns of S*M, MS*, SM* and M*S.
- (ii) Group 2. Females were mated twice, once with a male from the medium line and once with a male from the long line. Reciprocal matings were carried out giving mating patterns of M*L, ML*, LM* and L*M.
- (iii) Group 3. Females were mated twice, once with a male from the short line and once with a male from the long line. Reciprocal matings were carried out giving mating patterns of S*L, LS*, SL* and L*S.

In addition to the crosses described above, two groups of experimental controls were necessary in order to (i) determine the natural level of infertility within the population and (ii) measure the effectiveness of the sterilization technique. The inherent level of infertility within the population was estimated using double matings between two non-irradiated males (M versus M, L versus L and S versus S). The proportion of eggs developing from these crosses gives the level of natural infertility ($1-p$). The effectiveness of the irradiation technique was estimated using double matings of two irradiated males (we used only M* versus M* and L* versus L* in order to retain sufficient S* males for the competitive crosses). The proportion of eggs developing normally (z) as a result of these crosses will quantify the level of sterility induced by the irradiation.

In all of the double matings, none of the pairs of males that were taken from the same selected line (in the case of the control matings) were siblings. The females used in the sperm competition experiments were only taken from the medium selected line in order to reduce any female effect upon the outcome of sperm competition. Virgin females from each of the 10 families within the medium line were placed in 0.51 cages and covered with loose fitting plastic lids and provided with food and water *ad libitum*. A single male was placed in these cages depending on which experimental treatment the female was assigned to. Males and females mated readily in these conditions and, immediately after successful, spermatophore transfer, the female was removed from the cage and placed within a narrow plastic tube (which was perforated in order to provide ventilation) in order to prevent premature spermatophore removal (this technique was successful, with only 7% of pairings followed by premature spermatophore detachment; these failed matings were excluded from the analyses).

The female was removed from the tube after 60 min, the drained spermatophore disconnected with forceps and the female returned to her cage. The spermatophore attachment time was kept constant at 60 min because this is the time taken for a spermatophore to empty completely in *G. bimaculatus* (Simmons 1986), thus giving a degree of control over the sperm numbers for each mating. The second male was then

immediately introduced into the female's cage and the procedure repeated. The female was then returned to her cage for oviposition. The females were provided with *ad libitum* food and a 5 cm Petri dish containing damp absorbent cotton wool for oviposition. The oviposition dishes were replaced every 3 days in order to avoid faecal contamination, and eggs laid up to 12 days after the matings were examined. The cotton wool pads were inverted in the Petri dishes and covered with a lid and incubated for 12 days at $28 \pm 2^\circ\text{C}$, thereby allowing all fertilized eggs to develop normally. The oviposition dishes were stored at -20°C on the twelfth day of incubation until egg scoring. Eggs were scored after defrosting the oviposition dishes and sorting eggs from the cotton wool pads. Fertilized eggs were darker and larger with a segmented body and red eyespots visible through the egg membrane at $\times 10$ magnification.

(c) Calculation of sperm precedence

The proportions of developed versus undeveloped eggs from each female were used for calculating sperm precedence (P_2) following the procedure described by Boorman & Parker (1976). Any egg which develops normally within the experimental crosses will potentially have been fertilized by either a sperm from a non-irradiated normal male (S, M or L) or a sperm from an irradiated male that has not been completely sterilized (S*, M* or L*). Similarly, an embryo that fails to develop may have been fertilized by a sperm from an irradiated male or by a sperm from a non-irradiated male that subsequently dies for some other reason. We therefore control the P_2 values by correcting for both natural infertility and the incompleteness of sterilization. The proportion of eggs failing to develop from a normal-normal cross ($1-p$) and the proportion of developing embryos in an irradiated-irradiated cross (z) may be calculated from the proportions of eggs that fail to develop or that develop within these two control groups. Boorman & Parker (1976) have shown that the proportion of eggs fertilized by the irradiated male's sperm (P_R) is calculated from

$$P_R = (1 - x/p) + z/p \times \left(\frac{1 - (x/p)}{1 - (z/p)} \right), \quad (2.1)$$

where x is the observed proportion of developed eggs. If an irradiated male mates first then $P_2 = 1 - P_R$. Alternatively, if the irradiated male mates second then P_2 is simply P_R .

3. RESULTS

(a) Natural fertility and the effectiveness of sterilization

The control matings of two non-irradiated males showed a high level of fertility overall (mean developed eggs = $83 \pm 6.7\%$, $n = 28$ matings and $n = 5914$ eggs), which was comparable with that found by Simmons (1987) (mean hatch rate = $79.36 \pm 2.06\%$ and $n = 18$ matings). Therefore, $p = 0.83$ over the 12 day oviposition period and there was no evidence for differences between fertility on days 3, 6, 9 and 12 of this period ($F_{3,40} = 1.09$ and $p = 0.364$); the mean fertility percentages over these respective days are: day 3 = $90.0 \pm 1.01\%$, day 6 = $88.2 \pm 1.84\%$, day 9 = $93.6 \pm 0.29\%$ and day 12 = $87.3 \pm 1.22\%$.

On average, $9.10 \pm 4.04\%$ ($n = 2376$ eggs and $n = 14$ matings) of the eggs from irradiated-irradiated crosses developed within the 12 day oviposition period, which is higher than the value found by Simmons (1987) (mean

Table 1. Mean fertilization precedence values ($\pm$ s.e.) separated between each of the six possible combinations of matings.

(The P_2 value refers to the proportion of paternity attributable to the males marked by asterisks.)

mating combination	P_2 value	s.e.	n
short versus medium*	0.59	0.10	15
short versus long*	0.45	0.10	16
medium versus short*	0.55	0.09	16
medium versus long*	0.53	0.07	19
long versus short*	0.56	0.06	18
long versus medium*	0.29	0.10	13

hatch rate=0% and number of matings not specified). However, since we achieved some male fertility after irradiation, it is unlikely that sperm viability was compromised and we set z at 0.091. If irradiation is 100% effective we do not know to what extent the dose was greater than that required to simply render spermatozoal DNA non-fertile; if sterility is complete there may be additional and broader effects of irradiation.

(b) *Sperm competition experiments*

A total of 97 double matings were successfully included in the sperm precedence analysis. The P_2 values calculated for the second mates from each of the experimental crosses are given in table 1. Sperm size gives no clear advantage in conditions of sperm competition. The P_2 values for males that are predicted to have longer sperm than their competitors (M versus L, S versus L and S versus M crosses) were not significantly different from the P_2 values calculated for males that were predicted to have shorter sperm (L versus M, L versus S and M versus S crosses) (figure 1 and table 2).

It is possible that males whose sperm length was exaggerated in the long or short lines may be at a disadvantage in sperm competition against the population mean. In other words, the medium line that underwent neutral selection may be more successful in fertilization competitions because those gametes are phenotypically similar to the 'natural' population mean. However, there were also no differences in the P_2 values for males from the medium line in competition with males from either the short or long lines (figure 2 and table 3).

4. DISCUSSION

Since our selection design incorporated 10 independent families within each sperm-length line (Morrow & Gage 2001b) and because no sib pairings occurred, systematic differences between lines that are independent of sperm length are not likely to explain our results. However, there may be differences between males that are dependent on sperm length (such as genetic pleiotropisms), which we aim to investigate. However, despite this selectively engineered variance, our primary finding was that a male's sperm length does not influence his sperm competition success within 97 independent competitive matings and over the maximum 12 day oviposition period

Figure 1. Mean sperm precedence ($P_2 \pm$ s.e.) when the second male to mate came from a line that produced longer (first clear column) or shorter (second filled column) sperm than his rival in competition. The numbers of sperm precedence competitions for each mating order are shown above each column.

Table 2. ANOVA of the P_2 values for double matings where the second male to mate is predicted to have longer sperm compared with matings where the second mate is from a short sperm line.

source	d.f.	SS	MS	F	p
between	1	0.04	0.04	0.32	0.57
within	95	13.04	0.14	—	—
total	96	13.09	—	—	—

that our experiment measured. Sperm precedence is not affected when a male has longer or shorter sperm than his competitors. Furthermore, males producing sperm that are closer to the population median are also at no sperm competition advantage over competitors producing more divergent spermatozoa.

If sperm length is a consequential trait for sperm competition success, our findings could be explained by the particular nature of the experimental design. First, it is possible that there was insufficient divergence in sperm length for an effect to be manifested. However, we did achieve significant and directional selection of increasing and decreasing sperm lengths (Morrow & Gage 2001b). The generation that sired our male competitors had mean sperm lengths of 958 and 1001 μ m for the short and long lines, respectively. The four generations of selection showed linear increases and decreases in sperm length due to selection and we therefore predict that the males used in the competitions had diverged to an even greater degree. The sperm-length divergences that we achieved also showed very narrow and non-overlapping ranges with the standard errors being 4.15 and 5.15 for the short and long lines, respectively ($n = 50$ males per line). Thus,

Figure 2. Mean sperm precedence ($P_2 \pm \text{s.e.}$) when the second male to mate came from a line that produced more median (first clear column) or more diverged (second filled column) sperm than his rival in competition. The numbers of sperm precedence competitions for each mating order are shown above each column.

Table 3. ANOVA of the P_2 values for double matings where the second mate is from the medium (population mean) line versus a second mate from either the short or long (diverged) lines

source	d.f.	SS	MS	F	p
between	1	0.05	0.05	0.17	0.68
within	61	18.07	0.30	—	—
total	62	18.12	—	—	—

as a percentage of the means, the standard errors were only 0.43 and 0.51% for the short and long lines, respectively. Finally, previous work has shown that sperm length is male specific and is consistent between different spermatophores produced on different days (Morrow & Gage 2001a). Accordingly, we probably achieved sufficient divergence in sperm length for any effect to be manifested through controlled sperm competitions.

Our negative results could be explained if there was uncontrolled variance in the sperm numbers between different males' spermatophores. Sperm number is an important determinant of sperm competition success (Parker 1982; Letsinger & Gromko 1985; Simmons 1987). However, we controlled for the confounding variance in sperm number that is introduced by the premature removal of spermatophores by females through ensuring that all spermatophores had the same attachment duration. Furthermore, there is no evidence for any trade-off or differences in the numbers of sperm within the spermatophores of the three male lines so that, for example, males producing small sperm do not produce greater numbers of sperm (Morrow 2000). Accordingly, the variation in sperm numbers between spermatophores will be random and therefore will not lead to systematic errors.

Mating order is unlikely to explain our results since we employed a fully reciprocated experimental design that

also controls for differences in the competitiveness of irradiated and non-irradiated spermatozoa (Boorman & Parker 1976). We also reduced the influence of female effects by only using females from the medium sperm length line. Variance in sperm length may be consequential for fertilization and post-fertilization processes (Karr 1991; Pitnick & Karr 1998), but we found no evidence for sperm size-associated differences in fertilization success. Our results could be explained if sperm length is not an important male trait in sperm competition. However, it seems unlikely that sperm size is a neutral trait given the variation between taxa (e.g. Gage 1994; Briskie *et al.* 1997; Morrow & Gage 2000) and that fertilization is the focus of such intense selection. It is possible that our selection only achieved divergence in the total length of the sperm cell and it is the dimensions of the components within the spermatozoon that are significant for the cell's function. The volume of the cell is largely occupied by a pair of mitochondrial derivatives running the length of the cell in the spermatozoa of many arthropods (Jamieson *et al.* 1999). The function of these derivatives is not clearly understood, but they appear to be a significant structural component of the sperm cell and, therefore, may play an important role in sperm function and motility (Sivinski 1984). Variance in the size of the derivatives may have occurred independently of selection for the total cell length, thereby leading to no obvious effect of sperm length on sperm competition success.

Finally, it is possible that the significance of sperm size is manifested over a longer sperm competition period than the maximum 12 day period we examined. If sperm size relates to longevity (Gomendio & Roldan 1993) then extended sperm competition periods may select for males producing a sperm length that gives a cell an increased longevity. Currently it is not clear whether sperm size is positively or negatively related to a cell's longevity. Gomendio & Roldan (1993) suggested that, across mammals, longer sperm swim faster, and that increased velocity trades against longevity. However, these associations are yet to receive adequate experimental testing. Males' sperm length within Atlantic salmon (which varied significantly between individuals) did not relate to the longevity of sperm motility (Gage *et al.* 1998), but further studies are required. While longer flagella may generate greater velocities and place increased energy demands on ATP catalysis, it is not clear whether all spermatozoa are programmed for functioning according to similar rules. For example, it is possible that bigger sperm volumes allow greater endogenous reserves and/or increased mitochondria to be transported, which might enable increased longevity. While the results from this study do not show that sperm length influences sperm competition success over a 12 day fertilization period, we are currently exploring whether sperm length influences relative fertilization success over a longer period of sperm competition by exploring sperm precedence dynamics in relation to the competing males' relative sperm lengths.

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