

## SPERM SURVIVAL IN FEMALE STALK-EYED FLIES DEPENDS ON SEMINAL FLUID AND MEIOTIC DRIVE

CATHERINE L. FRY<sup>1</sup> AND GERALD S. WILKINSON<sup>2</sup>

Department of Biology, University of Maryland, College Park, Maryland 20742

<sup>1</sup>E-mail: cfry@wam.umd.edu

<sup>2</sup>E-mail: wilkinso@umd.edu

**Abstract.**—Sperm competition is common in many insect species; however, the mechanisms underlying differences in sperm precedence are not well understood. In the stalk-eyed fly, *Cyrtodiopsis whitei* (Diptera, Diopsidae), sperm precedence is influenced by the presence of sex chromosome meiotic drive. When drive-carrying males compete with non-driving males for fertilizations within a female, the number of progeny sired by drive males is significantly fewer than predicted by sperm mixing alone. Thus, drive males apparently suffer not only a reduction in the number of viable sperm produced, but also a reduction in sperm competitive ability. In this study, we manipulated the amount and source of seminal fluid and sperm received by females by interrupting copulations before sperm, but after seminal fluid, was transferred. We find that seminal fluid from another male influences the number of progeny sired by a drive-carrying male when both males mate with the same female. Sperm viability staining reveals that sperm from drive males are incapacitated by seminal fluid from other males within the female reproductive tract. These results suggest that multiple mating by females enables seminal fluid products to interact differentially with sperm and may reduce the transmission advantage of the drive chromosome.

**Key words.**—Accessory gland proteins, *Cyrtodiopsis whitei*, seminal fluid, sperm incapacitation, sperm precedence.

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Insects are predicted to have high levels of sperm competition because females tend to store sperm from multiple inseminations in specialized storage organs (Parker 1970). Nevertheless, relatively little is known about the mechanisms responsible for differences in sperm precedence. One of the most promising research directions in this area has been the study of seminal fluid products (Clark 2002). Recent evidence indicates that accessory gland proteins (Acps) present in seminal fluid may influence a male's fertilization success long after copulation has taken place (reviewed in Chapman 2001; Wolfner 2002; Gillott 2003). Accessory gland proteins can manipulate female physiology and behavior, mediate sperm storage (Tram and Wolfner 1999), and may also affect the outcome of sperm competition (Harshman and Prout 1994; Chapman et al. 2000). For example, *Drosophila melanogaster* males lacking the Acp36DE protein sired significantly fewer offspring as second mates in double matings (Chapman et al. 2000). In *D. simulans*, seminal fluid acts to both displace and incapacitate sperm from heterospecific males (Price et al. 1999), resulting in conspecific sperm precedence that persists regardless of the order of mating (Price 1997). In species where remating rates are high, selection on sperm competitive ability should be intense and proteins present in seminal fluid are likely targets of this selection.

Although studies on *Drosophila* have helped to elucidate mechanisms of sperm competition, the consequences of multiple inseminations are certainly not limited to this genus. Multiple mating is common in many species of insects, and females may gain direct benefits by copulating with more than one male. In a meta-analysis of 122 experimental studies, Arnqvist and Nilsson (2000) demonstrated that polyandrous females had increased lifetime offspring production. Indirect benefits for multiply mating females have also been proposed, typically in the form of genetic benefits that increase offspring fitness (reviewed in Yasui 1998). Sperm competition, although often considered an extension of male-

male competition, may therefore also be advantageous to females that produce genetically diverse broods.

In species that exhibit selfish genetic elements, such as meiotic drive, female mating behavior can influence offspring genotype. Stalk-eyed flies of the genus *Cyrtodiopsis* (family Diopsidae) offer an interesting example of the relationship between multiple mating and meiotic drive. Meiotic drive causes nonrandom segregation of alleles and males bearing a driving X chromosome produce dysfunctional Y-bearing sperm (Wilkinson and Sanchez 2001). This condition causes affected males to sire offspring with predominantly or exclusively female-biased sex ratios (Presgraves et al. 1997). The presence of meiotic drive can favor the evolution of multiple mating by females if driving (sex-ratio or SR) and nondriving (standard or ST) males differ in sperm competitive ability (Haig and Bergstrom 1995). In some species of *Cyrtodiopsis*, populations with higher frequencies of meiotic drive also exhibit higher frequencies of females mating with multiple males (Wilkinson et al. 2003).

The interaction between sperm from SR and ST males is more complex than predicted by simple sperm mixing. Because SR males are expected to produce half as many fertilization-competent sperm as ST males, they should sire approximately one-third of the offspring when the two males' sperm compete within a female's reproductive tract. However, in a reciprocal mating experiment involving *Cyrtodiopsis whitei*, SR males sired less than 10% of the progeny from a female jointly mated by an ST male, independent of mating order (Wilkinson and Fry 2001). This pattern of precedence suggests that both sperm number and sperm competitive ability are reduced in SR males. Therefore, we hypothesized that sperm competitive ability in *C. whitei* is influenced by seminal fluid. In this study we use interrupted copulations to test whether seminal fluid influences the outcome of sperm competition between SR and ST males. In addition, we use a sperm viability stain to evaluate the pos-

sibility that sperm precedence associated with meiotic drive is the result of incapacitation caused by seminal fluid products.

## MATERIALS AND METHODS

### *Fly Stocks*

We used two laboratory populations of *C. whitei* that were bred from flies originally collected near Chiang Mai, Thailand (1600 m elevation; 19°9'N, 98°7'E) and Ulu Gombak, Malaysia (350 m elevation; 3°19'N, 101°43'E) in January 1996. Flies from these two populations show heritable differences in body color but will mate freely with one another in the laboratory. The flies feed and oviposit on pureed corn treated with mold inhibitor (10% methylparaben). Laboratory populations are maintained on a 12 L:12 D cycle with at least 75% relative humidity.

### *Seminal Fluid Manipulation*

In *C. whitei* sperm are transferred via envelopes of proteins (spermatophores) secreted by the male accessory glands (Kotrba 1996). Copulations last approximately 60 seconds, but sperm are not transferred during the first 40 seconds of mating (Lorch et al. 1993). To demonstrate that interrupted copulations transfer seminal fluid but not sperm, we paired 46 males from the Gombak population with 46 females from the Chiang Mai population. Twenty-three of these pairs were allowed to copulate once for at least 40 sec. The remaining 23 pairs were interrupted 30 sec. after intromission. Immediately after copulation termination, females were dissected so that the contents of the spermatophore could be observed. We anaesthetized the females with CO<sub>2</sub> and placed them on a glass slide with one or two drops of PBS (pH = 7.4) close to the ovipositor. Under a dissecting microscope, we removed the last two abdominal segments with forceps and removed the reproductive tract directly into the PBS. Each reproductive tract was then examined under 100× magnification and scored for the presence or absence of a spermatophore. Spermatophores were subsequently scored for the presence or absence of sperm and seminal fluid.

### *Seminal Fluid Effects on Progeny Production*

To assess the effects of additional seminal fluid on a previous insemination, we conducted 46 sets of crosses each involving a pair of males multiply mated to either two or three Chiang Mai females. In 28 sets of these crosses, three females each received one full-length copulation (timed to the nearest second) with a male from the Gombak population. Approximately 24 h later, one of these females received additional seminal fluid from a different male by copulating with a Chiang Mai male for 30 sec. The second female received no additional copulations while the third female received a 30-sec copulation with the original Gombak male. These crosses included nine ST<sub>G</sub>-ST<sub>C</sub>, eleven SR<sub>G</sub>-ST<sub>C</sub>, six ST<sub>G</sub>-SR<sub>C</sub>, and two SR<sub>G</sub>-SR<sub>C</sub> male pairs, with subscripts indicating male population of origin. In the remaining 18 crosses we included only the first two types of female treatments and used six ST<sub>G</sub>-ST<sub>C</sub>, six SR<sub>G</sub>-ST<sub>C</sub>, four ST<sub>G</sub>-SR<sub>C</sub>, and two SR<sub>G</sub>-SR<sub>C</sub> male pairs. SR males were identified by assessing

their offspring sex ratios for deviation from 1:1 as described in Wilkinson and Fry (2001). Males scored as ST may fall into two categories: those that do not bear meiotic drive and those that are carrying both meiotic drive and a drive suppressor. Although we cannot distinguish between these possibilities based on progeny sex ratios, we assume that the phenotypes of ST males are comparable. After the interrupted copulations, each female was housed alone in a plastic cage (12 cm × 16 cm × 13.5 cm) and allowed to oviposit on pureed corn until offspring production ceased. Progeny from females receiving seminal fluid from Chiang Mai males were examined for body color to ensure that no interrupted copulations resulted in sperm transfer.

### *Seminal Fluid Effects on Sperm Viability*

To determine if additional seminal fluid influences survival of sperm from a previous insemination, we stained sperm stored by females with a viability indicator. We performed the same mating protocol involving two males and three females as described above with the following alteration: forty-eight hours after the first copulation, each female was dissected in order to remove the spermathecae and stain and count stored sperm. We used the Live/Dead Sperm Viability Kit (L-7011) from Molecular Probes (Eugene, OR) to assess the viability of sperm removed from females. We prepared the dye solution as follows: 40 μl PBS (pH = 7.4) was added to 2.5 μl propidium iodide and 5 μl SYBR-14 dilution (1 μl SYBR-14 plus 12 μl DMSO). Females were anaesthetized with CO<sub>2</sub> and the reproductive tract was removed as described above. The spermathecae were isolated from the rest of the reproductive tract and placed on a glass slide. We then added 15 μl of dye solution directly to the spermathecae, covered them with a cover slip, and gently crushed them with blunt forceps. This procedure releases all sperm stored in the spermathecae and mixes them with the dye solution. We then examined and counted sperm at 200× magnification under ultraviolet light using a Nikon Eclipse E600 microscope fitted with two fluorescence filter cubes (B-2E/C and G-2E/C from Nikon). Using these filter cubes, inviable sperm fluoresce red and viable sperm fluoresce green. We counted the total number of red and green sperm on each slide separately with each filter cube. We tested 26 crosses that included 15 ST<sub>G</sub>-ST<sub>C</sub>, six SR<sub>G</sub>-ST<sub>C</sub>, three ST<sub>G</sub>-SR<sub>C</sub>, and two SR<sub>G</sub>-SR<sub>C</sub> male pairs and used 78 Chiang Mai females.

### *Statistical Analyses*

We tested for differences in progeny production and sperm survival between SR and ST males using a nested ANOVA with females as a random effect nested within the meiotic drive phenotype of the Gombak male. Sperm survival was assessed by calculating the arcsine square-root proportion of viable sperm found in each female's spermathecae. Descriptive statistics are expressed as mean ± SE. All analyses were performed using JMP version 5.0 (SAS Institute, Cary, NC).

## RESULTS

### *Sex-Ratio Distortion*

Comparison of brood sex ratios produced by Chiang Mai and Gombak SR males chosen for this study revealed no

TABLE 1. Analysis of variance results for progeny production, number of sperm counted in spermathecae, and arcsin square root proportion of viable sperm. Seminal fluid source was nested in drive phenotype and treated as a random effect.

Source of variation	MS	df	F	P
<b>Progeny production</b>				
Drive phenotype	986.3	1	0.95	0.38
Seminal fluid source	1069	4	4.19	0.0033
Error	255.2	114		
<b>Number of sperm stored</b>				
Drive phenotype	191.9	1	0.6	0.46
Seminal fluid source	331.1	4	2.4	0.06
Error	0.02	72		
<b>Proportion viable sperm</b>				
Drive phenotype	0.68	1	1.3	0.32
Seminal fluid source	0.61	4	28.2	<0.0001
Error	0.02	72		

difference between the degree of progeny sex-ratio distortion ( $F_{1,33} = 1.0$ ,  $P = 0.33$ ). Gombak SR males produced  $0.15 \pm 0.02$  male progeny while Chiang Mai SR males produced  $0.18 \pm 0.03$  male progeny.

#### Seminal Fluid Manipulation

Copulations lasting more than 40 sec transferred both seminal fluid and sperm (21 copulations resulted in both sperm and seminal fluid transfer and two copulations resulted in only seminal fluid transfer). Copulations that were interrupted at 30 sec transferred only seminal fluid (22 copulations resulted in seminal fluid transfer and one copulation resulted in an empty spermatophore). Hence, interrupted copulations transfer seminal fluid but not sperm ( $\chi^2 = 38.7$ ,  $P < 0.0001$ ).

#### Seminal Fluid Effects on Progeny Production

Progeny production by females receiving additional seminal fluid differed dramatically according to the source of that seminal fluid, but did not differ between male drive phenotypes (Table 1). When a full-length mating by an SR Gombak male was followed by seminal fluid from a different male, the SR male sired significantly fewer progeny than if the first copulation was followed by either no additional seminal fluid or additional seminal fluid from the original Gombak male (Fig. 1). Copulation duration did not differ between SR and ST males ( $F_{1,114} = 1.0$ ,  $P = 0.36$ ) and did not differ among females ( $F_{4,114} = 0.7$ ,  $P = 0.57$ ). Examination of offspring body color confirmed that no interrupted copulations resulted in progeny production.

#### Sperm Viability after Interrupted Copulations

Sperm viability staining revealed that sperm from SR males are incapacitated by seminal fluid from ST males (Fig. 2). The proportion of viable sperm observed in spermathecae differed according to the source of additional seminal fluid (Table 1). However, total numbers of sperm did not differ across the three types of females (Table 1). Females mated singly to SR males stored as many sperm ( $32.1 \pm 3.9$ ) as females mated singly to ST males ( $34.7 \pm 2.9$ ). As above, copulation duration did not differ between SR and ST males

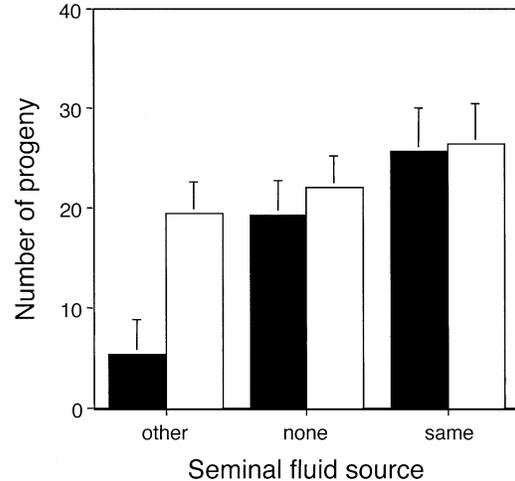


FIG. 1. Least squares mean ( $\pm$  SE) numbers of progeny produced by females receiving full-length copulations from either SR or ST Gombak males. Each female subsequently received additional seminal fluid from either a Chiang Mai male ("other"), no additional male ("none"), or the original Gombak male ("same"). Bar colors indicate the meiotic drive phenotype of the Gombak male (black, SR; white, ST).

( $F_{1,77} = 0.06$ ,  $P = 0.81$ ) and did not differ among females ( $F_{4,77} = 0.2$ ,  $P = 0.95$ ). No inviable sperm were observed within females with or without additional seminal fluid from the original Gombak male even when he carried an SR chromosome.

#### DISCUSSION

The results presented here indicate that sperm from SR males fertilize dramatically fewer eggs when exposed to the seminal fluid of a different male inside the spermathecae of a female. No reduction in progeny production was observed

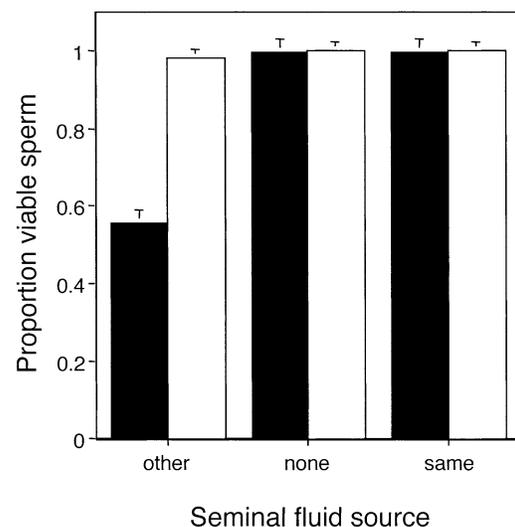


FIG. 2. Average proportion ( $\pm$  SE) of viable sperm within spermathecae of females that received full-length copulations from either SR or ST Gombak males. Sources of additional seminal fluid are identical to those in Figure 1. Bar colors indicate the meiotic drive phenotype of the Gombak male (black, SR; white, ST).

when a full-length copulation by an SR male was followed by additional seminal fluid from the original male. The effect of foreign seminal fluid does not appear to be the result of sperm displacement as described in *Drosophila* (Price et al. 1999, 2000) since all three types of females had comparable numbers of sperm stored in their spermathecae. Reduced progeny production by SR males also cannot be attributed to cryptic female choice of sperm because females mated singly stored equal numbers of sperm from SR and ST males. Females do not, therefore, discriminate between SR and ST sperm prior to storage.

The competitive inferiority of SR males described here and in Wilkinson and Fry (2001) cannot be the result of compromised sperm production. One of the key assumptions made when modeling the dynamics of drive is that SR males produce half as many viable sperm as ST males (Haig and Bergstrom 1995; Jaenike 2001). This appears to be true within the testes as developing sperm bundles in SR males contain approximately 50% inviable sperm (C. L. Fry, unpubl. data). However, we never observed inviable sperm in females mated singly to SR males (see Fig. 2), indicating that these sperm are either not transferred to females or not stored within spermathecae. Examination of sperm within spermathecae also revealed that a single type of sperm was present. Male *C. whitei* produce dimorphic sperm of distinct "long" and "short" size classes (Presgraves et al. 1999). Since the short sperm are unlikely to be involved in fertilization (Presgraves et al. 1997), alternative functions such as "cheap filler" or "sperm defense" have been proposed (Presgraves et al. 1999). However, since we did not observe any short sperm stored within spermathecae, the function of these sperm remains unclear.

Our counts of sperm in spermathecae indicate that female *C. whitei* do not retain many sperm from each copulation. After a single insemination females stored 34 sperm and produced 20 progeny, on average. Although females may eject spermatophores before they are completely empty (Kotrba 1991), these results suggest that males transfer remarkably few sperm in each ejaculate. In contrast, *D. melanogaster* females store 700–1000 sperm (reviewed in Bloch Qazi et al. 2003) per insemination and approximately 400 are used to fertilize eggs (Pitnick 1991). Male *D. pseudoobscura* pass as many as 25,000 sperm in a single ejaculate (Snook et al. 1994). Multiple mating by female *C. whitei* may be an adaptation to ensure that adequate numbers of sperm are available for fertilization. Females are able to store more sperm than males transfer in a single mating and Lorch et al. (1993) demonstrated that spermathecae are not filled after four copulations. However, females in captivity typically copulate two times per hour every morning (Wilkinson et al. 2003). Such promiscuity seems excessive if the primary goal of multiple matings is to fill spermathecae.

An additional explanation for the high rate of multiple mating is that it functions to counteract the effects of copulating with SR males by reducing the transmission of drive chromosomes. This possibility is consistent with a recent model, which suggests that meiotic drive may favor the evolution of female remating (Lorch and Chao 2003). Another model by Taylor and Jaenike (2002) further demonstrated that sperm competition such as that described here and in

Wilkinson and Fry (2001) can help to stabilize an SR polymorphism within some populations. Selection for multiple mating is not limited to meiotic drive; other selfish genetic elements such as maternal-effect lethals, transposable elements, and *Wolbachia* may also contribute to the evolution of polyandry (Zeh and Zeh 1996, 1997). A recent analysis of over 70 species of mammals also suggests that polyandry is consistent with the avoidance of genetic incompatibilities (Stockley 2003). In limited cases, remating may be a bet-hedging strategy employed when females are unable to reliably identify genetically compatible males (see Yasui 1998). In *C. whitei*, females exhibit a mating preference for males with large eyespan (Burkhardt and de la Motte 1988; Wilkinson et al. 1998). In *C. dalmanni*, a closely related congener that also exhibits meiotic drive (Presgraves et al. 1997), male eyespan is associated with meiotic drive (Wilkinson et al. 1998) because the X chromosome explains 30% of the variation in male eyespan (Wolfenbarger and Wilkinson 2001). Although we do not know if eyespan also predicts drive in *C. whitei*, females would be expected to mate multiple times whenever a phenotypic indicator of drive is not perfectly reliable.

The results of a previous reciprocal mating experiment indicate that 90% of the progeny produced by doubly mated females are sired by ST males whereas only 10% are sired by SR males (Wilkinson and Fry 2001). In contrast, sperm viability staining revealed that approximately 50% of the SR sperm stored by females were incapacitated by seminal fluid from ST males. Thus, the effect of seminal fluid on sperm survival does not appear to be as intense as the effect on progeny production. This discrepancy may reflect a difference in the timing of data collection rather than a difference in mechanism. Offspring from females used in the interrupted mating experiment were collected for 3–4 weeks until females had ceased laying eggs. In contrast, sperm to be treated with the viability indicator were removed from spermathecae 24 h after the introduction of additional seminal fluid. If a seminal fluid product is involved in incapacitation, the effects may accumulate over time as documented in *Drosophila*. In *D. melanogaster*, mating-induced changes in female physiology and behavior have short- and long-term stages. Acps alone have short-term effects but when combined with sperm can have long-term effects. This "sperm effect" is apparently caused by sperm serving as vehicles for Acps (Swanson 2003; Liu and Kubli 2003). If Acps mediate incapacitation in *C. whitei*, the presence of sperm may be necessary to observe the complete effect.

In conclusion, we find that drive-carrying males suffer reduced progeny production when their sperm are exposed to the seminal fluid of another male. The mechanism by which this occurs appears to be differential incapacitation of sperm via seminal fluid. Whether one or more accessory gland proteins are responsible for this effect warrants further research. Several *Drosophila* Acps have already been implicated in sperm competition, including Acp36DE, which functions in sperm storage (Chapman et al. 2000), and Acp62F, which influences sperm protection (Lung et al. 2002). Protease inhibitors such as Acp62F may be particularly likely candidates for mediating incapacitation since the failure to express these inhibitors may leave sperm susceptible to proteolytic deg-

radation (see Wolfner 2002). Acps in *C. whitei* have not yet been identified, but a recent study of *C. dalmanni* found that male mating frequency is dependent on accessory gland size (Baker et al. 2003). In addition, SR males of both *C. dalmanni* and *C. whitei* copulate less often than ST males (Wilkinson et al. 2003). Together, these results suggest that the characterization of accessory gland products may provide additional insight into the dynamics of X chromosome meiotic drive in these flies and possibly other species.

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