Developmental Biology 232, 105–114 (2001)
doi:10.1006/dbio.2000.0136, available online at http://www.idealibrary.com on IDEAL®

Analyses of Reproductive Interactions That Occur after Heterospecific Matings within the Genus *Caenorhabditis*

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Formation of zygotes in internally fertilizing organisms requires a number of successful interactions between oocytes and sperm within a receptive female reproductive tract. These interactions are usually assumed to be species-specific. For most species, it is either not possible to inseminate females with sperm from a different species or not possible to observe the consequences of such an insemination because the female is opaque. Nematodes of the genus *Caenorhabditis* are optically transparent and prior work indicates copulation between individuals of two different species is possible. We have used a series of vital stains and other cytological methods to analyze sperm after cross-species mating. We present here a series of analyses of the postcopulatory, prefertilization interactions among three *Caenorhabditis* species and find that reproductive biology is conserved, to varying degrees, among all three species. This approach allows investigation into which *in vivo* interactions between sperm and both oocytes and the somatic gonad have been maintained during the reproductive isolation that accompanies speciation. © 2001 Academic Press

Key Words: **reproductive isolation; fertilization; mating; sperm; oocytes.**

INTRODUCTION

Closely related species are usually anatomically similar and, generally, share similar reproductive strategies. However, reproductive isolation is a key feature of speciation and, in fact, species are considered distinct if they fail to successfully interbreed (Dobzhansky, 1935, 1937). Interspecies matings can fail prezygotically because of events preceding fertilization, or postzygotically due to events that occur after fertilization has taken place (reviewed by Coyne and Orr, 1998). Prezygotic isolation can be caused by courtship barriers that prevent copulation; barriers that prevent sperm from reaching the oocyte or any other (of many) mechanism that prevents sperm from fertilizing oocytes after insemination. Postzygotic barriers may involve hybrid lethality, hybrid sterility, reduced hybrid fitness, or reduced numbers of hybrid progeny when compared to conspecific broods. Postzygotic isolating mechanisms are associated with the formation of hybrid embryos and/or progeny that can be analyzed to discern the degree of developmental compatibility between the parental species. Among prezygotic mechanisms in most organisms, only precopulatory events are accessible to study because they occur externally and are readily observed. For these reasons, most studies of reproductive isolation have examined either precopulatory (sexual) or postzygotic isolation (reviewed by Coyne and Orr, 1998).

Isolating mechanisms that act after copulation but before fertilization are classically named gametic isolation (Dobzhansky, 1951). Many of the species used for these studies have internal fertilization, so they are understandably difficult to study. Nevertheless, experiments designed to analyze gametic isolating mechanisms have been performed in several organisms. Sperm competition/assortative fertilization has been studied in ground crickets *Allonemobius fasciatus* and *A. socius*, which occasionally mate heterospecifically (Gregory and Howard, 1994). When they do occur, these matings result in hybrids that are viable and fertile. However, if a female of either species is mated sequentially by a conspecific male and a heterospecific male, in any order, most of the progeny are sired by the conspecific male. In many cases, the heterospecific male sires no progeny at all, despite evidence of sperm transfer.

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Similar phenomena occur in grasshoppers (Hewitt *et al.,* 1989; Bella *et al.,* 1992) and flour beetles (Wade *et al.,* 1994).

Gametic isolation has been studied most extensively in *Drosophila* (reviewed by Coyne and Orr, 1998). In *Drosophila*, gametic isolation appears to be mediated primarily by the female (reviewed by Markow, 1997). For instance, *Drosophila* females can respond to heterospecific insemination by producing a vaginal mass named the "insemination reaction" (Patterson, 1947; Patterson and Stone, 1952). Such a mass can contain dead sperm and it either delays or prevents the female from remating (Patterson and Stone, 1952). The sequential insemination of a *Drosophila* female by two males of different species can result in sperm competition, but the competitive superiority of conspecific sperm is a function of the genotype of the inseminated female (Price, 1997; Clark *et al.,* 1999). A *Drosophila mojavensis* or *D. nigrospiracula* female will refrain from laying eggs after being inseminated by her brother, suggesting that gametic isolation prevents excessive inbreeding within these species (Markow, 1997). Consistent with this interpretation, other work in *Drosophila* has shown that the extent of reproductive isolation increases with the genetic/evolutionary distance between species (Coyne and Orr, 1989, 1998).

Most of the species described above are not amenable to detailed cytological analyses because they exhibit internal fertilization and are optically opaque. In contrast, rhabditid nematodes are optically transparent and well suited for *in vivo* analyses at the cellular level (e.g., Sulston *et al.*, 1983). Furthermore, the genetics and events of reproduction in *C. elegans* have been well characterized (reviewed by L'Hernault, 1997; Schedl, 1997) and there are a number of other species in this genus that are also suitable for laboratory experiments. Aside from the observation that successful hybridization is not observed between different *Caenorhabditis* species (Maupas, 1919; Nigon and Dougherty, 1949; Friedman *et al.,* 1977; Baird *et al.,* 1992), little is known about the cellular events that accompany reproductive isolation. We have developed new methods that take advantage of the genetics and cytology available in nematodes to examine postinsemination, prezygotic cellular events in more detail than has been possible in *Drosophila* or any other organism. Analyses of heterospecific matings between these closely related species have revealed that, to a surprising degree, reproductive interactions have been conserved during speciation in this genus.

MATERIALS AND METHODS

Worm Strains and Maintenance

All of the strains and species utilized in this study were maintained under standard conditions, as previously described (Brenner, 1974), and standard nomenclature is used throughout (Horvitz *et al.,* 1979). The following *C. elegans* strains and mutations were used in this study: *unc-13*(*e1091, e51*), *spe-4*(*q347*), *let-88*(*s132*), *spe-8*(*hc53*), *dpy-5*(*e61*), and *fog-1*(*q253ts*) LGI; *daf-4*(*e1364*) LGIII; *fem-1*(*hc17ts*) LGIV; *him-5*(*e1490*) LGV (Hodgkin, 1997); all of these are derived from the *C. elegans* reference wild-type strain Bristol N2 (Brenner, 1974). *him-5(e1490)* males were used as "wild-type" *C. elegans* males for matings. The strain BA626 [*sDp2*/*spe-8*(*hc53*) *dpy-5*(*e61*)] LGI was used as a source of morphologically marked *spe-8* hermaphrodites. Homozygous *spe-4*(*q347*) males were obtained by crossing *unc-13*(*e1091*) *spe-4*(*q347*) hermaphrodites by *spe-4*(*q347*) *dpy-5*(*e61*)/*let-88*(*s132*) *unc-13*(*e51*) males and selecting nonUnc male progeny, which had the genotype *spe-4*(*q347*) *dpy-5*(*e61*)/*unc-13*(*e1091*) *spe-4*(*q347*). Recombination in these balanced strains is not a serious problem because *spe-4* is less than 0.7 map units from *unc-13* (L'Hernault and Arduengo, 1992), and the lack of progeny resulting from crosses with these males confirmed their genotype (see Results). The restrictive temperature for the temperature-sensitive (ts) mutants used in this study is 25°, except for *daf-4*(*e1364*), which is nonDaf at 16° but Daf at 20° and 25°. *daf-4*(*e1364*) animals can be raised until the L4 stage at 16° and shifted to either 20° or 25° without showing a Daf phenotype because this is after the temperature-sensitive period of development (Golden and Riddle, 1984). This strain was used in some fluorescent sperm-tracking experiments (see below) because it has reduced gut autofluorescence compared to wild-type *C. elegans*, and this phenotypic trait is unaffected by growth temperature (K. Hill and S. W. L'Hernault, unpublished observation). The *C. briggsae* strains used were G16 (wild-type; Fodor *et al.,* 1983) and PB101 *cby-3* (*bd101*), which has a short and fat body phenotype similar to *C. elegans dpy* mutants. The *C. remanei* strain used was EM464, previously reported as "*C. sp. v* " in Baird *et al.* (1992) (S. Emmons, personal communication). *C. briggsae* and *C. remanei* strains were received from the *Caenorhabditis* Genetics Center. *C. elegans* and *C. briggsae* are hermaphroditic (XX) species, with rare males (XO) arising spontaneously through X chromosome nondisjunction, while *C. remanei* is a dioecious species. All mating experiments occurred on 6-cm agar plates seeded with a single spot of *E. coli* strain OP50 (Brenner, 1974).

Fluorescent Sperm Tracking

In preparation for sperm tracking experiments, L4 virgin males were picked to a fresh seeded plate and aged overnight at 16° in the absence of females or hermaphrodites. Aged, virgin males were picked into a glass depression slide containing 200 μl of a 50–70 μM SYTO17 (Molecular Probes, Eugene, OR) in TBS (10 mM Tris, 1 mM EDTA, 5 mM NaCl, pH 8). SYTO 17 is a fluorescent nucleic acid binding dye that labels mitochondria in nematode sperm but does not appear to affect sperm function. The depression slide was placed in a darkened humid chamber and incubated at 25° for 3 h. Fluorescently labeled males were then transferred to a fresh, seeded plate with a Pasteur pipette, allowed to recover, and only the vigorously active males were subsequently picked for mating experiments. Recipient females or hermaphrodites were picked as L4 larvae, and those that had undergone the L4/adult molt during the period when the males were incubating in the dye solution were selected for matings. Matings of 10 or 12 males to four females/hermaphrodites were set up and placed at 20° overnight in the dark. The following morning, recipient females or hermaphrodites were anaesthetized in either 0.25% propylene phenoxytol in TBS (Kimble and White, 1981) or 2.5 mM levamisole in TBS (Brenner, 1974) and mounted on 2% agar pads under coverslips. These worms were then examined by differential interference contrast (DIC) and by epi-fluorescence microscopy in the rhodamine channel for the presence and location of male-derived,

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fluorescently labeled sperm. In matings to *C. elegans* recipients, *daf-4*(*e1364*) hermaphrodites were used because their reduced gut autofluorescence facilitates the identification of fluorescent male-derived sperm within the reproductive tract (see above). Oocytes fertilized with fluorescent sperm gave rise to fertile adults, indicating that these methods do not interfere with normal *C. elegans* development (data not shown).

Hermaphrodite-Derived Spermatid Activation

Experiments in this section were performed essentially as described elsewhere (Singson *et al.*, 1999). Males and hermaphrodites for these experiments were picked and aged as described above. *C. elegans spe-8* hermaphrodites produce spermatids that fail to activate and are, consequently, self-sterile (L'Hernault *et al.*, 1988; Shakes and Ward, 1989). Insemination by wild-type males provides an as yet unidentified male-derived sperm activator in the seminal fluid that activates *spe-8* hermaphrodite-derived spermatids and restores self-fertility to mated *spe-8* hermaphrodites (Shakes and Ward, 1989). Therefore, the restoration of self-fertility in mated *spe-8* hermaphrodites can be used as a sensitive assay for the transfer of seminal fluid and the competence of the male-derived sperm activator. Recently molted individual adult *spe-8*(*hc53*) *dpy-5*(*e61*) hermaphrodites were mated to eight or 12 males and transferred to fresh plates daily until the hermaphrodite ceased laying. Age-matched sibs of the recipient hermaphrodites were picked individually as controls. *spe-8*(*hc53*) *dpy-5*(*e61*) hermaphrodite self-progeny are readily identified by their Dpy phenotype. All progeny produced by each hermaphrodite were counted, and only *spe-8* self-progeny were produced from any of these matings. *C. briggsae* and *C. remanei* males were mated to either *spe-8*(*hc53*) or *spe-8*(*hc53*) *dpy-5*(*e61*); all progeny of unmarked *spe-8*(*hc53*) hermaphrodites in these interspecific matings were *spe-8* self-progeny, based on their normal morphology and the observation that neither species is cross-fertile with *C. elegans* (Baird *et al.*, 1992; see below). *C. elegans spe-4*(*q347*) *unc-13*(*e1091*)/*spe-4*(*q347*) *dpy-5*(*e61*) males were crossed to *spe-8*(*hc53*) *dpy-5*(*e61*) hermaphrodites; no non-Dpy progeny were observed, and all progeny were scored as *spe-8* self-progeny because mutations in *spe-4* render males completely sterile (L'Hernault *et al.*, 1988; L'Hernault and Arduengo, 1992; Arduengo *et al.*, 1998).

Ovulation Studies

Males and females or hermaphrodites were picked and aged as described above. Restrictively raised *C. elegans fem-1*(*hc17ts*) genetic females (spermless hermaphrodites; Nelson *et al.*, 1978) were mated individually to six or eight *C. briggsae* wild-type, *C. remanei* wild-type, or *C. elegans fog-1*(*q253ts*) *him-5*(*e1490*) males at 25° for a period of 3 days and transferred to fresh plates daily. Age-matched *fem-1*(*hc17ts*) sibs were picked individually to plates as controls. All oocytes laid by both mated and unmated *fem-1*(*hc17ts*) females were counted by removing them from the growth plate, and the mean numbers of oocytes laid by each group were compared to determine whether mating increased oocyte production. Matings of *C. elegans* and *C. briggsae* males to *C. remanei* females were performed in an identical manner at 20°, except that matings of *C. briggsae* males to *C. remanei* females were terminated after 24 h because extensive cross-fertility was observed. For these interfertile matings of *C. briggsae* males to *C. remanei* females, unfertilized oocytes were counted and removed from the growth plate, while shelled embryos were counted but left undisturbed. After 24 h, embryos and larvae were counted to determine the percentage of hybrid embryos that hatched.

Sperm Competition Experiments

Sperm competition experiments were performed essentially as previously described (Singson *et al.*, 1999). Successful competition by inseminated, male-derived sperm is indicated by a reduction in the number of self-progeny produced by mated hermaphrodites, compared to unmated controls. Successful sperm competition does not require that male-derived sperm be able to fertilize oocytes (Singson *et al.*, 1999), and the reduction in number of hermaphrodite self-progeny may still occur when no outcross progeny are produced. Males and hermaphrodites were picked and aged as described above. Single newly molted adult *C. elegans dpy-5*(*e61*) hermaphrodites were mated to six *C. briggsae* or *C. remanei* males at 20° for a period of 24 h. Age-matched *dpy-5* hermaphrodites were picked to plates individually as controls. For each series of heterospecific matings to *C. elegans dpy-5*, a number of conspecific control crosses by *C. elegans him-5*(*e1490*) males were performed in parallel. After 24 h, adults were removed and the plates were shifted to 25° to accelerate maturation of the progeny. All progeny were counted and scored as either hermaphrodite self-progeny (Dpy) or outcross progeny (non-Dpy). The numbers of hermaphrodite self-progeny from unmated *dpy-5* hermaphrodites were compared to the numbers of hermaphrodite self-progeny from each category of mating to determine the ability of male-derived sperm from each species to compete with the endogenous *C. elegans* hermaphrodite-derived sperm. Experiments assessing sperm competition within *C. briggsae* hermaphrodites were performed in an identical manner, using either wild-type (*C. elegans* and *C. remanei* matings) or morphologically marked *cby-3*(*bd101*) (*C. briggsae* matings) *C. briggsae* hermaphrodites. In matings to *C. elegans him-5*(*e1490*) males, all progeny were scored as *C. briggsae* self-progeny because these species are not cross-fertile. No dead eggs or abnormal larvae were observed in these matings. In matings of *C. remanei* males to *C. briggsae* hermaphrodites, some embryos did not hatch within 24 h of being laid at 25°, and these were scored as outcross progeny, because no dead eggs were produced by unmated *C. briggsae* controls.

Data Analyses

All data analyses were performed using the software program Microsoft Excel 98. Statistical analysis was performed using Student's t test, two-tailed, assuming unequal variance.

RESULTS

Male-Derived Sperm Activation and Spermathecal Targeting

Fertilization in *C. elegans* and related nematodes occurs within the hermaphrodite or female sperm storage organs, the spermathecae (Ward and Carrel, 1979; Schedl, 1997; McCarter *et al.*, 1999). Inseminated, male-derived sperm are deposited just inside the vulva and must crawl to the spermathecae in order to participate in fertilization. Sperm migration requires that sessile spermatids undergo activation to become motile spermatozoa (Ward and Carrel, 1979;

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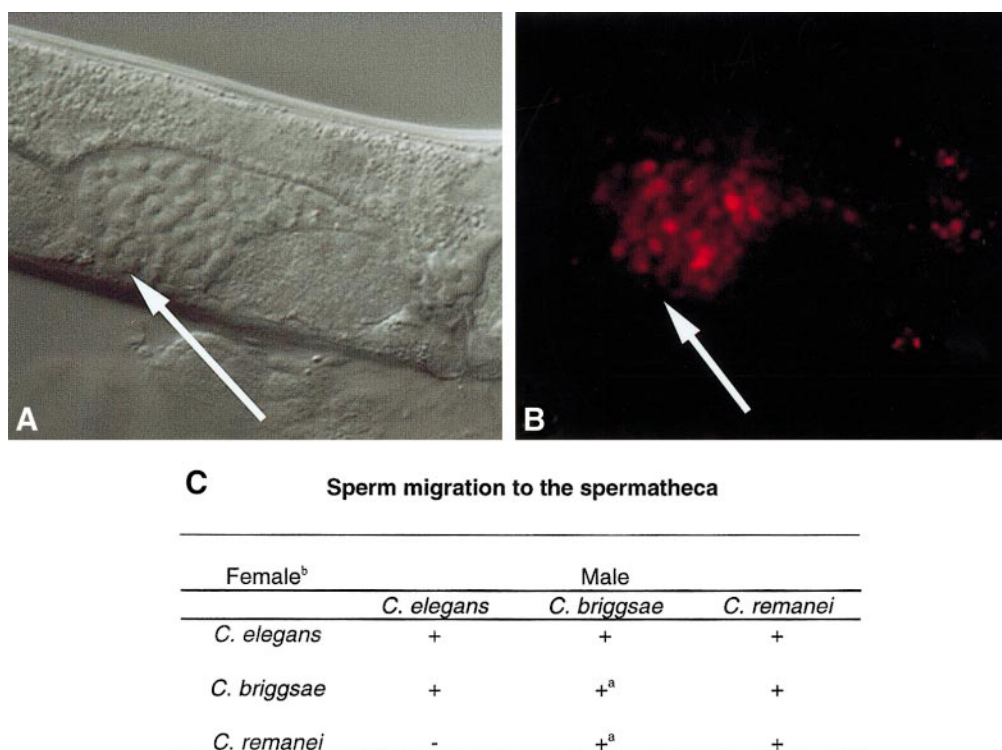

C Sperm migration to the spermatheca

Female[b]	Male		
	C. elegans	*C. briggsae*	*C. remanei*
C. elegans	+	+	+
C. briggsae	+	+[a]	+
C. remanei	-	+[a]	+

FIG. 1. Sperm migration/targeting to the spermatheca in con- and heterospecific crosses of *Caenorhabditis*. Males labeled with the vital fluorescent dye SYTO17 were mated to unlabeled recipient hermaphrodites or females. The following day, fluorescence microscopy was used to determine the position of labeled sperm within the recipient. (A, B) *C. remanei* sperm within the spermatheca of a *C. briggsae* hermaphrodite. These images are representative of typical positive sperm targeting results. The number of labeled sperm within the spermatheca, however, varies widely with the number of copulations and efficiency of sperm transfer. (A) Nomarski DIC; (B) SYTO17 fluorescence; (C) table summarizing spermathecal targeting results in all pairwise matings of *C. elegans*, *C. briggsae*, and *C. remanei*. [a] Inferred from con- or heterospecific fertility. [b] *C. elegans* and *C. briggsae* hermaphrodites were used in sperm targeting crosses.

Ward *et al.*, 1983; L'Hernault *et al.,* 1988; Shakes and Ward, 1989; Lamunyon and Ward, 1994; reviewed by L'Hernault, 1997).

We developed a live fluorescent sperm tracking assay (similar to that used in Ward and Carrel, 1979) to assess the insemination, activation, and spermathecal targeting of male-derived sperm in con- and heterospecific matings between *C. elegans*, *C. briggsae*, and *C. remanei*. Young adult males of each species were soaked in the fluorescent dye SYTO17, which labels sperm but does not affect their function, and then mated to unlabeled, recipient hermaphrodites or females (see Materials and Methods). After approximately 12–16 h, recipient hermaphrodites or females were anaesthetized and examined by DIC and fluorescence microscopy to determine if any labeled sperm were present, and where such sperm were located within the recipient reproductive tract (Figs. 1A and 1B). In most con- and heterospecific matings, male-derived sperm successfully migrated to the spermatheca in the recipient animals (Fig. 1C). This indicates the signal(s) that attracts inseminated sperm to the spermatheca was conserved during speciation within the genus *Caenorhabditis*. The lone exception was *C. elegans* males placed with *C. remanei* females, where despite observed copulation, no sperm were ever seen anywhere within the bodies of recipient females (Fig. 1C).

Activation of Hermaphrodite-Derived Sperm

Like male-derived sperm, hermaphrodite-derived sperm must be activated to participate in fertilization (Ward and Carrel, 1979; Ward *et al.*, 1983; L'Hernault *et al.,* 1988; Shakes and Ward, 1989; Lamunyon and Ward, 1994; reviewed by L'Hernault, 1997). Spermatids produced by unmated *C. elegans spe-8* hermaphrodites do not activate, and these animals are self-sterile (L'Hernault *et al.,* 1988; Shakes and Ward, 1989). However, the presence of an unidentified activating substance in competent seminal fluid will activate hermaphrodite-derived *spe-8* spermatids and restore self-fertility (Shakes and Ward, 1989). We set up matings to assess if the seminal fluid from *C. elegans*, *C. briggsae*, or *C. remanei* males can activate *C. elegans spe-8*(*hc53*) hermaphrodite-derived spermatids [see Materi-

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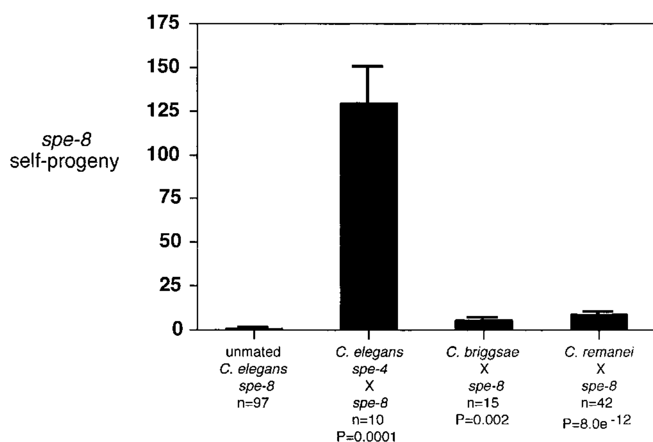

FIG. 2. Analysis of the ability of heterospecific seminal fluid to activate *C. elegans spe-8* hermaphrodite-derived spermatids *in vivo*. For experimental details, see Materials and Methods. *spe-8 dpy-5* self-progeny in conspecific crosses could be identified by their Dpy (short and fat) phenotype; however, no outcross progeny were observed in these experiments. Progeny were counted for the entire reproductive span of each *spe-8* hermaphrodite. Bars indicate mean ± SE.

als and Methods and Singson *et al.,* (1999) for detailed descriptions of this assay]. We found that heterospecific mating of either *C. briggsae* or *C. remanei* males to *C. elegans spe-8* hermaphrodites significantly increased self-fertility over that of unmated *spe-8* hermaphrodite controls (Fig. 2). Therefore the male-derived sperm activation pathway has been conserved among *C. elegans*, *C. briggsae*, and *C. remanei*. However, heterospecific matings were less efficient at activating *C. elegans spe-8* spermatids than mating to *C. elegans spe-4* males (Fig. 2), which ejaculate competent seminal fluid but no sperm (L'Hernault *et al.,* 1988; L'Hernault and Arduengo, 1992; Arduengo *et al.,* 1998).

Stimulation of Ovulation

In *C. elegans*, mutant hermaphrodites that either make no sperm (genetic females, e.g., *fem-1*) or that make only abnormal spermatocytes that arrest early in spermatogenesis (e.g., *spe-4*) produce oocytes at a much lower rate than do wild-type hermaphrodites (Fig. 3A; L'Hernault *et al.,* 1988; McCarter *et al.,* 1999). Inseminating these mutant hermaphrodites with wild-type, male-derived sperm restores the rate of oocyte maturation and ovulation to wild-type levels (McCarter *et al.,* 1999). Similar to *C. elegans*, unmated *C. remanei* females ovulate less than five oocytes during their entire life (Fig. 3B), but once inseminated by *C. remanei* males, will produce many progeny.

To examine the ability of male-derived sperm to stimulate ovulation in heterospecific matings, we mated either *C. briggsae* or *C. remanei* males to restrictively raised *C. elegans fem-1(hc17ts)* genetic females. The *C. elegans* females used in these matings laid significantly more oocytes on the growth plate during a 3-day period than did their unmated siblings (Fig. 3A). Mating these females to *fog-1* males did not stimulate ovulation, which acts as a control for copulation in the absence of sperm transfer because these phenotypic males do not engage in spermatogenesis (Barton and Kimble, 1990). This illustrates that the pathway responsible for triggering *C. elegans* ovulation can respond to either *C. briggsae* or *C. remanei* spermatozoa. Despite the observed stimulation of ovulation in these heterospe-

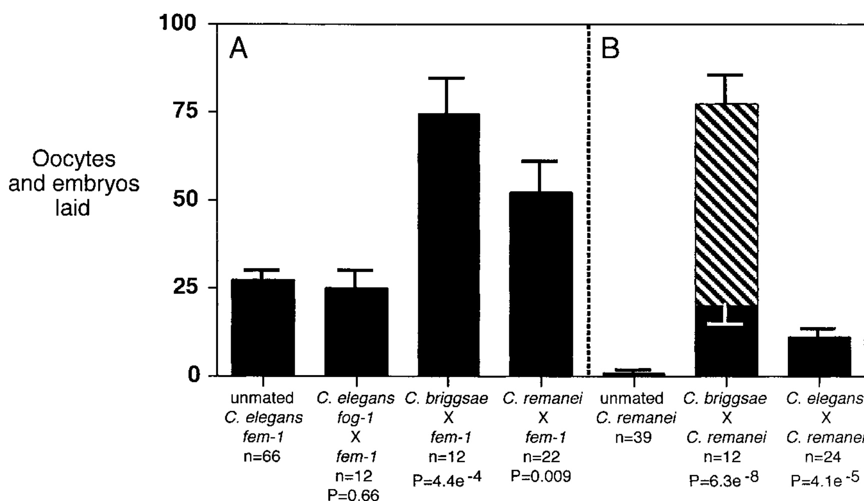

FIG. 3. Stimulation of ovulation by heterospecific sperm. Matings to restrictively raised *C. elegans fem-1(hc17ts)* genetic females (A) and *C. remanei* females (B) are shown with unmated female controls. Oocytes and/or progeny laid were counted for 3 days. Bars indicate mean ± SE. Solid bars = oocytes; cross-hatched bar = progeny.

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cific matings, no shelled eggs were ever seen (Fig. 3A), confirming that fertilization does not occur between *C. briggsae* or *C. remanei* sperm and *C. elegans* oocytes.

In a similar experiment, *C. elegans* or *C. briggsae* males were mated to *C. remanei* females and, in both cases, mated females laid significantly more oocytes than did their unmated sibs (Fig. 3B). The matings to *C. elegans* males resulted only in the laying of unfertilized *C. remanei* oocytes (Fig. 3B). Matings of *C. remanei* females to *C. briggsae* males resulted in the laying of a large number of shelled eggs (embryos; Fig. 3B, cross-hatching), and some unfertilized oocytes (Fig. 3B). Although the mating of *C. briggsae* males to *C. remanei* females resulted in abundant cross-fertility, very few of the hybrid embryos (~6%) survived embryogenesis. All hybrid larvae exhibited variable defects in morphogenesis and, if they reached adulthood, sterility (data not shown), consistent with prior results (Baird *et al.,* 1992).

Sperm Competition

In the self-fertile hermaphroditic nematodes *C. elegans* and *C. briggsae*, reproduction is primarily a single-animal event. However, outcrossed progeny occur when inseminated, male-derived sperm successfully out-compete endogenous hermaphrodite-derived sperm and fertilize oocytes (Ward and Carrel, 1979; Lamunyon and Ward, 1995). In broods from hermaphrodites mated to wild-type males, the number of self-progeny is diminished. If the male-derived sperm are fully competent to fertilize, the number of outcrossed progeny approximates the reduction in number of self-progeny, so that the total number of progeny remains the same (Fig. 4A). This phenomenon of suppression of hermaphrodite self-fertility by the presence of male-derived sperm holds true for *C. briggsae* as well (Fig. 5A).

When *C. briggsae* (Fig. 4B) or *C. remanei* (Fig. 4C) males were mated to *C. elegans* hermaphrodites, the mated *C. elegans* hermaphrodites produced no fewer self-progeny than the unmated control hermaphrodites. This result shows that, although they reach the spermatheca, neither *C. briggsae* nor *C. remanei* male-derived sperm are able to compete with the endogenous *C. elegans* hermaphrodite-derived sperm for access to oocytes. Similarly, when *C. elegans* males were mated to *C. briggsae* hermaphrodites (Fig. 5C), no reduction in *C. briggsae* self-fertility was observed, confirming that sperm competition has not been conserved between these two species. Matings of *C. remanei* males to *C. briggsae* hermaphrodites (Fig. 5B) resulted not only in a significant reduction in *C. briggsae* hermaphrodite self-fertility, but also in the production of a small number of dead embryos, which were not seen in the unmated controls (Fig. 5B, cross-hatching). This shows that *C. remanei* males mated to *C. briggsae* hermaphrodites are cross fertile, but to a lesser extent than the reciprocal mating (compare Fig. 3B to Fig. 5B). No abnormal larvae or adults were produced by *C. briggsae* hermaphrodites after they were mated to *C. remanei* males, suggesting that hybrids from these crosses never survive embryogenesis.

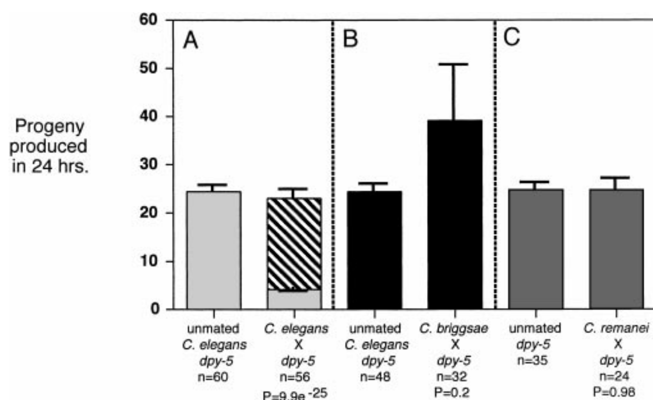

FIG. 4. Analysis of competition for oocytes between male-derived sperm and endogenous *C. elegans* hermaphrodite-derived sperm. Successful competition by male-derived sperm is indicated by a reduction in hermaphrodite self-fertility. Each experiment (A, B, or C) consisted of plates containing either male/hermaphrodite matings or age-matched, nonmated hermaphrodite controls, and experimental and control plates were identically handled to ensure valid statistical comparisons. Bars indicate mean ± SE. Solid bars = hermaphrodite self-progeny; crosshatched bar = outcross progeny. (A) Competition by *C. elegans* male-derived sperm with *C. elegans* hermaphrodite-derived sperm. (B) Competition by *C. briggsae* male-derived sperm with *C. elegans* hermaphrodite-derived sperm. (C) Competition by *C. remanei* male-derived sperm with *C. elegans* hermaphrodite-derived sperm.

A determining factor in sperm precedence appears to be size; larger sperm outcompete smaller sperm (Lamunyon and Ward, 1998). *C. elegans* male-derived sperm are consistently larger (~50%) than hermaphrodite-derived sperm and appear to use their associated increase in speed and vigor to displace the hermaphrodite-derived (or smaller male-derived) sperm from the distal end of the spermatheca, thereby gaining first access to passing oocytes (Lamunyon and Ward, 1995, 1998; Singson *et al.,* 1999). Spermatids dissected from *C. briggsae* males are not obviously different in size from those of *C. elegans* males, while sperm from *C. remanei* males are relatively enormous, having approximately two times the diameter of *C. elegans* male-derived sperm (Fig. 6).

DISCUSSION

Nematode reproduction can be divided into five stages. Inseminated spermatids undergo activation (Fig. 7A) and resulting spermatozoa migrate through the uterus to the spermatheca, where fertilization takes place (Fig. 7B). The female responds to the presence of sperm by triggering oocyte maturation (Fig. 7C). Male-derived spermatozoa must compete with the hermaphrodite's endogenous sper-

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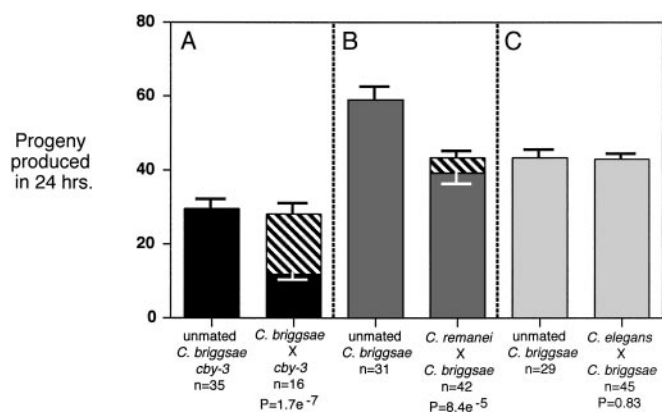

FIG. 5. Analysis of competition for oocytes between male-derived sperm and endogenous *C. briggsae* hermaphrodite-derived sperm. Successful competition by male-derived sperm is indicated by a reduction in hermaphrodite self-fertility. Each experiment (A, B, or C) consisted of plates containing either male/hermaphrodite matings or age-matched, nonmated hermaphrodite controls, and experimental and control plates were identically handled to ensure valid statistical comparisons. Bars indicate mean ± SE. Solid bars = hermaphrodite self-progeny; crosshatched bars = outcross progeny. (A) Competition by *C. briggsae* male-derived sperm with *C. briggsae* hermaphrodite-derived sperm. (B) Competition by *C. remanei* male-derived sperm with *C. briggsae* hermaphrodite-derived sperm. (C) Competition by *C. elegans* male-derived sperm with *C. briggsae* hermaphrodite-derived sperm.

matozoa for access to oocytes (Fig. 7D). Finally, a spermatozoon fuses with an oocyte during fertilization (Fig. 7E). We have shown that the interactions preceding sperm/oocyte contact are partially compatible among three *Caenorhabditis* species (Table 1), consistent with prior work (e.g., Baird *et al.,* 1992). However, our techniques allow examination of gametic interactions in significantly more detail than any prior work in nematodes or other phyla. For instance, after males inseminate a female/hermaphrodite of another species, fluorescently labeled spermatozoa were frequently observed to migrate into the spermatheca, suggesting the signal(s) that attract spermatozoa to that organ have been conserved.

The basal ovulatory rate of *C. elegans* hermaphrodites is low unless sperm are in the reproductive tract (Ward and Carrel, 1979; McCarter *et al.,* 1999). Sperm at any developmental stage are sufficient to stimulate *C. elegans* oocyte maturation, and a persistent, high ovulatory rate requires the continuous presence of sperm (McCarter *et al.,* 1999). *C. briggsae* and *C. remanei* are sufficiently similar to *C. elegans* that their sperm stimulate *C. elegans* oocyte maturation. Recent results suggest that secreted major sperm protein from *C. elegans* sperm triggers oocyte maturation (M. A. Miller and D. I. Greenstein, personal communication). This protein is highly conserved among nematodes (Klass *et al.,* 1984; Bennett and Ward, 1986; Ward *et al.*, 1988; Scott *et al.*, 1989a,b), and this might explain why sperm can stimulate ovulation across species lines.

Like *C. elegans*, *C. remanei* females restrict ovulation when sperm are absent. Mating *C. remanei* females to *C. briggsae* males increases ovulation approximately 75-fold, and *C. briggsae* sperm fertilize most of these oocytes. Mating *C. elegans* males to *C. remanei* females causes an 11-fold increase in ovulation, but none are fertilized. Our data suggest that insemination must be rare because fluorescent *C. elegans* sperm were not observed in *C. remanei* females following attempted copulation. Perhaps insemination is inhibited because the *C. elegans* male has difficulty either finding or interacting with the *C. remanei* vulva (reviewed by Emmons and Sternberg, 1997).

Seminal fluid from either *C. briggsae* or *C. remanei* males activated *C. elegans spe-8* hermaphrodite-derived spermatids into spermatozoa. These spermatozoa fertilize oocytes, and the resulting brood size is a measure of activation success (Shakes and Ward, 1989; Singson *et al.,* 1999). By this technique, cross-species activation was not as efficient

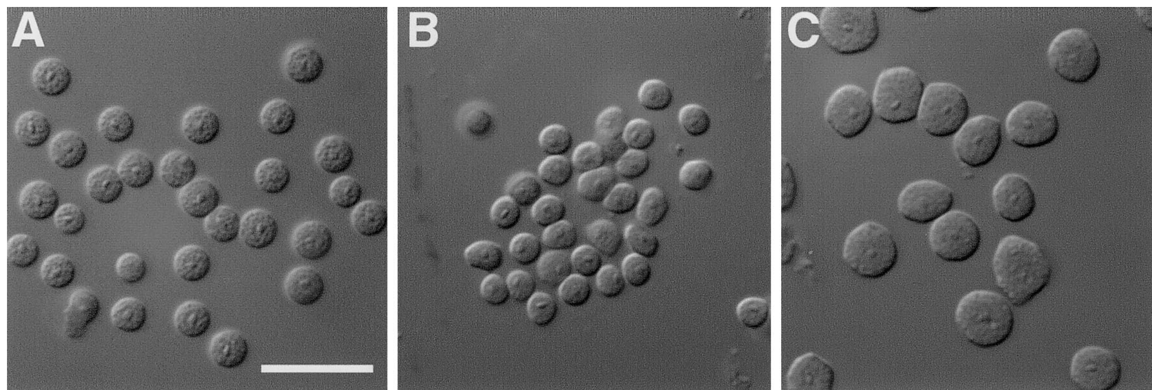

FIG. 6. Size comparison of *C. elegans*, *C. briggsae*, and *C. remanei* male-derived sperm. (A) *C. elegans*; (B) *C. briggsae*; (C) *C. remanei*. Scale bar, 10 μm.

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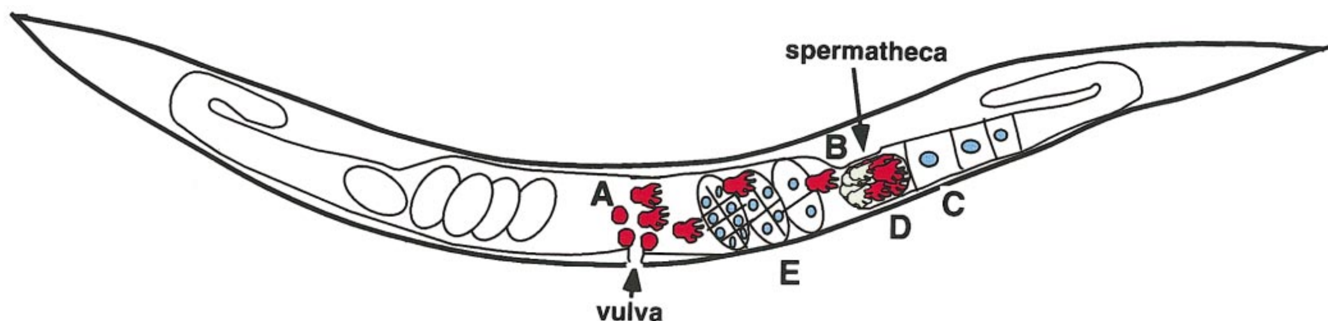

FIG. 7. Stages of gametic interactions in *Caenorhabditis*. A schematic of a *Caenorhabditis* hermaphrodite reproductive tract is shown. The gonad is doubly reflexed and symmetrical; only one side (on the right) is shown in detail in this figure. (A) Activation of male-derived spermatids to become motile spermatozoa (in red) with the ability to fertilize. (B) Migration of inseminated male-derived sperm through the uterus to the spermatheca. (C) Stimulation of ovulation by the presence of sperm. (D) Sperm competition; smaller, hermaphrodite-derived sperm (in green) are shown displaced from the distal portion of the spermatheca by larger, male-derived sperm (in red). (E) Fertilization. Oocyte (at C) and embryonic blastomere (at E) nuclei appear in blue.

as within-species activation. Perhaps seminal fluid transfer is inefficient during heterospecific mating or the activating component(s) have diverged sufficiently to reduce their biological activity in this assay.

In *C. elegans* and *C. briggsae,* male-derived spermatozoa compete with hermaphrodite-derived spermatozoa in order to fertilize oocytes. Successful competition by male-derived spermatozoa in hermaphrodites shifts the brood composition from self to outcross progeny, but the offspring produced in a given time remains the same (Ward and Carrel, 1979; Lamunyon and Ward, 1995; Singson *et al.,* 1999; this investigation). Sperm competition does not require fertilization because fertilization-incompetent *C. elegans* mutants produce spermatozoa that compete effectively (Singson *et al.,* 1999). Inseminated sperm from either *C. briggsae* or *C. remanei* reach the spermatheca in *C. elegans* hermaphrodites, yet they cannot compete with *C. elegans* hermaphrodite-derived sperm. Since *C. remanei* sperm are much larger than either *C. elegans* or *C. briggsae* sperm, this shows that sperm size per se does not confer a competitive advantage. Sometimes, *C. briggsae* or *C. remanei* fluorescent, male-derived spermatozoa were concentrated at the proximal end of the *C. elegans* spermatheca. In such cases, the distally located hermaphrodite-derived spermatozoa would contact oocytes prior to spermatozoa from males (data not shown).

TABLE 1

Summary of the Conservation of Reproductive Interactions among *C. elegans, C. briggsae,* and *C. remanei*

Reproductive interaction	*C. elegans* male			*C. briggsae* male			*C. remanei* male		
	Female			Female			Female		
	C. elegans	*C. briggsae*	*C. remanei*	*C. elegans*	***C. briggsae***	*C. remanei*	*C. elegans*	*C. briggsae*	***C. remanei***
Sperm activation	**+**	NP	N/A	+	**NP**	N/A	+	NP	**N/A**
Spermathecal targeting	**+**	+	−[a]	+	**+**	+	+	+	**+**
Stimulation of ovulation	**+**	NP	+	+	**+**	+	+	NP	**+**
Sperm competition[b]	**+**	−	N/A	−	**+**	N/A	−	+	**N/A**
Fertilization	**+**	−	−	−	**+**	+	−	+	**+**

Note. Conspecific interactions are bold and are assumed to be fully compatible. Female refers to hermaphrodite, female, or genetic female, as appropriate. N/A, not applicable; NP, analysis not possible due to reproductive physiology or lack of suitable mutant strains.

[a] This result may be due to failure of insemination.

[b] Competition with endogenous, hermaphrodite-derived sperm.

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Male-derived sperm impose no reproductive cost on a hermaphrodite if they have no competitive advantage. Matings between *C. elegans* and *C. briggsae* are reproductively neutral because the heterospecific sperm do not affect hermaphrodite self-fertility. However, mating between *C. briggsae* and *C. remanei* results in either dead or sterile hybrid progeny (*C. remanei*) or reduced hermaphrodite self-fertility (*C. briggsae*) (also see Baird *et al.,* 1992). Heterospecific matings between *C. remanei* and *C. elegans* stimulates ovulation and these oocytes are wasted. Reinforcement theory (e.g., Dobzhansky, 1940; Butlin, 1989; Liou and Price, 1994; Kelly and Noor, 1996) says sympatric species exhibiting postzygotic isolation will rapidly develop premating isolation. Presumably, selection acts against dead or sterile hybrids, as has been shown in *Drosophila* (reviewed by Coyne and Orr, 1989, 1998). Strong premating isolation does not exist in *Caenorhabditis*, perhaps because they do not frequently encounter one another in the wild. However, when *C. elegans* or *C. briggsae* hermaphrodites are placed with males of both species, conspecific mating preferentially occurs, suggesting the presence of premating isolation (K. Hill and S. W. L'Hernault, unpublished observations).

Total reproductive isolation involves several incomplete reproductive barriers at a variety of steps (e.g., Coyne and Orr, 1998). Early postcopulatory events are qualitatively conserved among the three *Caenorhabditis* species examined, but the degree to which function is maintained varies with the assay. For instance, the *C. remanei* male-derived spermatid activator is more efficient than that of *C. briggsae* at activating *C. elegans spe-8* spermatids. This suggests that *C. remanei*, rather than *C. briggsae*, is more closely related to *C. elegans*. In contrast, ovulation in *C. elegans* was more highly stimulated after mating to *C. briggsae,* rather than *C. remanei* males. The late-stage events of sperm competition and fertilization are conserved between *C. briggsae* and *C. remanei*, but not between either of those species and *C. elegans*. This suggests that *C. briggsae* and *C. remanei* are more closely related to each other than either is to *C. elegans*, consistent with prior work based on gene sequence comparisons (Fitch *et al.,* 1995; Fitch and Thomas, 1997). The patterns of reproductive interaction are distinct for each species pair and might provide useful tools for classifying evolutionary relationships among nematode species.

ACKNOWLEDGMENTS

This work was supported by NSF Grant IBN-9808847 to S.W.L. K.L.H. was partly supported by a National Research Service Award from the NIH. The authors acknowledge Drs. William G. Kelly, John Lucchesi, Barry Yedvobnick, Grant MacGregor, and John Logsdon and all the members of the L'Hernault lab for useful discussions.

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Received September 6, 2000
Accepted December 1, 2000
Published online March 5, 2001

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