FASTER FERTILIZATION RATE IN CONSPECIFIC VERSUS HETEROSPECIFIC MATINGS IN HOUSE MICE

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Barriers to gene flow can arise at any stage in the reproductive sequence. Most studies of reproductive isolation focus on premating or postzygotic phenotypes, leaving the importance of differences in fertilization rate overlooked. Two closely related species of house mice, *Mus domesticus* and *M. musculus*, form a narrow hybrid zone in Europe, suggesting that one or more isolating factors operate in the face of ongoing gene flow. Here, we test for differences in fertilization rate using laboratory matings as well as in vitro sperm competition assays. In noncompetitive matings, we show that fertilization occurs significantly faster in conspecific versus heterospecific matings and that this difference arises after mating and before zygotes form. To further explore the mechanisms underlying this conspecific advantage, we used competitive in vitro assays to isolate gamete interactions. Surprisingly, we discovered that *M. musculus* sperm consistently outcompeted *M. domesticus* sperm regardless of which species donated ova. These results suggest that in vivo fertilization rate is mediated by interactions between sperm, the internal female environment, and/or contributions from male seminal fluid. We discuss the implications of faster conspecific fertilization in terms of reproductive isolation among these two naturally hybridizing species.

Speciation can, in principle, result from reproductive isolation arising at any stage in the reproductive process. All else being equal, barriers that occur earlier in the reproductive sequence will have a greater effect on overall reproductive isolation (Coyne and Orr 2004). For example, strong postzygotic isolation may contribute only weakly to overall isolation if barriers operating at earlier stages make the formation of hybrid zygotes unlikely. In vertebrates, many studies of speciation have focused on hybrid sterility and inviability, both postzygotic phenotypes. In cases where the process of speciation is incomplete and there is still potential gene flow among taxa, it is possible that fertilization rate differs in conspecific versus heterospecific matings, thus contributing to isolation in the face of gene flow.

Mechanisms that operate after mating, but before zygotes are formed, may be especially important in contributing to iso-lation for several reasons. Genes whose proteins participate in the events leading up to fertilization tend to diverge more rapidly than average (Wyckoff et al. 2000; Swanson and Vacquier 2002; Torgerson et al. 2002; Waterston et al. 2002; Jansa et al. 2003; Swanson et al. 2003; Castillo-Davis et al. 2004; Dorus et al. 2004; Gibbs et al. 2004; Nielsen et al. 2005; Clark et al. 2006; Turner and Hoekstra 2006; Kelleher et al. 2007; Dean et al. 2008), and elevated divergence may disrupt proper fertilization. Furthermore, females of many species mate with more than one male during their fertile period (reviewed in Birkhead and Pizzari 2002). If females frequently mate with both conspecific and heterospecific males, paternity could be biased toward conspecific offspring if conspecific males fertilize faster.

Two closely related species of house mice, *Mus musculus* and *M. domesticus* (also referred to as subspecies *M. musculus*

musculus and *M. musculus domesticus* in the literature) represent the best studied mammalian model of speciation. These species diverged about 500,000 years ago (She et al. 1990; Boursot et al. 1993; Suzuki et al. 2004; Salcedo et al. 2007) and secondary contact occurred in Europe around 3,000–6,000 years ago (Auffray et al. 1990). The European hybrid zone shows sharp transitions between *M. musculus* alleles in the east and *M. domesticus* alleles in the west, with mixed genotypes occurring in a narrow cline (Dod et al. 1993; Munclinger et al. 2002; Payseur et al. 2004; Macholán et al. 2007; Teeter et al. 2007). The sharp transition in allele frequency suggests that one or more isolating factors prevent gene flow between the two species. The possibility that differences in fertilization rate contribute to isolation has not been tested before.

Here, we show that fertilization rate is significantly faster in conspecific versus heterospecific matings. However, in vitro sperm competition experiments showed that *M. musculus* sperm always outcompeted *M. domesticus* sperm, regardless of which species donated ova. By comparing in vivo and in vitro results, we suggest that this difference is due to interactions between sperm, the internal female environment and/or male seminal fluid.

Materials and Methods
MOUSE STRAINS USED
Inbred-derived strains
We chose four inbred strains of mice: WSB/Eij and LEWES/EiJ represent *M. domesticus*, PWK/PhJ and CZECHII/EiJ represent *M. musculus*. All four strains were derived from natural populations outside of the European hybrid zone, and all have the standard karyotype ($2n = 40$). All strains were initially purchased from the Jackson Laboratory (Bar Harbor, ME).

Progeny of intraspecific crosses (LEWES/EiJ ♀ × WSB/Eij ♂ for *M. domesticus*; PWK/PhJ ♀ × CZECHII/EiJ ♂ for *M. musculus*) were used in all experiments to avoid confounding effects of inbreeding while maintaining the benefits of a reproducible genotype. Hereafter, DOM and MUS refer to intraspecific F1 progeny of *M. domesticus* and *M. musculus*, respectively. In all crosses, males and females were paired for approximately 1 week and then separated so that females gave birth in isolation. Approximately 21 days postpartum, offspring were weaned. Male progeny were weaned with one individual per cage to avoid dominance interactions among brothers; grouped males have reduced fertility compared to singly caged males (Snyder 1967). Males were considered sexually mature at 60 days of age. Females were weaned with up to four individuals per cage and used in experiments at approximately 5 weeks of age. All mice were maintained at the University of Arizona Central Animal Facility in accordance with IACUC regulations.

Wild-derived mice
To corroborate patterns observed with DOM and MUS genotypes, we collected wild *M. domesticus* from Tucson, Arizona. Each mouse was caught at least 100 m from all other mice to avoid collecting relatives. As above, we used F1 progeny of these mice, referred to as DOM$_{WILD}$, in all experiments.

LABORATORY MATINGS
At approximately 5 weeks of age, females were induced to ovulate with standard techniques (Nagy et al. 2003). Females were intraperitoneally injected with 2.5–5.0 units of Pregnant Mare's Serum Gonadotropin (CalBiochem, San Diego, CA), a follicle stimulating hormone. Approximately 48 h later, females were injected with 2.5–5.0 units of Human Chorionic Gonadotropin (hCG, CalBiochem), a luteinizing hormone. Immediately following administration of hCG, females were individually paired with a male that was 60–90 days old. Following 20 h of pairing, females were sacrificed and the ova-containing cumulus masses were dissected from oviducts into a 250 μl drop of phosphate buffered saline (PBS). Ova were dissociated from cumulus cells by adding 250 units of hyaluronidase (Sigma, St. Louis, MO) and incubating for a few minutes. Copulatory plugs, if present, were removed and weighed. Copulatory plug mass is positively, although not significantly, correlated with the number of sperm ejaculated (Ramm and Stockley 2007).

After sperm and ova fuse, the second polar body is extruded, followed by the formation of two pronuclei (corresponding to sperm and egg). Matings were inferred to be successful if (1) at least one ovum had two polar bodies and/or two pronuclei, (2) a copulatory plug was present, and/or (3) sperm and/or seminal contents were observed in the female reproductive tract. Only successful matings were included in the analyses below. Fertilization rate was quantified as the proportion of ova with two polar bodies and/or two pronuclei after 20 h of mating. The difference in conspecific and heterospecific fertilization rate was then normalized by conspecific fertilization rate (i.e., [conspecific fertilization rate – heterospecific fertilization rate]/conspecific fertilization rate). A score of 0 indicates no difference in fertilization rate, > 0 indicates faster conspecific versus heterospecific fertilization, and < 0 indicates faster heterospecific versus conspecific fertilization. An example calculation is given in Table 1.

IN VITRO SPERM COMPETITION ASSAYS
We developed in vitro sperm competition assays to further isolate any differences observed in the laboratory matings. In vitro methods offer a powerful means to remove the influence of the internal female environment, and to eliminate interactions between sperm and male seminal fluid. Furthermore, sperm count can be carefully controlled.

Table 1. In vivo fertilization rate.

Male[1]	Female[1]	Fertilized	Not	P (fertilized)	P^2	Normalized difference[3]
(A) Inbred-derived males and females						
DOM (2)	DOM (4)	44	16	0.73	10^{-6}	0.52
DOM (4)	MUS (5)	80	147	0.35		
MUS (3)	MUS (8)	204	21	0.91	10^{-10}	0.39
MUS (3)	DOM (5)	44	35	0.56		
(B) Inbred-derived males $\times$ wild females						
DOM (2)	DOM$_{WILD}$ (5)	41	2	0.95	10^{-4}	0.32
MUS (2)	DOM$_{WILD}$ (6)	49	27	0.64		
(C) Wild males $\times$ inbred-derived females						
DOM$_{WILD}$ (6)	DOM (17)	152	121	0.56	10^{-16}	0.60
DOM$_{WILD}$ (6)	MUS (7)	73	254	0.22		

[1]Numbers in parentheses indicate the number of individual mice sampled.

[2]Fisher's exact test probability of independence between the number of ova fertilized or not, comparing each pair of rows.

[3]The difference in conspecific and heterospecific fertilization rate (CFR and HFR, respectively).

This score is calculated as (CFR-HFR)/CFR and theoretically ranges from −1 to 1. For example, in the first two rows the difference in fertilization rate=(0.73−0.35)/0.73=0.52. A score of 0 would indicate no difference in conspecific versus heterospecific fertilization rate.

Scores>0 indicate faster conspecific fertilization rate.

Females were induced to ovulate as described above, and cumulus masses were dissected 12 h after administration of hCG. All dissections were made in Mouse Vitro Fert (MVF, Cook Australia, Eight Mile Plains, Australia) under embryo-tested mineral oil (Sigma) that had been equilibrated overnight in 5% CO2 at 37 C. After the initial dissection, cumulus masses were pipetted into a fresh equilibrated drop of MVF to reduce cellular waste and returned to the incubator.

Sixty day old males were sacrificed and the caudal end of the epididymis (where mature sperm are stored prior to ejaculation) plus the vas deferens (through which mature sperm travel prior to and during ejaculation) were dissected and placed in equilibrated MVF. The caudal end of the epididymis was sliced once longitudinally with a 28G needle, and the vas deferens was stripped. The sperm dissection was then returned to the incubator.

After 1 h of incubation, the tissues were removed from the sperm dissection drop which was swirled to mix sperm. A small sample was diluted in 1% sodium citrate and heat shocked at 70 C for 2 min to quantify sperm with a hemacytometer. Approximately 3.6×10^6 non–heat-shocked sperm were incubated in a 75 μl drop of equilibrated MVF with 400 nM Mitotracker Green (Invitrogen, Carlsbad, CA) for 20 min. Mitotracker Green stains mitochondria in the sperm midpiece, and has been used previously in reproductive biology research (Sutovsky et al. 2003). After incubation, sperm dilutions were centrifuged at 500 g for 2 min, and supernatants removed with a pipettor. This step was meant to remove residual dye and DMSO (the dye buffer). To be consistent, other subsets of 3.6×10^6 sperm, which remained undyed, were also incubated and centrifuged alongside the dyed sample. Following centrifugation, sperm were resuspended in 70 ul equili-

brated MVF, then a sample was diluted in 1% sodium citrate, heat shocked at 70°C for 2 min, and requantified with a hemacytometer as described above.

Two different assays were performed. In a set of control assays, a subset of dyed sperm from one male was competed against undyed sperm from this same male. This experiment generated the expected proportion of ova fertilized by dyed sperm in the absence of genetic differences. To achieve a proportion of 0.50, it was necessary to use approximately 0.5×10^5 and 2.0×10^5 undyed and dyed sperm, respectively. Therefore, a total of 2.5×10^5 total sperm were added to cumulus masses, then gently pipette-mixed. After 4 h of incubation, ova were washed through a series of equilibrated MVF droplets to remove excess sperm and cellular waste. After an additional 3 h, ova were examined under fluorescence microscopy. Any ova with two pronuclei were scored for the presence or absence of a green streak caused by stained mitochondria in the sperm midpiece. Scoring was done blind.

In a set of competitive assays, dyed sperm from the same aliquot used in the control assays were competed against undyed sperm from a different male. The shift in the proportion of ova fertilized by dyed sperm was used to quantify conspecific sperm precedence. An example calculation is given in Table 2.

Results
LABORATORY MATINGS
Two major results emerged from laboratory matings. First, fertilization was significantly faster in conspecific versus

Table 2. In vitro sperm competition assays.

Assay type[1]	Ova donor[2]	Sperm donor[2]		2-pronuclei ova		P (Dyed)	P[3]	Conspecific precedence[4]
		Dyed	Not	Dyed	Not			
(A) Inbred-derived females and males								
Control	DOM (31)	DOM (8)	DOM (8)	131	124	0.51	10^{-11}	−0.55
Competitive	DOM (36)	''	MUS (9)	67	221	0.23		
Control	DOM (9)	MUS (2)	MUS (2)	54	56	0.49	10^{-7}	0.59
Competitive	DOM (23)	''	DOM (5)	150	42	0.78		
Control	MUS (19)	DOM (8)	DOM (8)	141	186	0.43	10^{-9}	−0.48
Competitive	MUS (33)	''	MUS (11)	93	319	0.23		
Control	MUS (8)	MUS (5)	MUS (5)	96	137	0.41	10^{-6}	0.47
Competitive	MUS (19)	''	DOM (7)	187	121	0.61		
(B) MUS females, MUS and wild-derived males								
Control	MUS (3)	MUS (1)	MUS (1)	34	42	0.45	10^{-17}	1.15
Competitive	MUS (6)	''	DOM$_{WILD}$ (1)	122	5	0.96		

[1]Control: Dyed sperm from a male was competed against undyed sperm from the same male. Competitive: This same dyed sperm was competed against undyed sperm from a second male.

[2]Numbers in parentheses indicate the number of individual mice sampled.

[3]Fisher's exact test probability of independence between the number of ova fertilized or not, comparing each pair of rows.

[4]The shift in the proportion of dyed ova between control and competitive experiments. For example, from the first two rows, conspecific precedence=(0.23−0.51)/0.51=−0.55, suggesting dyed DOM sperm performs 55% worse than expected when in competition with undyed MUS sperm.

heterospecific matings. Second, MUS males showed higher overall fertilization ability than DOM males.

Fertilization was significantly faster in conspecific versus heterospecific matings

After 20 h of mating, DOM males fertilized 73% of DOM ova, compared to 35% of MUS ova fertilized (Table 1A). This difference in conspecific versus heterospecific fertilization rate was highly significant (Fisher's exact test [FET] $P < 10^{-6}$). Similarly, MUS males fertilized 91% of MUS ova, compared to 56% of DOM ova (FET, $P < 10^{-10}$).

Faster conspecific fertilization was also observed using genetically heterogeneous, wild-derived *M. domesticus* (DOM$_{WILD}$). Four DOM$_{WILD}$ female progeny were weaned from each of 3 litters derived from unrelated parents (a total of 12 female progeny). From each set of four full sisters, two were mated to DOM males and two were mated to MUS males. DOM males fertilized 95% of DOM$_{WILD}$ ova, whereas MUS males fertilized 64% of DOM$_{WILD}$ ova (FET, $P < 10^{-4}$; Table 1B).

Faster conspecific fertilization was also observed with DOM$_{WILD}$ males. Two DOM$_{WILD}$ male progeny were weaned from each of 3 litters (a total of six male progeny). All DOM$_{WILD}$ males fertilized more DOM ova than MUS ova. Conspecific fertilization rate was significantly higher in the pooled data (FET, $P < 10^{-16}$) (Table 1C), as well as five of six individual comparisons (five of six FET, $P < 0.05$).

Interestingly, DOM females were fertilized more slowly than DOM$_{WILD}$ females (Table 1A vs. 1B), but DOM males fertilized ova faster than DOM$_{WILD}$ males (Table 1A vs. 1C). Explanations for this pattern are not obvious, but the strong laboratory selection in the two parental strains of DOM mice (LEWES and WSB) may have affected male and female reproductive parameters in different and complex ways.

The faster conspecific fertilization observed in both directions was the result of mechanisms operating after mating. If differences were due to premating isolation, such as behavioral dysfunction or delays in heterospecific matings, we would expect reduced heterospecific mating success. However, 100% of both conspecific and heterospecific matings were successful. To further explore whether the differences in Table 1 might be due to delays in heterospecific compared to conspecific matings, we analyzed matings after 12 h instead of 20 h, crossing MUS females to MUS or DOM males. Ova display two pronuclei about 8 h after fertilization (Nagy et al. 2003), so allowing mice to mate for 12 h provided an appropriate test of this alternative. There was no difference in success of conspecific matings after 12 h (nine of 12 crosses successful) versus heterospecific matings (eight of eleven successful) (FET, $P = 1$).

Differences in fertilization rate were not correlated with copulatory plug weight, a very indirect proxy for ejaculate volume and the number of sperm transferred (Ramm and Stockley 2007). In conspecific matings, median copulatory plug weight was 22.8 mg, compared to heterospecific matings, in which median plug weight

was 33.0 mg (Wilcoxon Rank Sum Test, $P = 0.23$). The results were the same if DOM and MUS males were analyzed separately. It is interesting that males produced a slightly (but not significantly) larger plug when mated to heterospecific females. Although outside the scope of the present study, a similar pattern has also been observed in *Drosophila*, in which heterospecific matings result in larger insemination reactions (Knowles and Markow 2001), presumably due to incompatible biochemical interactions between male and female proteins.

MUS males fertilized faster than DOM males

In conspecific matings, 91% of ova were fertilized by MUS males, compared to 73% of ova by DOM males (FET, $P < 10^{-3}$) (Table 1A). In heterospecific matings, 56% of ova were fertilized by MUS males, compared to 35% of ova by DOM males (FET, $P = 0.31$) (Table 1A). These observations might be explained in part by differences in sperm swimming speed between the two species (see below).

IN VITRO SPERM COMPETITION ASSAYS

The faster conspecific fertilization predicts that conspecific sperm should fertilize faster than heterospecific sperm in a competitive context. Interestingly, this result was not observed; instead, MUS sperm consistently outcompeted DOM sperm in an in vitro context regardless of which female donated ova (Table 2A). For example, when dyed DOM sperm competed against undyed DOM sperm from the same male, 51% of fertilized eggs were sired by dyed sperm. When these same dyed sperm competed against undyed MUS sperm, 23% of ova were fertilized by dyed sperm. Therefore, dyed DOM sperm fertilized 55% fewer DOM ova when in competition with MUS sperm (conspecific precedence = [0.23–0.51]/0.51 = −0.55) (FET, $P < 10^{-11}$). Conversely, dyed MUS sperm fertilized 59% more DOM ova when in competition with DOM undyed sperm (FET, $P < 10^{-7}$). The same pattern was observed with MUS females. Dyed DOM sperm fertilized 48% fewer MUS ova when in competition with undyed MUS sperm (FET, $P < 10^{-9}$), whereas dyed MUS sperm fertilized 47% more MUS ova when in competition with undyed DOM sperm (FET, $P < 10^{-6}$).

The competitive superiority of MUS sperm in an in vitro context was corroborated by competing a MUS male against a DOM$_{WILD}$ male (Table 2B). Dyed MUS sperm fertilized 115% more MUS ova than expected when in competition with undyed DOM$_{WILD}$ sperm (FET, $P < 10^{-17}$, Table 2A).

MUS sperm swim faster than DOM sperm

To explore mechanisms that might explain the competitive advantage of MUS sperm in vitro, we reanalyzed data from Good et al. (2007) and discovered that MUS sperm swim faster than DOM sperm. Good et al. (2007) measured one aspect of sperm motility (the number of live sperm that swim through a defined area within 100 sec) and sperm count (the number of sperm in a heat shocked sample), using the same DOM and MUS genotypes studied here. Dividing motility by sperm count gives a rough approximation of swimming speed. MUS sperm were significantly faster than DOM sperm (MUS median = 6.5 motile sperm/sperm/mL, DOM median = 4.2, $n = 10$ individuals each genotype, Wilcoxon Rank Sum Test, $P = 0.02$). Faster sperm are expected to penetrate both the cumulus mass and the zona pellucida more quickly (Yanagimachi 1969; Stauss et al. 1995; Suarez and Ho 2003), which might explain not only the greater competitive ability of MUS sperm in vitro, but also the higher fertilization ability of MUS males in the noncompetitive matings described above.

Discussion

The evolution of reproductive isolation is of central importance to the study of speciation. Although house mice are a major model system for speciation research, only premating and postzygotic phenotypes of isolation have been investigated. In this study, we demonstrated that fertilization occurs significantly faster in conspecific matings compared to heterospecific matings. This difference in fertilization rate might contribute to reproductive isolation that occurs after mating but before zygotes are formed.

CONSPECIFIC VERSUS HETEROSPECIFIC FERTILIZATION RATE

Differences in reproductive performance pose an important barrier to gene flow between a wide variety of internally fertilizing taxa (Coyne and Orr 2004). In some insects, males transfer more sperm in conspecific versus heterospecific matings, leading to increased reproductive success (Gregory and Howard 1994; Knowles and Markow 2001; Price et al. 2001). When females mate with both conspecific and heterospecific males, the resulting progeny are often biased towards conspecific sires (Hewitt et al. 1989; Gregory and Howard 1994; Robinson et al. 1994; Wade et al. 1994; Price 1997; Howard et al. 1998; Howard 1999; Dixon et al. 2003; Chang 2004; Fricke and Arnqvist 2004; Rugman-Jones and Eady 2007). Faster conspecific fertilization has been observed in many vertebrates (Gray 1958; Lopyrin and Loginova 1963; Gray 1972; Hanada and Chang 1972; Maddock and Dawson 1974; West et al. 1977; Fukuda et al. 1979; Lambert 1984; Roldan et al. 1985; Roldan and Yanagimachi 1989; Slavík et al. 1997; Kouba et al. 2001; Kochhar et al. 2002; Birkhead and Brillard 2007), but never in species pairs known to hybridize in nature. In fact, copulation is mechanically impossible in most vertebrate studies, so the potential for differences in fertilization rate to contribute to reproductive isolation in nature remains unknown.

DOES FASTER CONSPECIFIC FERTILIZATION RATE CONTRIBUTE TO REPRODUCTIVE ISOLATION?

Faster conspecific fertilization rate might reduce gene flow between sympatric species or between parapatric species in areas where they meet. The precise strength of such a barrier will depend on several (currently unknown) features of the mating system.

Differences in fertilization rate would be irrelevant if premating isolation is complete. However, ecological divergence probably does not contribute to current reproductive isolation, as both *M. domesticus* and *M. musculus* are human commensals that occupy seemingly identical habitat types. Similarly, premating isolation is generally very weak, with *M. musculus* females showing slight preferences for odors of conspecific males (Smadja et al. 2004; Ganem et al. 2005; Smadja and Ganem 2005). *M. musculus* females were only ∼4% more likely to engage in sexual behavior with a conspecific versus a heterospecific male, whereas *M. domesticus* females displayed no detectable preference (Smadja and Ganem 2002). Likewise, we show no evidence for premating isolation in the present study. Hence, gene flow does not seem to be strongly inhibited by ecological or behavioral barriers in house mice.

Differences in fertilization rate would not pose a strong barrier to gene flow if females mate with a single male during a single estrus cycle and heterospecific males are capable of fertilizing ova, as observed here. Without sperm competition, slower heterospecific sperm may eventually fertilize an equal number of ova. However, sperm competition is probably very common in mice; in North American and Australian populations of *M. domesticus*, females carry a multiply sired litter in at least 20% of all pregnancies, which represents an underestimate of the true frequency of multiple mating (Dean et al. 2006). It seems likely that multiple mating is also frequent in the European hybrid zone, where females presumably have the opportunity to mate with both conspecific and heterospecific males. All else being equal, faster fertilizing conspecific sperm should gain the majority of fertilizations.

This prediction could be complicated if there were complex interactions among male ejaculates. In *Drosophila*, seminal fluid from one male can incapacitate sperm from another male (Harshman and Prout 1994). However, in order for such complex interactions to counteract the conspecific fertilization advantage observed here, heterospecific seminal fluid would have to incapacitate conspecific sperm in both directions of the cross. This scenario seems unlikely.

In cases of multiple mating, the time between matings may influence paternity (Ginsberg and Huck 1989). In cases in which this time interval is long and the conspecific male is first to mate, the faster conspecific fertilization observed here could result in strong reproductive isolation. In the extreme case, conspecific sperm may fertilize the majority of ova before another male mated, making even the most complex interactions between male ejaculates irrelevant. On the other hand, if the heterospecific male is first to mate, the relatively modest differences in fertilization rate documented here might not lead to substantial reproductive isolation.

The strength of reproductive isolation caused by conspecific fertilization rate advantage will also depend on the precise genetic architecture responsible for these phenotypes. Individuals in the center of the European hybrid zone are often mixtures of *M. domesticus* and *M. musculus* genomes (Macholán et al. 2007). In this scenario, it is difficult to predict faster conspecific fertilization advantage because pure species are not mating. However, due to the narrowness of the hybrid zone, it is possible that individuals from more "pure" parental populations encounter each other.

In sum, the strength of reproductive isolation caused by conspecific fertilization advantage will depend on many unknown parameters. Intuitively, the faster fertilization rate observed in conspecific matings should contribute at least some reproductive isolation as many of the necessary conditions seem to be present. On the other hand, the finding that even heterospecific sperm fertilized a significant portion of oocytes suggests that the barrier may not be strong under some conditions. Further studies, especially of wild mice from geographic regions where both species are found, will help in understanding the relative strength of conspecific fertilization advantage compared to the much better studied forms of postzygotic isolation.

CONTRASTING IN VIVO AND IN VITRO EXPERIMENTS

One striking feature of this study is the difference between the noncompetitive in vivo experiments, where conspecific fertilization occurred more rapidly than heterospecific fertilization in both directions, and the competitive in vitro experiments, where MUS sperm outcompeted DOM sperm regardless of which species donated ova. The in vitro experiments leave out two crucial features of reproductive biology, which could shed light on the mechanism of conspecific fertilization advantage.

First, the internal female environment was excluded from in vitro experiments. Many genes are specifically upregulated in mouse oviducts in response to the presence of sperm (Georgiou et al. 2005), and some of these gene products may act on sperm as a means of cryptic female choice. The female environment has also been shown to affect fertilization outcomes in *Drosophila* (Clark and Begun 1998; Clark et al. 1999, 2000).

Second, male seminal fluid was excluded from in vitro experiments because sperm were isolated directly from the epididymis. In mammals, seminal fluid has several important impacts on reproductive success, including increased pregnancy rate (Pang et al. 1979; Queen et al. 1981; Peitz and Olds-Clarke 1986; Carballada and Esponda 1999), litter size (Pang et al. 1979), developmental rate (Peitz and Olds-Clarke 1986; O et al. 1988), and rate of oocyte

penetration (Henault et al. 1995; Henault and Killian 1996). Some seminal proteins bind to sperm or comigrate with sperm through the uterus (Irwin et al. 1983; Robinson et al. 1987; Carballada and Esponda 1997, 1998) and affect sperm motility (Peitz and Olds-Clarke 1986; Agrawal and Vanha-Perttula 1987; Peitz 1988) and capacitation (Huang et al. 2000; Kawano and Yoshida 2007). These proteins can also suppress the female immune responses toward sperm (Peitz and Bennett 1981; Anderson and Tarter 1982; Thaler 1989). Disruption at any of these levels could potentially underlie the conspecific fertilization advantage.

Conclusions

The study of reproductive isolation requires a full understanding of potential isolating factors operating at all stages of the reproductive process. Here we have documented that conspecific matings result in faster fertilization compared to heterospecific matings, and this difference arises in the postmating, prezygotic arena. Faster conspecific fertilization should lead to at least some form of reproductive isolation among these naturally hybridizing species of house mice, although details of the relevant parameters remain unknown. By contrasting our in vivo mating data with artificial in vitro sperm competition experiments, we suggest that the mechanism of conspecific fertilization advantage lies in the interactions between sperm, the internal female environment, and/or contributions from male seminal fluid. Identification of both female- and male-derived proteins may provide insights into the reproductive biology and reproductive isolation among house mice.

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