

SPERM COMPETITION PROMOTES ASYMMETRIES IN REPRODUCTIVE BARRIERS BETWEEN CLOSELY RELATED SPECIES

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Reproductive barriers between closely related species are often incomplete and asymmetric, but the evolutionary significance of these well-known phenomena remains unsolved. We test the hypothesis that the degree of gametic incompatibility in reciprocal crosses is associated to levels of sperm competition because this selective force favors both increased sperm competitiveness and ovum defensiveness. Using three species of *Mus* with high, intermediate, and low levels of sperm competition, we examined fertilization rates in competitive and noncompetitive contexts. We found that the influence of sperm competition upon sperm competitiveness is as strong as it is upon ovum defensiveness, revealing an effect upon female gametes so far overlooked. As a result, fertilization success was strongly related to differences in sperm competition levels between species providing sperm and ova, thus generating major asymmetries in reciprocal crosses. When placed in competition, conspecific sperm maintained levels of fertilization success similar to those found in noncompetitive contexts, at the expense of the success of heterospecific sperm. When only heterospecific sperm competed, species with highest levels of sperm competition outcompeted others and asymmetries were exacerbated. We conclude that sperm competition explains both the degree of gametic isolation and the degree of asymmetries between closely related species.

KEY WORDS: Fertilization, ova, sperm competition, speciation, spermatozoa.

New species arise when reproductive barriers separate formerly interbreeding populations (Mayr 1942), but little is known about how such barriers originate. Related species rarely show complete reproductive isolation, which is precisely what would be expected if the process of speciation involves acquiring reproductive barriers gradually; when populations begin to diverge they will go through intermediate stages where isolation will be incomplete. However, reproductive barriers are often asymmetrical, a phe-

nomenon that has been reported in many taxa (Yanagimachi 1977; Roldan and Yanagimachi 1989; Yanagimachi 1994; Birkhead and Brillard 2007; reviewed by Coyne and Orr 2004), but which remains largely unexplained (Kaneshiro 1980; Arnold et al. 1996; McCartney and Lessios 2002). The study of closely related species will reveal how incipient reproductive barriers appear, their directionality in the early stages of divergence, and the selective pressures promoting such changes.

Most studies have focused on premating (behavioral) barriers that prevent copulation, and on postzygotic barriers that cause

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hybrid sterility or inviability (Coyne and Orr 2004). Little attention has been paid to barriers that act between copulation and fertilization (also known as “gametic isolation”). When males and females from different species copulate, fertilization success may be low because of poor transfer, storage, or transport of sperm (Gregory and Howard 1994; Price et al. 2001). When gametes interact, failure of fertilization (Palumbi and Metz 1991; Vacquier 1998; Levitan 2002) may be due to differences in proteins present in sperm and ova that are needed for gamete recognition (Palumbi and Metz 1991; Swanson and Vacquier 1998; Vacquier 1998). The fertilization success of heterospecific males is not always low, but decreases considerably when in competition with conspecific gametes (Howard 1999).

Reproductive proteins evolve rapidly in many taxa (Swanson and Vacquier 2002), and have a major influence upon individual reproductive success in natural environments (Levitan and Ferrell 2006). Theoretical models show that sexual selection can promote rapid divergence in reproductive traits and lead to speciation (Gavrilets 2000). When ejaculates from rival males compete to fertilize ova (i.e., sperm competition) there will be strong selection on males to improve ejaculate competitiveness (Birkhead and Pizzari 2002). Females will respond by evolving defensive mechanisms in ova, to counteract the increased risk of polyspermy. Sperm will in turn undergo selection to overcome these defenses, leading to a perpetual arms race in which male gametes evolve to become ever more competitive, and female gametes evolve to become ever more defensive.

If gametic isolation is a byproduct of sexual selection, one major but so far overlooked implication is that the success of heterospecific gamete interactions will be asymmetrical (Birkhead and Brillard 2007). This is because antagonistic coevolution as a result of sperm competition will select for enhanced sperm competitiveness in males and increased ovum defensiveness in females. Thus, the outcome of crosses between a species with high levels of sperm competition and another with low levels of sperm competition will differ depending on the directionality of the cross: high sperm competitiveness \times low ovum defensiveness will lead to high fertilization success, whereas low sperm competitiveness \times high ovum defensiveness will lead to low fertilization success. The main prediction is that the greater the difference between levels of sperm competition for the species supplying the sperm (from now on “levels of sperm competition for sperm,” which is an index of sperm competitiveness) and levels of sperm competition for the species supplying the ova (from now on “levels of sperm competition for ova,” which is an index of ovum defensiveness), the higher the fertilization success. We define “sperm competitiveness” as an increase in fertilization success and “ovum defensiveness” as a decrease in fertilization success.

The aim of this study is to test the hypothesis that fertilization success in reciprocal crosses is the result of the com-

bined effects of levels of sperm competition on sperm and ova. In addition, we also investigate fertilization success in reciprocal crosses when there is competition between species. Previous studies (Gomendio et al. 2006) were carried out to find a suitable model. This is a fundamental issue because the model has to include phylogenetically close species, which nevertheless differ substantially in sperm competition levels. Only in this way could we minimize the already well-known effects of phylogeny and be able to detect the effects of sperm competition in the early stages of divergence. To find a suitable model we carried out background work on 10 species of *Mus*, which covered aspects of male and female reproductive physiology (Gomendio et al. 2006), as well as molecular work to obtain a detailed phylogeny (in this article). Once this was done, we carefully selected the three species that were phylogenetically close and covered the full range of low, intermediate, and high levels of sperm competition. Three species are needed to evaluate (1) the influence of conspecificity versus heterospecificity, (2) the effect of competition between conspecific/heterospecific sperm, and (3) the effect of competition between two different types of heterospecific sperm. We chose not to include more species because it would add noise to the system because a stronger phylogenetic component would be included, and would not provide any new insights because the effects of sperm from more heterospecific species competing at the same time would not have answered any of the questions addressed by this study and would represent a very unrealistic scenario.

Thus, we chose as our model *Mus spicilegus* with high levels of sperm competition, *M. spretus* with intermediate levels, and *M. musculus* with low levels of sperm competition. These species are ideal to study fertilization success because previous work has shown that sperm competition has selected for an increase in the relative number of spermatozoa produced and also in sperm's functional capacity (Gomendio et al. 2006). Thus, in these species sperm competition has favored the evolution of spermatozoa that may perform better at fertilization and this improved ability is likely to increase the risk of polyspermy. We carried out three series of in vitro fertilization experiments controlling for sperm numbers in which we examined: (1) all possible combinations of reciprocal crosses between these three species in the absence of competition between species; (2) sperm from the three species in competition to fertilize ova of each of the three species (conspecific and two types of heterospecific sperm competed for fertilizations); (3) sperm from heterospecific species competing for fertilizations.

Materials and Methods

ANIMALS

Adult males and females of three species of Murid rodents (*Mus musculus musculus* (hereinafter, *M. musculus*), *M. spicilegus*

and *M. spretus*) were purchased from the Institut des Sciences de l'Evolution-Montpellier, CNRS-Université de Montpellier II. These three species were chosen because they show high, intermediate, and low levels of sperm competition (Gomendio et al. 2006). Animals were kept using standard laboratory mouse conditions in an environmentally controlled room with a 14 h light:10 h darkness photoperiod. Mice were weaned when they were 4 weeks old. After weaning, females were housed together whereas males were kept individually. Husbandry conditions followed EU recommendations and Spanish legislation. Animals were provided with food and water, which were available ad libitum. Mice were 12- to 20-week old when they were used for experiments. In total 56 females and 53 males were used in the experiments (*M. musculus*: 16 females, 17 males; *M. spretus*: 11 females, 19 males; *M. spicilegus*: 29 females, 17 males).

OOCYTE COLLECTION AND IN VITRO MATURATION

To obtain a large number of oocytes from each female, females were stimulated following the methodology described in Martín-Coello et al. (2008). Immature oocytes were collected from females of the three species 48 h after intraperitoneal administration of 5 IU PMSG (Martín-Coello et al. 2008). In vitro maturation of oocytes was performed as described (Kawamura et al. 2005) with some modifications (Martín-Coello et al. 2008). Ovaries were dissected and placed in M2 medium (Sigma, Madrid, Spain) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Madrid, Spain). Cumulus-oocytes-complexes (COCs) were released by follicular puncture carried out with the aid of a pair of 30G needles attached to 1 mL syringes under a stereomicroscope. Only immature oocytes surrounded by compact-cumulus cells were selected for culture. After several washes in M2, groups of 20–50 COCs were placed in 4-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) containing the in vitro maturation medium, covered with 250–300 μ l of mineral oil and cultured at 37°C under 5% CO₂ in air with maximum humidity for 17 h. A volume of 500 μ l of Minimum Essential Medium-alpha, supplemented with Earle's salts (MEM-alpha, Gibco, Madrid, Spain), and with the addition of 10 μ g streptomycin sulphate/mL, 75 μ g penicillin G/mL and 5% (v/v) heat-inactivated FBS, was used as maturation medium. Meiosis progression to metaphase II (MII) was confirmed by the presence of a polar body at the end of the incubation period. In total 56 females were used in the experiments, which produced 1001 oocytes; from these, 879 oocytes matured in vitro and were used in several replicates in different experimental series.

EXPERIMENT 1. IN VITRO FERTILIZATION BY CONSPECIFIC OR HETEROSPECIFIC SPERMATOZOA IN THE ABSENCE OF COMPETITION BETWEEN SPECIES

Epididymides were removed from males of the three mouse species (*M. musculus*, *M. spretus*, and *M. spicilegus*) and were placed in 1 mL drops of Human Tubular Fluid medium (HTF) (Quinn et al. 1985; Martín-Coello et al. 2008) under oil and incubated at 37°C under 5% CO₂/air for 10 min to allow sperm dispersion into the medium. After incubation, epididymal tissue was removed and sperm suspensions were incubated at 37°C under 5% CO₂/air for capacitation (Martín-Coello et al. 2008). In vitro fertilization (IVF) was carried out in 500 μ l droplets of HTF medium (Kawamura et al. 2005; Martín-Coello et al. 2008). Oocytes were matured in vitro, assessed for progression to MII, and mature oocytes were coincubated with spermatozoa from each of the three different mouse species. Mature oocytes from two to four females of each species were pooled and divided into three groups, which were placed in HTF medium in different dishes. Each of the three groups of oocytes from the same species was inseminated with a final concentration of 0.8–1 \times 10⁶ sperm/mL of precapacitated spermatozoa from one of the three mouse species (i.e., each of the three groups of oocytes from the same species was inseminated with sperm from one male of each of the three different species in separate trials). For each possible combination (e.g., oocytes species A \times spermatozoa species A, oocytes species A \times spermatozoa species B, oocytes species A \times spermatozoa species C, etc.) the experiments were repeated three times. In each of the three experiments we used a pool of mature oocytes from different females as well as spermatozoa from different males of each species. The total sample size for this experiment consisted of 24 females (*M. musculus* $N = 6$ females that produced 139 mature oocytes, *M. spretus* $N = 6$ females that produced 106 mature oocytes, *M. spicilegus* $N = 12$ females that produced 110 mature oocytes). A larger number of females of *M. spicilegus* was necessary to obtain a similar number of mature oocytes. The number of males was 27 (nine from each species).

Gametes were coincubated for 4 h at 37°C under 5% CO₂/air. This period was enough for sperm to penetrate, fuse with the oolema, decondense, and form a male pronucleus. After this period of incubation, the remaining cumulus cells and attached spermatozoa were removed by washing the oocytes in and out of a fine pipette. After removing cumulus cells and attached sperm, oocytes were washed three times with HTF medium. Finally, oocytes were placed in 150 μ l drops of HTF medium and the fertilization rates were assessed by recording the presence of pronuclei using phase contrast microscopy. Fertilization rate was expressed over the number of mature oocytes coincubated with spermatozoa.

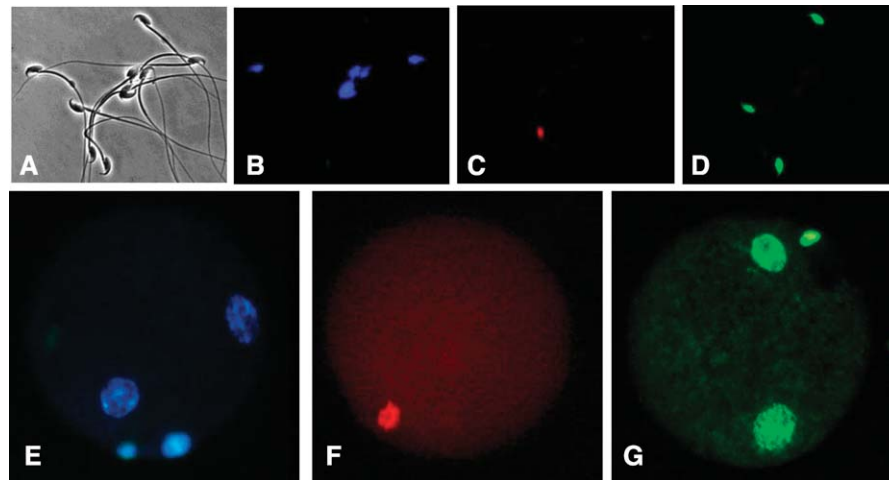


Figure 1. Competitive fertilization in mice (*Mus*). (A–D) Spermatozoa were stained with different DNA fluorochromes (Hoechst 33342, SYTO orange, SYTO green), mixed in equal numbers, and examined using phase contrast optics or fluorescence microscopy with different filter sets (one for each fluorochrome). As can be seen, there is no cross-staining of spermatozoa with the different fluorochromes. (A) phase contrast, (B) Hoechst 33342, (C) SYTO orange, (D) SYTO green. (E–G) Oocytes fertilized by spermatozoa pre-labeled with different fluorochromes. (E) Fertilization by a spermatozoon pre-labeled with Hoechst 33342, (F) Fertilization by a spermatozoon pre-labeled with SYTO orange, (G) Fertilization by a spermatozoon pre-labeled with SYTO green. Note that when sperm cells were stained with SYTO orange only the male pronucleus is stained whereas in the other two cases the female pronucleus is also stained.

EXPERIMENTS 2 AND 3. IN VITRO FERTILIZATION BY CONSPECIFIC AND HETERO SPECIFIC SPERMATOZOA UNDER COMPETITIVE CONDITIONS

To examine the fertilization of oocytes from each mouse species by conspecific or heterospecific spermatozoa in direct competition, sperm cells from each species were differentially stained to identify the species of the fertilizing spermatozoon. To this end, DNA fluorochromes that would stain live cells but that would not alter sperm function were used.

Labeling of spermatozoa with DNA fluorochromes

Spermatozoa were obtained from males of the three species (*M. musculus*, *M. spretus*, and *M. spicilegus*). Epididymides were placed in 1 mL drops of a Hepes-buffered modified Tyrode's medium (mT-H) (Shi and Roldan 1995) under oil and incubated at 37°C for 10 min under air to allow sperm to swim out and disperse in the medium. Spermatozoa were stained with the DNA fluorochromes Hoechst 33342 (Sigma), SYTO 24 green, or SYTO 82 orange (the latter two from Molecular Probes, Invitrogen, Leiden, The Netherlands). A stock solution of 50 µg Hoechst 33342/mL was prepared by dissolving the fluorochrome in PBS without Ca²⁺ or Mg²⁺. Stock solutions of 5 mM SYTO green and SYTO orange in DMSO, as supplied by the manufacturer, were diluted in PBS without Ca²⁺ or Mg²⁺ to a working concentration of 100 µM. Final concentrations used for sperm labeling were: 2.5 µg Hoechst 33342/mL, 1 µM SYTO 24 green, and 15 µM SYTO 82 orange. Spermatozoa were labeled by incubation with fluorochrome during 20 min at 37°C and excess dye was removed by centrifugation

at 300 × g for 8 min. Sperm cells were examined using phase contrast optics and epifluorescence with a mercury excitation beam passed through a 380 nm filter and fluorescence emission with a DM 400 dichroic mirror (Hoechst 33342), a 490 nm filter and fluorescence emission with a DM 505 dichroic mirror (SYTO green), and a 560 nm filter and fluorescence emission with a DM 575 dichroic mirror (SYTO orange).

These fluorochrome concentrations and staining conditions allowed for an intense staining of spermatozoa (Fig. 1). No cross-staining of spermatozoa occurred when sperm suspensions pre-labeled with different fluorochromes were mixed (Fig. 1). We also verified that spermatozoa retained the fluorochrome for several hours and that it did not leak out of the sperm cells. Staining with the different fluorochromes, or exposure of spermatozoa to solvent (DMSO), at the concentrations and staining conditions described above, did not have any effect on sperm viability as assessed by sperm survival during incubation at 37°C. No differences were found in percentages of motile spermatozoa at different times of incubation for the different fluorochromes and in the three mouse species. Moreover, the staining procedure did not affect the ability of spermatozoa to interact with oocytes at fertilization.

In vitro fertilization under competitive conditions

Epididymides were removed from males of three mouse species (*M. musculus*, *M. spretus*, and *M. spicilegus*) and placed in different 1 mL drops of mT-H under oil and incubated at 37°C for 10 min under air to allow sperm dispersion. Epididymal tissue was then removed and spermatozoa from each species were stained

with 2.5 μg Hoechst 33342/mL; 1 μM SYTO 24 green, or 15 μM SYTO 82 orange by incubation with the fluorochrome for 20 min at 37°C. Excess dye was removed by centrifugation at 300 $\times g$ for 8 min and resuspension of spermatozoa in HTF medium. Spermatozoa were then capacitated at 37°C under 5% CO_2 /air as previously described (Martín-Coello et al. 2008). IVF was carried out in 500 μl droplets of HTF medium (Kawamura et al. 2005; Martín-Coello et al. 2008). In vitro matured oocytes from each species (collected and incubated as described above) were placed in HTF medium. As in the previous experiment, oocytes from two to four females of each one of the three species were pooled. In one experimental series (Experiment 2) each pool of oocytes from the same species was independently coincubated with a mixture of prelabeled spermatozoa from the three species (*M. musculus*, *M. spretus*, and *M. spicilegus*); final concentration for each species was 0.8–1 $\times 10^6$ spermatozoa/mL. This experimental procedure was repeated twice for each possible combination of species using different males and females. The sample size for experiment 2 was 20 females and 18 males (*M. musculus*: 5 females (124 mature oocytes), 6 males; *M. spretus*: 5 females (115 mature oocytes) and 6 males, *M. spicilegus*: 10 females (106 mature oocytes) and 6 males)). In another experimental series (Experiment 3) oocytes from either *M. musculus* or *M. spicilegus* were inseminated with a mixture of prelabeled heterospecific spermatozoa (final concentration of 0.8–1 $\times 10^6$ spermatozoa/mL for each species). The same experimental design as in experiment 2 was followed. In this experiment the sample size consisted of 12 females and 8 males (*M. musculus*: 5 females (104 mature oocytes), and 2 males; *M. spretus*: 4 males, *M. spicilegus*: 7 females (75 mature oocytes) and 2 males).

In both experiments, gametes were coincubated for 4 h at 37°C under 5% CO_2 /air. At the end of incubation, cumulus cells and attached sperm were removed by washing the oocytes in and out of a fine pipette. Then, oocytes were washed three times with HTF medium. Finally, oocytes were placed in 150 μl drops of HTF medium and fertilization rates were assessed based on the presence of stained pronuclei (Fig. 1) as visualized at 400 \times magnification using phase contrast optics and epifluorescence with settings described above. Fertilization rates for each species were expressed in relation to the number of mature oocytes, and also in relation to the number of oocytes fertilized.

BIOINFORMATICS AND STATISTICAL ANALYSES

Experiment 1. Fertilization by conspecific or heterospecific spermatozoa in the absence of competition between species

Test 1. We tested with General Linear Models (GLM) if sperm competition level of donor species explained fertilization success in heterospecific crosses, controlling by phylogeny and the number of oocytes used. We calculated an index of phylogenetic

similarity between each pair of species as one minus the phylogenetic distance between the two species. Conspecific crosses were given a score of one. Genetic distances were obtained using gene sequences available in GenBank for seven species of *Mus* and including *Apodemus sylvaticus* as an outgroup; mean pairwise genetic distances were obtained using the MEGA 4.0 program (Tamura et al. 2007) (Table 1). Relative testes size has been shown to be a reliable indicator of levels of sperm competition in this group of species (Gomendio et al. 2006). We used residual values of the rodent regression equation of Kenagy and Trombulak (Kenagy and Trombulak 1986), expected testis mass = 0.031 \times (body mass)^{0.77}, to obtain relative testes size. Thus, we calculated the relative testes size for both the species providing spermatozoa and the species providing ova. Because fertilization rates were obtained using different number of oocytes in each experiment, we also included this variable in the analysis.

To test for the role of sperm competition, we predicted that fertilization rate should be proportional to the difference between the level of sperm competition for spermatozoa (an index of sperm competitiveness) and the level of sperm competition for ova (an index of ovum defensiveness). Thus, we tested with GLMs whether phylogeny, differences in levels of sperm competition, and the number of oocytes used explained fertilization rates in noncompetitive contexts. For each cross we calculated the difference between relative testes size for the species providing spermatozoa and relative testes size for the species providing ova. We performed this analysis both with ($n = 27$) and without conspecific crosses ($n = 18$).

In all GLMs, we standardized the data and partitioned the variance to quantify for each predictor the explained variance, and to separate the real effect of predictors from the effect of interactions between predictors. *P*-values were obtained, randomizing 1000 times, using restricted permutations and permuting exchangeable units (Anderson and Braak 2003; Gotelli and Ellison 2004).

Test 2. We tested significance of the Spearman correlation using Monte Carlo (Gotelli and Ellison 2004) and randomizing 1000 times.

Experiment 2. Fertilization by conspecific and heterospecific spermatozoa under competitive conditions

Test 3. Our null hypothesis was that the fertilization rate of conspecific sperm is the same with (Experiment 2) or without heterospecific competitors (Experiment 1). To test this, we used Pearson chi-square (Anderson and Braak 2003), $\chi^2_{obs} = \Sigma [(observed - expected)^2/expected]$. The observed value was the total number of oocytes fertilized by each conspecific sperm in a competitive context (Experiment 2), and the expected value was calculated as the product of the total number of oocytes used for each cross and

Table 1. Phylogenetic distances in rodents. Mean pairwise genetic distances and their standard error values (lower and upper triangle, respectively) among eight species of rodents (genera *Mus* and *Apodemus*). Distances represent the mean number of nucleotide substitution per site according to the TN93+gamma model. SE were computed using 1000 bootstrap pseudoreplications. Species and data marked with an asterisk were used in this study. Sequences used for analyses, downloaded from GenBank, were as follows: *M. musculus*, AB033711, AY260481, AY260493, DQ021492, AY160024, AY160011, AY159989, AY215075, AF039064, U89287, AY490639, AY057791, U70654, AY057804, X61217, AY057800, AY057746, AY057761, SY057776; *M. macedonicus*, AB125805, AY260490, AY260503, DQ021496, AY160013, AY160003, AB125829, AF413622, U89290, AY490627, AY057794, L29547, AY057808, X61213, L05899, AY057750, AY057765, AY057780; *M. spicilegus*, AJ698882, AY260483, AY260495, DQ021494, AY160014, AY160004, AY159980, AB125835, AF413621, U89291, AY490632, AY057795, L29550, AY057809, X61216, L05578, AY057751, AY057766, AY057781; *M. spretus*, AB033708, AY260485, AY260497, DQ021493, AY160015, AY160002, AY159978, AB125836, AF413620, U89289, AY490631, AY057796, L29544, AY057810, X61215, M84362, AY057752, AY057767, AY057782; *M. famulus*, AJ698884, AF413623, AY490634, AJ279442, AJ698872; *M. cookii*, AB125802, AY260486, AY260498, DQ021498, AY160016, AY160000, AY159976, AB125828, AF413625, U89284, X85946, L29549, AY05813, AY057822, L05581, AY057755, AY057770, AY057785; *M. pahari*, AB096855, AY260488, AY260500, DQ021500, AB125844, X84383, L29543, AY057814, AY057821, L05579, AY057756, AY057771, AY057786; and *A. sylvaticus*, AB032863, AB164041, AF445762, AY490614, AJ311126, AF159395.

	<i>M. musculus*</i>	<i>M. macedonicus</i>	<i>M. spicilegus*</i>	<i>M. spretus*</i>	<i>M. cookii</i>	<i>M. famulus</i>	<i>M. pahari</i>	<i>A. sylvaticus</i>
<i>M. musculus</i>		0.0016	0.0018	0.0017	0.0024	0.0044	0.0032	0.0079
<i>M. macedonicus</i>	0.0175		0.0008	0.0013	0.0020	0.0032	0.0036	0.0067
<i>M. spicilegus</i>	0.0176*	0.0087		0.0013	0.0019	0.0036	0.0032	0.0065
<i>M. spretus</i>	0.0212*	0.0185	0.0189*		0.0023	0.0049	0.0037	0.0068
<i>M. cookii</i>	0.0396	0.0392	0.0384	0.0387		0.0061	0.0038	0.0074
<i>M. famulus</i>	0.0564	0.0541	0.0538	0.0573	0.0745		0.0087	0.0076
<i>M. pahari</i>	0.0744	0.0696	0.0689	0.0727	0.0653	0.1093		0.0082
<i>A. sylvaticus</i>	0.1386	0.1214	0.1226	0.1276	0.1268	0.1355	0.1112	

the fertilization rates of each conspecific sperm obtained in the first experiment. Significance of the χ^2_{obs} statistic corresponded to its relative position in a frequency histogram of simulated χ^2_{sim} . For each repeated cross, we simulated the number of ova fertilized by each conspecific sperm and calculated $\chi^2_{sim} = \sum [(simulated - expected)^2 / expected]$. To simulate the number of fertilized ova in each cross, we randomized for each oocyte the success of being fertilized with the fertilization rate of the corresponding conspecific sperm obtained in the first experiment. Therefore, simulated chi-square will correspond to deviation from the expected success due to chance. We repeated this step 1000 times to obtain a frequency distribution of 1000 χ^2_{sim} . We also estimated the power of this test ($1 - \beta$) calculating the probability of committing Type II error (β), i.e., to falsely accept the null hypothesis. To this end, we randomized the total number of oocytes fertilized by each conspecific sperm in each repeated cross using random fertilization rates for each conspecific sperm, and we tested the significance with the above test, were $\chi^2_{random} = \sum [(random - expected)^2 / expected]$. This was repeated 100 times. The power of the test was 0.99.

Test 4. Asymmetry was the difference between the fertilization rate of the cross with sperm species A \times ova species B and its reciprocal (sperm species B \times ova species A). To test if asymmetries of heterospecific crosses differ between competitive (Experiment 2) and noncompetitive (Experiment 1) contexts we used Pearson chi-square statistic (Pearson 1900), described for Test

3. Observed values were asymmetries observed in competitive contexts (Experiment 2), and expected values were asymmetries found in the first experiment, after correcting fertilization rates to maintain the observed ratio of success between competitors (both conspecific and heterospecific sperm) (e.g., adjusted fertilization rate of *M. spretus* sperm against *M. musculus* ova from Table 1 was = $67.4 / (67.4 + 85.4 + 75.6) = 0.3$). Significance was tested as described for Test 3. To simulate asymmetries, we randomized the number of fertilized oocytes in each cross with the adjusted fertilization rate of each spermatozoa. We estimated the power of the test creating random data with random fertilization rates for each sperm. The power of the test was 0.96 (see Test 3 for further details).

Test 5. We used GLM to test if the success of conspecific sperm, when in competition with heterospecific sperm, increases with increasing levels of sperm competition for the species. As the number of oocytes used varied among crosses, we included this factor in the analysis. We standardized data and partitioned the variance as in Test 1. *P*-values were obtained using Monte Carlo (Gotelli and Ellison 2004), randomizing 1000 times ($n = 7$).

Experiment 3. Fertilization by heterospecific spermatozoa under competitive conditions

Test 6. We tested if sperm from the species with higher levels of sperm competition has more success fertilizing ova than the other

heterospecific sperm. We applied a replicated goodness-of-fit test (G-Statistic) (Sokal and Rohlf 1995). The expected values for a noncompetitive context were the fertilization rates of Experiment 1.

Test 7. To test if the fertilization rate of heterospecific spermatozoa differs depending on the presence or absence of conspecific sperm (Experiments 2 and 3, respectively), we calculated the following statistic based on Pearson chi-square statistic (Pearson 1900), $\chi_{\text{obs}} = \sum [(\text{observed} - \text{expected})/\text{expected}]$. Observed values were the number of oocytes fertilized by each heterospecific spermatozoa in the presence of conspecific (Experiment 2) and, expected values were calculated as the product of the number of oocytes used and the fertilization rate of heterospecifics observed in crosses with no conspecific spermatozoa (Experiment 3). Significance of χ_{obs} statistic was tested as described for Test 3. To simulate the number of oocytes fertilized by each heterospecific sperm in the presence of conspecific sperm in each cross, we randomized for each oocyte the success of being fertilized by each heterospecific sperm with the fertilization rates observed in crosses with no conspecific sperm. The power of the test was 0.99 (see Test 3 for further details).

Test 8. To test if asymmetry of the heterospecific cross *M. musculus* \times *M. spicilegus* differs in competitive (Experiment 3) and noncompetitive (Experiment 1) contexts, we used the statistic described in Test 7. Observed values were the asymmetry observed in competitive context (Experiment 3, Table 3) and expected values were the asymmetry found in Experiment 1 (Table 1) after correcting fertilization rates to maintain the observed ratio of success between competitors. Significance was tested as described for Test 3. To simulate asymmetries, we randomized the number of fertilized oocytes with these adjusted fertilizations rates. The power of the test was 0.99 (see Test 3 for further details).

Statistical analyses and randomizations of tests described above were written in Matlab language.

Results

EXPERIMENT 1. FERTILIZATION BY CONSPECIFIC OR HETEROSPECIFIC SPERM IN THE ABSENCE OF COMPETITION BETWEEN SPECIES

Oocytes from *M. musculus*, *M. spretus*, and *M. spicilegus* were coincubated with spermatozoa from these species in either conspecific or heterospecific reciprocal crosses. Fertilization success in conspecific crosses was high in all species (Table 2). In heterospecific crosses, sperm from species with higher levels of sperm competition achieved higher fertilization success (i.e., higher “sperm competitiveness”) (R^2 , sperm competition level of sperm donor species: $R^2 = 0.531$, $P = 0.001$; phylogeny: $R^2 = 0.067$, $P = 0.280$). On the other hand, ova from species with higher levels of sperm competition had lower fertilization success in het-

Table 2. Fertilization by conspecific or heterospecific spermatozoa in the absence of competition between species. Oocytes from *Mus musculus*, *M. spretus*, or *M. spicilegus* were matured in vitro and were subsequently coincubated in vitro with either conspecific spermatozoa or with each of the heterospecific spermatozoa. Sample size: $N=27$ males, $N=24$ females (*M. musculus*: nine males and six females that produced 139 mature oocytes, *M. spretus*: nine males and six females that produced 106 mature oocytes, and *M. spicilegus*: nine males and 12 females that produced 110 mature oocytes). Results show percentages of fertilization (No. of fertilized oocytes/No. of mature oocytes \times 100) for each combination of gametes. In parenthesis, levels of sperm competition (SC) of the different species are given.

Oocytes of	Percentage of fertilization with spermatozoa of		
	<i>M. musculus</i> (low SC)	<i>M. spretus</i> (medium SC)	<i>M. spicilegus</i> (high SC)
<i>M. musculus</i> (low SC)	85.4%	67.4%	75.6%
<i>M. spretus</i> (medium SC)	45.7%	81.1%	58.8%
<i>M. spicilegus</i> (high SC)	32.3%	44.7%	84.2%

erospecific crosses (i.e., higher “ovum defensiveness”) (sperm competition level of ovum donor species: $R^2 = 0.549$, $P = 0.001$; phylogeny: $R^2 = 0.027$, $P = 0.376$). Interestingly, the influence of levels of sperm competition upon fertilization success rates for sperm (“competitiveness”) and ova (“defensiveness”) showed similar levels (variance explained by sperm competition level of donor species was 53.1% for sperm and 54.9% for ova). Thus, fertilization success in heterospecific crosses is the result of the combined effect of sperm competition levels upon both sperm and ova. Because sperm competition increases sperm competitiveness and ovum defensiveness to a similar extent, fertilization success is probably best explained as the difference in sperm competition levels between the species providing the sperm and the species providing the ova. As shown in Table 2, the success of “high sperm competition sperm” with “low sperm competition ova” was nearly as high as that seen in conspecific crosses. In all other combinations, fertilization success decreased as the difference between the level of sperm competition for male and female gametes became smaller. Asymmetries in fertilization success were greatest when sperm and ova from the highest and lowest sperm competition species were tested in reciprocal crosses, but were also present in all other cases. We tested whether phylogenetic distance and levels of sperm competition considered jointly explain the results obtained (Test 1 in Material and Methods) (Table 3). Phylogeny explained 52.3% of the variance ($P = 0.001$), whereas the difference between sperm competition levels for sperm and

Table 3. Influence of sperm competition, phylogeny, and number of oocytes on fertilization rates in noncompetitive contexts (GLM). (A) Including conspecific crosses. (B) Excluding conspecific crosses. SS is the sum of squares. Explained variance (% Variance) presents the real effect of predictors without any effect of interactions among predictors. *P* values were calculated with restricted permutations and permutating the exchangeable units.

	SS	Percent Variance	<i>P</i>
(A) Including conspecific crosses			
Sperm competition (difference between sperm and ova)	8.10	31.1	0.003
Phylogeny	13.61	52.3	0.001
Number of oocytes	0.14	0.5	0.713
error	0.96		
(B) Excluding conspecific crosses			
Sperm competition (difference between sperm and ova)	7.59	69.0	0.001
Phylogeny	0.06	0.6	0.762
Number of oocytes	0.07	0.7	0.756
error	0.26		

ova explained 31.1% of the variance (*P* = 0.001) (Table 3A). However, when conspecific crosses were excluded, phylogeny only accounted for 0.6% of the variance (*P* = 0.762), whereas the difference between sperm competition levels for sperm and ova explained 69.0% of the variance (*P* = 0.001) (Table 3B). In heterospecific crosses, fertilization rate was strongly correlated with the difference between sperm competition levels for sperm and ova (*r* = 0.98, *P* = 0.001, *n* = 18, Test 2 in Material and Methods) (Fig. 2).

EXPERIMENT 2. FERTILIZATION BY CONSPECIFIC AND HETERO SPECIFIC SPERM UNDER COMPETITIVE CONDITIONS

Oocytes from *M. musculus*, *M. spretus*, and *M. spicilegus* were each coincubated with a mixture of equal numbers of spermatozoa from the three species. To identify the species of the fertilizing sperm, DNA fluorochromes that stain live cells were used to prelabel spermatozoa (Fig. 1). Staining with these DNA fluorochromes did not alter sperm performance (see Materials and Methods).

When oocytes from each species were coincubated with a mixture of equal numbers of spermatozoa from the three mouse species, we found that conspecific sperm outcompeted heterospecific sperm, and no decrease in the fertilization success of conspecific sperm was observed as a consequence of such competition ($\chi^2_{obs} = 0.73$, *P* = 0.232, Test 3 in Material and Methods) (Table 4A). Conspecific sperm precedence resulted in decreased fertilization success for heterospecific sperm when compared to noncompetitive contexts. The success of heterospecific sperm dropped to zero values for sperm of low competitiveness against ova of

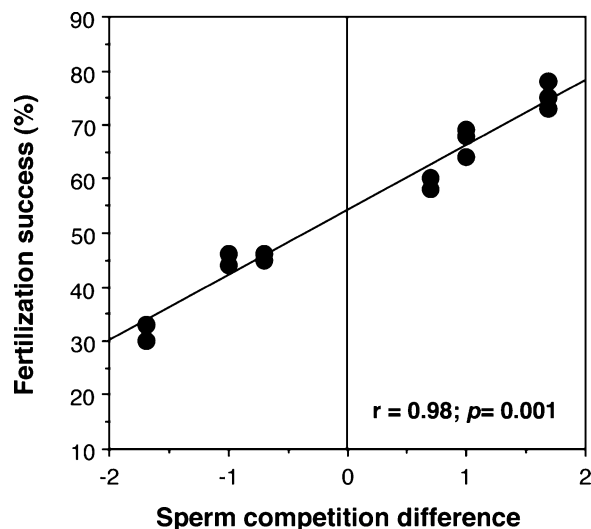


Figure 2. Sperm competition among donor species and fertilization success. Relationship between fertilization rates and differences in sperm competition levels (“sperm competition difference”) between *Mus* species supplying sperm and ova (*r* = 0.98, *P* = 0.001). Fertilization success was examined in the absence of competition between sperm from different species.

intermediate and high defensiveness, and sperm of intermediate competitiveness against ova of high defensiveness. The asymmetries of heterospecific crosses did not differ from those found in a noncompetitive context ($\chi^2_{obs} = 0.02$, *P* = 0.934, Test 4 in Material and Methods). Thus, heterospecific sperm lost out when in competition with conspecific sperm, but the rules explaining fertilization success remained the same (i.e., fertilization success was the result of the difference between levels of sperm competition for sperm and levels of sperm competition for ova). When we considered only the proportion of ova that were fertilized, the success of conspecific sperm increased with increasing levels of sperm competition (*R*² = 0.478, *P* = 0.029, Test 5 in Material and Methods) (Table 4A).

EXPERIMENT 3. FERTILIZATION BY HETERO SPECIFIC SPERM UNDER COMPETITIVE CONDITIONS

Oocytes from *M. musculus* and *M. spicilegus* (the species with low and high levels of sperm competition) were each coincubated with a mixture of equal numbers of heterospecific spermatozoa (i.e., *M. spretus* plus *M. spicilegus*, and *M. musculus* plus *M. spretus*, respectively). To identify the species of the fertilizing sperm, DNA fluorochromes were used to prelabel spermatozoa.

Results showed (Table 4B) that, for oocytes of the two species, spermatozoa from the species with higher levels of sperm competition outcompeted the other heterospecific species. In addition, the difference in fertilization success between the two heterospecific species was higher than that found in noncompetitive contexts (Experiment 1). This is true for both ova with

Table 4. Fertilization with competition between species. (A) Fertilization by conspecific and heterospecific spermatozoa under competitive conditions. In vitro matured oocytes from *Mus musculus*, *M. spretus*, or *M. spicilegus* were each coincubated with a mix of equal numbers of spermatozoa from the three species. Spermatozoa from each species were prelabeled with Hoechst 33342, SYTO green, or SYTO orange to identify the fertilizing spermatozoon. Two independent experiments were performed for each possible combination using different males and females. Sample size: $N=18$ males, $N=20$ females (*M. musculus*: 6 males and 5 females that produced 124 mature oocytes, *M. spretus*: 6 males and 5 females that produced 115 mature oocytes, *M. spicilegus*: 6 males and 10 females that produced 106 mature oocytes). (B) Fertilization by heterospecific spermatozoa under competitive conditions. Oocytes from *Mus musculus* were incubated with a mix of spermatozoa from *M. spretus* and *M. spicilegus*, whereas oocytes from *M. spicilegus* were incubated with a mix of spermatozoa from *M. musculus* and *M. spretus*. Spermatozoa from each species were prelabeled with different fluorochromes to identify the fertilizing spermatozoon. Two separate experiments were performed for each combination of species using oocytes and spermatozoa from different males and females. Sample size: $N=8$ males, $N=12$ females (*M. musculus*: 2 males and 5 females that produced 104 mature oocytes; *M. spretus*: 4 males; *M. spicilegus*: 2 males and 7 females that produced 75 mature oocytes). Results show actual percentages of fertilization (No. of fertilized oocytes/No. of mature oocytes $\times 100$). In brackets, percentages of oocytes fertilized by spermatozoa from each species in relation to the total number of fertilized oocytes. In parentheses, levels of sperm competition (SC) of the different species are given.

Oocytes of	Percentage of fertilization with spermatozoa of		
	<i>M. musculus</i> (low SC)	<i>M. spretus</i> (medium SC)	<i>M. spicilegus</i> (high SC)
(A)			
<i>M. musculus</i> (low SC)	79.0% [83.0%]	4.8% [5.1%]	11.3% [11.9%]
<i>M. spretus</i> (medium SC)	0% [97.0%]	86.1% [3.0%]	2.6%
<i>M. spicilegus</i> (high SC)	0%	0%	83.9% [100%]
(B)			
<i>M. musculus</i> (low SC)	-	18.3% [22.1%]	64.4% [77.9%]
<i>M. spicilegus</i> (high SC)	9.3% [20.6%]	36.0% [79.4%]	-

low defensiveness ($Gp_1 = 23.18$, $P = 0.001$) and with high defensiveness ($Gp_1 = 6.16$, $P = 0.013$, Test 6 in Material and Methods), and may be so because competition between heterospecific sperm lowers the success of the least competitive species below values obtained in the absence of competition. As expected,

the success of the most competitive species remained similar to that found in noncompetitive contexts. In addition, the success of heterospecific sperm was higher in the absence of conspecific sperm (Experiment 2 vs. Experiment 3) ($\chi_{\text{obs}} = -3.54$, $P = 0.001$, Test 7 in Material and Methods). Finally, when comparing the degree of asymmetry in reciprocal crosses between the species with high levels of sperm competition and the species with low levels, we found that it was higher in the competitive context (Experiment 3) than in the noncompetitive context (Experiment 1) ($\chi_{\text{obs}} = 1.21$, $P = 0.001$, Test 8 in Material and Methods).

Discussion

This study examined fertilization success in crosses between closely related species of *Mus* to investigate if the degree of gametic incompatibility and of asymmetries in reciprocal crosses is the result of levels of sperm competition in species providing sperm and ova. Our results show that the influence that sperm competition has in increasing the fertilization success of sperm (increased sperm competitiveness) is as strong as its influence in decreasing the fertilization success of ova (increased ovum defensiveness). As a result of the contrasting effects of sperm competition upon sperm and ova, fertilization success in heterospecific crosses is the result of the difference between levels of sperm competition for males (an index of sperm competitiveness) and levels of sperm competition for females (an index of ovum defensiveness) supplying the gametes. This leads to asymmetries in all reciprocal crosses, which are greatest in crosses between the species with highest and lowest levels of sperm competition.

Sperm from species with high levels of sperm competition are more effective fertilizers than sperm from species with low levels of sperm competition, but even so, the coevolution of sperm-ovum interactions within a species gives an advantage to conspecific sperm, regardless of the level of sperm competition within a species. It is likely that such mechanisms are unrelated to traits that make sperm competitive, or ova defensive, and instead are related to processes that facilitate rapid sperm-ovum recognition and fusion.

In competitive contexts, conspecific sperm outcompeted heterospecific sperm that, as a consequence, lowered their success. In addition, conspecific sperm were relatively more successful in species with higher levels of sperm competition, so females from these species were less likely to be fertilized by heterospecific sperm. Fertilization success in heterospecific crosses also depended on whether sperm were competitive enough to overcome female defenses. Finally, when heterospecific sperm competed in the absence of conspecific sperm, the species with highest levels of sperm competition achieved a success rate similar to that found in noncompetitive contexts, and the success of the less competitive one dropped. Thus, sperm competition between

heterospecific sperm exacerbated the asymmetries in reproductive barriers.

Our findings support the prediction that increased levels of sperm competition favor an increase in sperm competitiveness and in ovum defensiveness, which has important implications for the degree and directionality of reproductive barriers. Thus, increases in sperm competitive ability favored by sexual selection swiftly lead to a defensive response in ova, suggesting that the increased risk of polyspermy is a considerable cost to females. Most work has focused on the effects of post-copulatory sexual selection upon ejaculate competitiveness, showing that it favors an increase in sperm numbers and in the proportion of viable sperm, as well as changes in sperm morphology and dimensions that enhance sperm swimming velocity (reviewed by Birkhead and Pizzari 2002; Gomendio et al. 2007; Gomendio and Roldan 2008). Recent work has shown that competition between rival ejaculates continues at the site of fertilization, affecting the functional capacity of spermatozoa (Gomendio et al. 2006). In contrast, no work so far has focused on the effects of sperm competition levels upon ovum defensiveness, so little is known about the specific traits involved. Our findings suggest that ovum traits involved in “defensiveness” are also under strong selection, and therefore should be detected at the incipient stages of speciation. Because the influence of sexual selection upon ovum traits has not been envisaged before, this issue has so far been ignored. Understanding ovum defensiveness should be a major task for future work, because it will improve our understanding of reproductive barriers between species, and should be taken into account in studies investigating sperm–ovum interactions.

An unforeseen consequence of the contrasting effects of sperm competition upon sperm and ova, is that among species with high levels of sperm competition, males produce sperm that are competitive enough to overcome the ovum barriers of closely related species, whereas females produce ova that are defensive enough to avoid fertilization by closely related species. Thus, sperm competition does not merely promote divergence in reproductive traits, as theoretical models predict, but rather enhances the ability of sperm to fertilize heterospecific females, while at the same time decreases the risk of heterospecific fertilization for females. These findings support the prediction that during the early stages of speciation increased male competitiveness will act as a major catalyst to speciation by increasing selection on female resistance (Parker and Partridge 1998). Thus, females will act as a force favoring reproductive isolation, and males as a force against it.

Studies on marine invertebrates have also shown that sperm density has an influence on both male and female gametes. Thus, among external fertilizers sperm-limited species have eggs that are easily fertilized and long-lived sperm, whereas species living under conditions of sperm competition have small and “sperm-

resistant” eggs and fast competitive sperm (Levitan 2002). Furthermore, these gamete traits selected for best performance under different environments are associated with susceptibility to heterospecific fertilization, so that in sperm-limited environments in which eggs are easily fertilized they are also more likely to be fertilized by heterospecific sperm (Levitan 2002). Finally, species that produce easily-fertilized eggs are more susceptible to polyspermy, whereas species producing eggs that require higher concentrations of sperm to be fertilized are more resistant to polyspermy (Levitan et al. 2007). Thus, the effects of sperm competition on male and female gamete traits that have consequences for the likelihood of heterospecific fertilization seem to affect taxa with very different mating strategies (internal and external fertilizers) suggesting that it may be a widespread phenomenon. In addition, the costs of polyspermy in relation to levels of egg “defensiveness” have already been shown. These studies, however, have not systematically addressed the issue of asymmetries in reciprocal crosses.

Asymmetries in gametic isolation have been found in taxa as diverse as sea urchins (McCartney and Lessios 2002) and mammals (Yanagimachi 1977, 1994; Roldan and Yanagimachi 1989). Hybridization experiments often report asymmetries both in birds (Birkhead and Brillard 2007) and in mammals (Roldan and Yanagimachi 1989), which could also be the result of asymmetries in fertilization success. Because sperm competition has been found to be an ubiquitous and powerful selective force, it may have played a major role in creating asymmetric gametic isolation during speciation in a wide range of taxa. The best-known examples of asymmetries in reproductive isolation come from studies at the premating (behavioral) level (Kaneshiro 1980; Arnold et al. 1996; Coyne and Orr 2004). Whether sperm competition can also explain behavioral asymmetries in mating preferences deserves future study.

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