

# Infertile Matings and Sperm Competition: The Effect of “Nonsperm Representation” on Intraspecific Variation in Sperm Precedence Patterns

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**ABSTRACT:** In theoretical and experimental approaches to the study of sperm competition, it is often assumed that ejaculates always contain enough sperm of good quality and that they are successfully transferred and used for fertilization. However, this view neglects the potential effects of infertility and sperm limitation. Permanent or temporal male infertility due to male sterility, insemination failures, or failures to fertilize the ova implies that some males do not achieve sperm representation in the female reproductive tract after mating. A review of the literature suggests that rates of nonsperm representation may be high; values for the proportion of infertile matings across 30 insect species vary between 0% and 63%, with the median being 22%. I simulated  $P_2$  (the proportion of offspring fathered by the second male to copulate with a female in a double-mating trial) distributions under a mechanism of random sperm mixing when sample sizes and rates of male infertility varied. The results show that nonsperm representation can be responsible for high intraspecific variance in sperm precedence patterns and that it can generate misleading interpretations about the mechanism of sperm competition. Nonsperm representation might be a common obstacle in the studies of sperm competition and postcopulatory female choice.

**Keywords:** infertility, sperm competition, sperm precedence, nonsperm representation, cryptic female choice, sperm limitation.

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Sperm competition, when sperm from more than one male compete to fertilize available eggs from a single female (Parker 1970), has been a major selective force shaping

many aspects of sexual reproduction, including mate guarding, frequency and duration of copulation, genitalia morphology, testes size, sperm numbers, ejaculate quality, and sperm size (Smith 1984; Birkhead and Møller 1998; Simmons 2001). A strong theoretical basis for analyzing sperm competition has been developed over recent years (e.g., Parker 1990a, 1990b, 1998; Parker et al. 1997; Ball and Parker 1998; Mesterton-Gibbons 1999b), and empirical studies have generally supported the predictions generated by theoretical models (reviewed in Smith 1984; Birkhead and Møller 1998; Simmons 2001).

Patterns of sperm use are typically inferred from the mean specific  $P_2$  value (the mean of the proportions of eggs fertilized by the second male in a double-mating trial; Boorman and Parker 1976). Intermediate values are usually taken as indicative of sperm mixing, while extreme values are assumed to be the result of mechanisms for obtaining sperm precedence such as sperm stratification, sperm removal, or sperm displacement by sperm flushing (Birkhead and Møller 1992; Simmons and Siva-Jothy 1998; Simmons 2001). More recently it has been acknowledged that intraspecific variation in the proportion of offspring fathered by the last male to copulate with a female provides great insight into the mechanisms involved in sperm use (Lewis and Austad 1990; Cook et al. 1997; Simmons and Siva-Jothy 1998; Harvey and Parker 2000; Simmons 2001). In insects, for example, almost all species show moderate to high variability in  $P_2$ , with the intraspecific variance in  $P_2$  being as high as the interspecific variance (Simmons 2001). Extreme values of  $P_2$ , including 0 and 1 (complete first-male or last-male sperm precedence, respectively), have been found in 35 of 80 species for which the range of  $P_2$  values is known (Simmons 2001). The order Lepidoptera is a special case because a large number of species present bimodal distributions with the two modes occurring at these extreme values (e.g., Retnakaran 1974; LaMunyon and Eisner 1993a; Svård and McNeil 1994; Cook et al. 1997).

Intraspecific variation has usually been regarded as the

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result of male effects, of female effects that include male  $\times$  female interactions, or of the outcome of sexual conflict (Parker 1984; Simmons and Parker 1992; Sakaluk and Eggert 1996; Stockley 1997; Birkhead and Møller 1998; Simmons and Siva-Jothy 1998; Clark et al. 1999; Miller and Pitnick 2002; Nilsson et al. 2003). However, in a great number of species, the variance around  $P_2$  values is not explained in a satisfactory way, and phenotypic variables usually explain little of the total variation. Only two studies have attempted to predict the expected  $P_2$  variance taking into account random effects related to the mechanism of sperm competition. Cook et al. (1997) suggested that variation in the numbers of sperm delivered during mating by the competing males can be used to predict the expected variance around the mean value of  $P_2$ . In addition, Harvey and Parker (2000) showed that random effects relating to the degree of sperm mixing within the female tract can generate high intraspecific variation in  $P_2$  if the ejaculates break into packets and these packets mix randomly (instead of the mixing by individual sperm). However, a simpler process that has received no attention may be responsible for part of the high intraspecific variance and some typical platykurtic or bimodal  $P_2$  distributions found in experimental studies. This is based on purely random effects resulting from male sterility and functional infertility, which leads to females either not having sperm stored from one or more of their mates or having sperm unable to fertilize eggs. For convenience, I have named this “non-sperm representation” (henceforth NSR), which represents a rate that characterizes male populations and includes the rate of male sterility, the rate of insemination failures, and the rate of complete fertilization failures following single matings. No study has theoretically or experimentally examined how the complete failure to fertilize eggs by the males affects the distributions of  $P_2$  values. Here I show how by simple random effects NSR can lead to a high  $P_2$  variance under a mechanism of random sperm mixing. More importantly, the shape of the distribution obtained when NSR occurs can lead to misleading interpretations about the mechanism of sperm competition operating in the species. This problem can be further exacerbated when the sterile-male technique is used to obtain  $P_2$  values. The main causes promoting NSR are reviewed, as well as the extent of NSR in insects and other animals.

#### Causes of Nonsperm Representation

NSR as defined here includes male sterility and functional infertility, the latter describing instances when a nonsterile male does not fertilize the eggs. These include intromission failures, insemination failures, failures in the proper positioning of the sperm within the female tract, or failures in fertilization. The main causes of NSR are, first, insemination failures, including male sterility, male sperm depletion, male modulation of ejaculation investment, and mechanical infertility; and, second, postinsemination failures or fertilization failures arising from ejaculates of low quality or from female-mediated barriers to fertilization.

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#### *Insemination Failures*

*Male Sterility.* Males may have no spermatozoa in the ejaculate (azoospermia) or no ejaculate (aspermia). Male sterility has been extensively studied in humans, where it is one of a large array of causes for male infertility (Smith 1984; Iammarrone et al. 2003). It can be argued that sterility is likely to be rare in natural animal populations as there would be a strong selective force against infertility. However, as Sheldon (1994) points out, there is no requirement for males to be permanently incapable of producing sperm or for such sterility to have a genetic basis in order for them to be functionally infertile.

*Male Sperm Depletion, Modulation of Ejaculation Investment, and Sperm Limitation.* Males may become sperm depleted after few matings, therefore affecting female fertility. It has been noted in some species that the most preferred male may become sperm depleted and therefore convey low fertilization benefits to subsequent mates (Warner et al. 1995; Jones 2001; Preston et al. 2001). Male sperm depletion following mating has been documented among other taxa, in mammals (Gibson and Jewell 1982; Preston et al. 2001), birds (Birkhead and Fletcher 1995; Pizzari et al. 2003; and see Gray 1997), mites (Radwan 1997), lepidopterans (Royer and McNeil 1993; Foster and Ayers 1996), dipterans (Smith et al. 1990; Pitnick and Markow 1994; Prowse and Partridge 1997; Jones 2001), and coleopterans (Eady 1995; Savalli and Fox 1999). A decline in male fertilization success following mating may not necessarily mean sperm depletion because males may ejaculate strategically depending on the risk of sperm competition (Gage 1991; Cook and Gage 1995; Gage and Barnard 1996; Oppliger et al. 1998; Wedell and Cook 1999a, 1999b; Schaus and Sakaluk 2001; García-González and Gomendio 2004) or depending on the “quality” or “condition” of the female (Engqvist and Sauer 2003; Pizzari et al. 2003). This modulation in ejaculate allocation that results from the nontrivial cost of sperm production (Dewsbury 1982; Nakatsuru and Kramer 1982; Van Voorhies 1992; Pitnick et al. 1995; Pitnick 1996; Olsson et al. 1997) can translate to female sperm limitation, that is, low sperm numbers to be used for fertilization (Wedell et al. 2002; Pizzari et al. 2003). Sperm limitation in its extreme case can be a form of NSR for some males if, for instance, copulating males do not release sperm depending on the status (mated vs.

virgin) of the female (see Bukowski and Christenson 1997; for a review, see Wedell et al. 2002).

*Mechanical Infertility.* Insemination failures can arise as a result of mechanical infertility, which represents failure in sperm transfer and includes failure in penetration, failures in ejaculation, and male infections that prevent insemination, all of which have been well documented in a variety of internal fertilizers (Sheldon 1994; Eberhard 1996; Iamarrone et al. 2003). For instance, in species such as lepidopterans that produce spermatophores, there can be frequent failures in spermatophore formation, failures in transferring the spermatophore, and failures in spermatophore alignment (Callahan and Chapin 1960; Labine 1966; Brower 1975; Drummond 1984; Oberhauser 1989; Tamhankar 1995).

#### *Postinsemination Failures*

*Ejaculates of Low Quality.* Apart from nonviable sperm, an ejaculate can contain abnormal sperm, old sperm, immotile sperm, low concentration of sperm, or sperm with genetic abnormalities (e.g., Dziuk 1996; Siva-Jothy 2000). In mammals, it is well known that spermatozoa must undergo biochemical changes before interacting with the egg, whereas after binding they must undergo the acrosome reaction, a process essential for fertilization (Yanagimachi 1994). Successful fertilization in mammals is also dependent on the species-specific recognition, adhesion, and fusion between sperm and egg. All or most of the spermatozoa in an ejaculate may have diminished capabilities for fertilizing the egg if some of the factors and mechanisms regulating sperm functioning or gamete interaction fail. Humans, livestock, and species with conservation concerns are some of the taxa in which these causes have been studied most extensively. For example, an array of sperm problems in humans can be related to genetic disorders (Bhasin et al. 2000; Seshagiri 2001; Maduro and Lamb 2002; Saleh et al. 2003), and in endangered species, inbreeding has been seen to have negative effects on a great number of ejaculate features that influence fertilization success (Roldan et al. 1998).

*Female Barriers and Cryptic Female Choice.* A general characteristic of animals with internal fertilization is that the sperm face female physical and/or physiological barriers (Roldan et al. 1992; Birkhead et al. 1993; Eberhard 1996; Neubaum and Wolfner 1999). These barriers may have evolved by natural selection (to avoid fertilization by defective sperm or infection by pathogens) or in polyandrous species also by sexual selection because it would allow

females to exercise cryptic female choice (defined as non-random paternity biases resulting from female morphology, physiology, or behavior that occur after coupling; Eberhard 1996; Pitnick and Brown 2000). Sperm discarding or dumping by females or the flowback of semen in mammals (see reviews in Birkhead et al. 1993; Eberhard 1996) can lead to NSR. These mechanisms, either under female control (Pizzari and Birkhead 2000) or resulting from passive female sperm depletion or simple sperm mortality (Tsubaki and Yamagishi 1991), may lead to some males accruing zero paternity following copulation. In addition, in humans it is known that the production of male or female antisperm antibodies results in the destruction of gametes, sperm immobilization, prevention of egg penetration, or embryo development (Shushan and Schenker 1992; Shibahara et al. 2003). It is not clear whether this immune infertility can be considered a nonselective female barrier or female choice, but the consequences for NSR may be important if it plays a role in other animals.

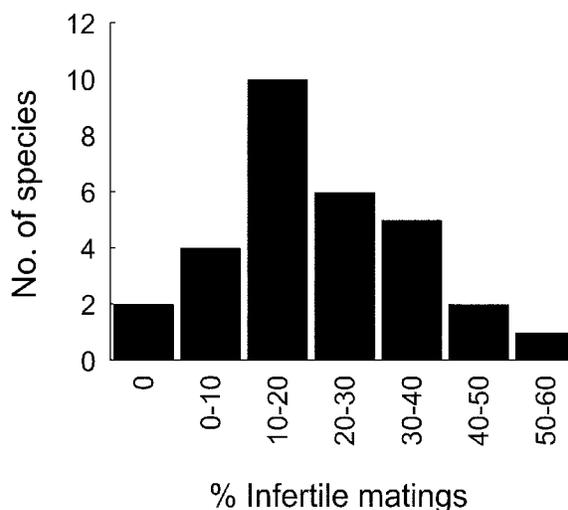
Cryptic female choice to avoid the negative effects of genetic incompatibility (Zeh and Zeh 1996, 1997) may potentially result in the failure of an entire set of embryos sired by a specific male. As such, this “extreme” cryptic female choice could not be considered NSR as defined here. However, this study deals with the effects of infertility on the interpretation of sperm precedence patterns, and the effects of extreme cryptic female choice on the patterns of  $P_2$  are the same as those produced by NSR. Other causes of infertility related to events occurring after fertilization such as embryo death resulting from polyspermy (Eberhard 1996; Morrow et al. 2002) could also compromise the interpretation of sperm competition mechanisms in the same way as NSR (see “Discussion” and Gilchrist and Partridge 1997).

#### **Lack of Insemination or Fertilization in Natural Populations: The Extent of “Nonsperm Representation”**

I present some examples of the extent of NSR taken from species in which there are data on the fertility of matings. I have focused on insects, the group that has most often been surveyed in studies on sperm competition. Ridley (1988) reported proportions of infertile matings for insects and found that across 11 species, the mean proportion of “impotent matings” was around 27%, ranging from 0% to 50%. However, Ridley’s (1988) review included those cases in which mated females had low fertility and therefore are not relevant for an estimation of infertile matings and NSR. Drummond (1984) reviewed rates of unsuccessful matings (failures to inseminate the females or failures to fertilize the eggs) in various species of Lepidoptera. However, sample sizes are not cited in Drummond’s

(1984) review, and it includes some cases in which rates of infertility were taken from matings involving individuals from different strains. For these reasons, I have reviewed the species cited by these authors as well as other species for which data on complete infertile matings have been reported, and I include some more recent studies. The review is not intended to summarize infertility in insects, but it aims to illustrate that infertile matings are more common than generally assumed and to offer a real range of proportions of infertile matings for examining the effects of plausible rates of NSR on the intraspecific variation in sperm precedence values. Data for infertile matings can be found in the literature as the number of once-mated females that do not lay eggs, the number of once-mated females that lay only infertile or nonviable eggs, and the number of single matings in which there is no sperm transference. Ideally, data regarding the first two groups are the most appropriate to assess the extent of NSR because sperm transference failures represent only the minimum threshold for the occurrence of NSR. However, this last category is the only available estimation of infertile matings in some species. Proportions of infertile matings in insects (49 estimates across 30 species distributed in eight orders) are shown in table A1 in the online edition of the *American Naturalist*. Figure 1 shows the frequency of the proportion of infertile matings calculated for the species in table A1. As can be seen in figure 1 and table A1, infertile matings are frequent in insect species. Values for the proportion of infertile matings vary between 0% and 63%, the median being 22%.

An estimation of NSR can also be gathered from infertile double or triple matings if we have information about the extent of female-based infertility. For instance, in the grain beetle *Tenebrio molitor*, Worden and Parker (2001) showed that 25% of the once-mated females did not produce any offspring, but this infertility rate was reduced when females mated with two males (10% of 20 females). Although in this species infertility estimates from double-mated females is relatively low, in reality it represents an NSR rate of approximately 32% (probability of a female mating with two males that both attain NSR would therefore be  $0.32 \times 0.32 = 0.1$ , that is, 10% of infertile double matings), assuming that female-based infertility does not occur in *T. molitor*. This assumption seems to be correct since the infertility rate was reduced to 0% ( $n = 21$  females) when females were mated to five males. In a separate experiment, 25% of the females mated once to the same male did not produce any offspring ( $n = 44$  females), and this proportion was reduced to 5% when females mated to the same male four times ( $n = 43$  females) or 0% ( $n = 43$  females) if the females mated to four different males. The data provided by Worden and Parker (2001) support the notion that NSR in this species is clearly high,



**Figure 1:** Frequency of the proportion of infertile matings calculated for 30 insect species distributed across eight orders (see table A1 in the online edition of the *American Naturalist*). The proportion of infertile matings was calculated in the majority of cases as the proportion of once-mated females that were not successfully inseminated or that produced only infertile eggs. The average proportion of infertile matings has been used when different estimates are available for the same species. Specific values for the proportion of infertile matings for each species and further details can be seen in table A1.

that female-based infertility is low, and that infertile matings do not necessarily result from infertile males. Another example is the earwig *Euborellia plebeja*, for which the proportion of 32 females mated three times with the same male and that laid only infertile eggs was 19% and the proportion of noninseminated females that mated with three different males was 6% ( $n = 32$  females). Assuming that no female was infertile in the triple-mating trials, NSR for this species would be as high as 39%.

NSR may be also important in other arthropods. Newcomer et al. (1999) found in the pseudoscorpion *Cordylorchernes scorpioides* that 19% of 77 females that mated with only one male failed to give birth to any offspring. Although Zeh et al. (1998) found in this species that in 11% of matings the male produced defective spermatozoa, the result by Newcomer et al. (1999) arose not because of infertile males but because of embryo failure that probably resulted from genetic incompatibility between maternal and paternal genomes (Zeh and Zeh 1996, 1997). The same process could explain to some degree infertile matings in other species although little is known (see above and “Discussion” for remarks about the potential convergent effects of NSR and cryptic female choice over  $P_2$  values).

Infertile matings have been reported in other taxa. In

humans, it is widely known that infertility affects 13%–18% of couples and presents a high incidence of male reproductive problems (Maduro and Lamb 2002; Iammarrone et al. 2003). Iammarrone et al. (2003) list 40 main causes for infertility in humans. Although many of the causes identified in humans can be species specific, many of them could be present in other mammals and other taxa (e.g., Laing 1970; Wolf et al. 2000). For instance, Hoogland (1998) reports that 7% of 87 once-mated prairie dog females (*Cynomys gunnison*) that were studied under natural conditions failed to give birth. As for reptiles, only one study has estimated the frequency of infertile males in natural populations, this being around 5% (only one male with permanent sterility among 22 males evaluated; Olsson and Shine 1997). In birds, it has been shown that males in many species can suffer functional infertility (reviewed in Sheldon 1994). In a recent review, Morrow et al. (2002) found that the rate of infertility in natural bird populations, measured as the number of eggs that fail to hatch, is often high (around 15%). However, little is known about the extent of complete infertility, that is, when all the eggs fail to hatch following copulation, although the evidence presented by Sheldon (1994) and Morrow et al. (2002) suggests that it can be high. One of the few bird studies in which male infertility has been measured in the wild was carried out on red-winged blackbirds *Agelaius phoeniceus*. In this species, Gray (1997) found that 9% of 65 territorial males showed permanent sterility, while 21% of 47 territorial males exhibited temporary infertility because they did not sire offspring in a given season but fathered offspring in the preceding or following year. This study shows that reduced fertility and temporary infertility is more common than permanent sterility in this species, as has been suggested for avian populations (Birkhead and Møller 1992). However, both are important for the consequences on the outcome of sperm competition. In the Japanese quail *Coturnix japonica* between 30%–40% of 233 single matings produced no fertile eggs even after insemination had been successful (Adkins-Regan 1995). Postcopulatory female choice or genetic incompatibility is a candidate to explain this failure in fertilization because fertilization success was highly correlated if the same female was mated with the same male a second time but uncorrelated if she was mated with a different male or if the same male was mated with different females. In a separate experiment by Adkins-Regan and MacKillop (2003), the percentage of matings in which all the eggs laid were infertile was 16%, 22%, 37.5%, or 60%, depending on external factors.

In summary, evidence suggests that the extent of NSR can be high in natural populations. The mentioned examples show that NSR may be sufficiently important in some instances to explain variance in the output of sperm

competition. In addition, a general trend to discard the data of females laying no fertile eggs when  $P_2$  studies are carried out is suspected because data on infertile matings are assumed noninformative for the patterns of sperm precedence. The inclusion of these data in future studies could further manifest the presence of NSR in other species and at the same time provide valuable information to understand the patterns of sperm precedence revealed by  $P_2$  values.

### Methods

The rationale of the methodology lies in the calculation of the probability of obtaining  $P_2$  distributions with a particular shape, mean, and standard deviation (SD) that derive from populations of males that differ in the capacity to have sperm representation. This probability is calculated for random sperm mixing, that is, when the sperm mix randomly in the female sperm store and each sperm has an equal chance of being used for fertilization; this is the “fair raffle” principle, in which the fertilization success of each male is related to the number of sperm in the fertilization set (Parker et al. 1990). The probability of obtaining a given  $P_2$  distribution (or a given mean and standard deviation for  $P_2$ ) under a given rate of NSR is compared with that obtained from populations in which all the males attain sperm representation (i.e., at least one sperm is transferred and utilized to fertilize an egg). Two assumptions are made: first, that there is no sampling error when obtaining each  $P_2$  value (i.e., from each female a large number of progeny is screened for paternity) and second, that no sloppy sperm mixing (Harvey and Parker 2000) is operating (i.e., it is the individual sperm of the males and not sperm packets that mix in the sperm storage organ).

Random sperm mixing is taken as the sperm competition mechanism operating in the species, which predicts no  $P_2$  values of 0 or 1 provided that there is sperm representation. Different  $P_2$  sample sizes (also named  $nP_2$ ) and NSR rates (also named %NSR) are simulated to investigate the effects of varying these two factors on the probability of obtaining specific  $P_2$  distributions.

The methodology used provides  $P_2$  values that would be obtained under natural conditions because  $P_2$  values are calculated from the relative fertilization success of each male in a double-mating trial. Any given  $P_2$  value is calculated as  $r_2/(r_1 + r_2)$ , where  $r_2$  is number of sperm present from the second male to mate with the female and  $r_1$  is the number from the first male on a scale from 0 to 1. This conforms to the fair raffle principle of Parker et al. (1990) and presents the following two advantages. First, this approach reflects the fact that the output of  $P_2$  depends on the random pairing of males that differ in sperm num-

bers. The success of a second male depends not only on the sperm numbers successfully transferred by him but also on the number of sperm successfully transferred by the first male. Second, this approach allows investigation of the effect of NSR in  $P_2$  distributions in a “real” way, by entering the rate of NSR in the original population of males and not directly in the final  $P_2$  values (see next subsection). The most direct consequence of this procedure is that it is valid for any given species-specific range of sperm numbers that are in competition for fertilization.

Three different rates of NSR have been investigated, 0%, 10%, and 30%, using four different sample sizes for obtaining  $P_2$  values, 10 ( $n$  males = 20), 20 (40 males), 30 (60 males), and 100 (200 males). Each set of the %NSR- $nP_2$  (rate of NSR and sample size for the values of  $P_2$ ) combination was simulated 300 times; thus, a total of 3,600 simulations were performed.

*Example for the Procedure Using a Sample Size of 100  $P_2$  Values per Distribution and a Rate of NSR = 10%*

First, the distributions under a rate of NSR = 0% were calculated. Random numbers from a uniform distribution were allocated to each of the 200 males competed. Random numbers range between 0 and 1 but do not include these extreme values (NSR = 0%) and represent the relative sperm representation (=relative fertilization success) of each of the males. Values for  $P_2$  were calculated as described above from randomly paired males. The mean and SD were calculated for the 100  $P_2$  values obtained. This procedure was repeated 300 times, each time following random number allocation to the males and randomization of the pairs of males. From the distribution of the 300 mean  $P_2$  values and SDs, the probability of obtaining distributions of a given mean  $P_2$  value or SD was calculated.

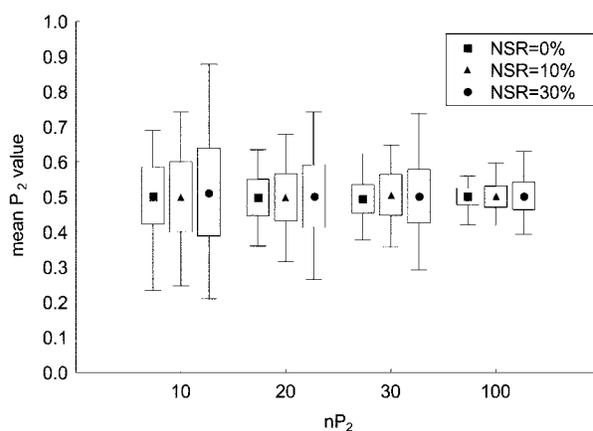
Second, the %NSR in the male population was introduced as 10% in this example. This means that 20 of the 200 males used to calculate single distributions of  $P_2$  values do not attain sperm representation (i.e., their sperm, if it exists, is not used to fertilize the eggs of a particular female). For each simulation, 180 random numbers between 0 and 1 (excluding 0 and 1) plus 20 values equal to 0 (no sperm representation for 20 males) were generated. The  $P_2$  values were calculated following randomization of pairings within the simulation. As mentioned previously, no values of 0 or 1 were entered directly as  $P_2$  values, but they result from the random pairing of males, as it would be in experimental data. When both the first male and the second male had a reproductive success of 0, the value of  $P_2$  could not be calculated, and this represented the case in which females used in experiments do not lay eggs or none of the eggs laid hatch as a consequence of nonsperm

representation (see “Discussion” for this specific case among those cases in which the sterile-male technique is used to investigate the pattern of sperm precedence). The probability of the occurrence of this event (both males in competition having nonsperm representation) depends on the rate of NSR in the population as well as on the number of  $P_2$  values. For instance, that probability for this specific example (100  $P_2$  values and 10% of NSR) is 0.01 (the probability of mating a female with a first male with nonsperm representation, i.e., 0.1, multiplied by the probability of mating that female with a second male with nonsperm representation, 0.1). Therefore, on average, 99 values of  $P_2$  out of 100 matings should be obtained or 29,700  $P_2$  values from overall 30,000 simulated matings (300 simulations  $\times$  100 values of  $P_2$  for each simulation).

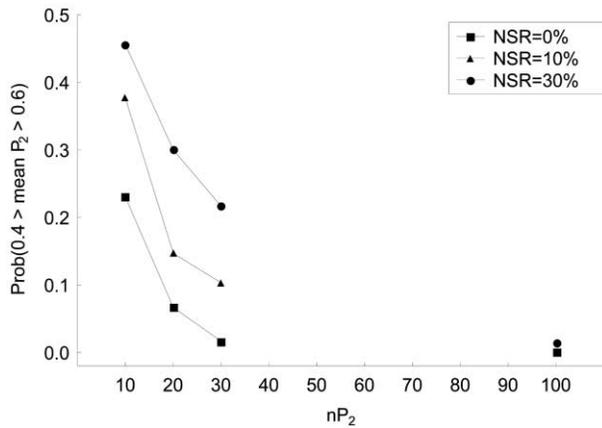
After calculating the  $P_2$  values of the 300 distributions simulated under 10% NSR, the mean  $P_2$  and SD of each distribution were obtained. Probabilities of obtaining a given mean  $P_2$  or SD were extracted from the distribution of these statistics. Independent  $t$ -tests were carried out to examine differences in the distribution of SDs obtained from populations with NSR = 0% and those with NSR > 0%.

## Results

As expected by the simulation of random sperm mixing, the overall mean  $P_2$  values for each of the 12 sets of 300 simulations did not differ from 0.5. However, the individual mean  $P_2$  value for each distribution varied from 0.21 to 0.88 (table A2 in the online edition of the *American Naturalist*; fig. 2), which strongly indicates that there is at



**Figure 2:** Effect of varying the %NSR and the sample size of  $P_2$  values ( $nP_2$ ) over the mean  $P_2$  value obtained. The mean, SD (box), and minimum and maximum values (lines) for the mean  $P_2$  values obtained in each set of simulations are shown.



**Figure 3:** Probability of obtaining a distribution of  $P_2$  values with a mean either  $< 0.4$  or  $> 0.6$  depending on the rate of nonsperm representation and the sample size of  $P_2$  values ( $nP_2$ ).

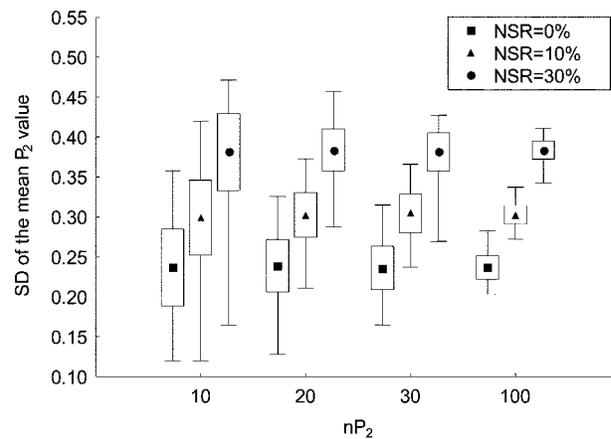
least some chance for the interpretation that another sperm precedence pattern different from sperm mixing was occurring, when in fact random sperm mixing is operating. This error is especially important when either a biased mean  $P_2$  value and a low SD is obtained or a mean  $P_2$  value near 0.5 and high SD associated with a bimodal distribution is obtained.

Obviously, the probability of obtaining extreme mean  $P_2$  values, high SDs, or bimodal distributions for  $P_2$ , which lead to the mistake of inference of mechanisms of sperm precedence other than random sperm mixing, depends on the  $nP_2$  and the %NSR. This probability may be very low, and thus the effect of NSR may be negligible. However, in some instances, this probability was high enough to raise concern over the negative effects of NSR on the interpretation of  $P_2$  distributions. For instance, in figure 3, I have generated the shape of the curves for the probability of obtaining mean  $P_2$  values either  $< 0.4$  or  $> 0.6$ . A mean  $P_2$  value  $< 0.4$  or  $> 0.6$  can lead to an interpretation of first- or last-male sperm precedence. Figure 3 shows that the probability of this mistake increases as  $nP_2$  decreases and %NSR increases. From this figure it can be seen that for  $nP_2 = 30$  and NSR = 30%, we will obtain a distribution with a biased mean  $P_2$  with a probability of 0.2. This problem can be serious if we consider that the mean  $P_2$  value can be as high as 0.74 under the conditions of NSR = 30% and  $nP_2 = 30$  (see fig. 2) or 0.88 for NSR = 10% and  $nP_2 = 10$ , with the chances of having the mean  $P_2 < 0.4$  or  $> 0.6$  in this latter case being almost 1 of 2 ( $p = 0.5$ ).

The second problem of NSR relates to changes in the shape of the distribution without changing the mean  $P_2$  value. The overall mean SD for each of the 12 sets of 300

simulations increases as the rate of NSR increases (table A2; fig. 4), and the individual SD values for each distribution can range between 0.12 and 0.47. There are highly significant differences for the SD obtained depending on the rate of NSR in all the four different sets of  $P_2$  sample sizes simulated (in all the cases,  $p \ll 0.001$  for the difference between SDs obtained under NSR  $> 0\%$  and those obtained under NSR = 0%,  $t$ -test for independent samples,  $df = 598$  in all cases; see table A2). The range of variation in both mean and SD for  $P_2$  depending on the %NSR and the sample size of the  $P_2$  values is highly informative because usually a great part of the interpretation of sperm precedence patterns relies on the mean  $P_2$  value obtained, and the rest relies on the variance around this value. Probabilities for obtaining high SDs that indicate how NSR affects the output of a  $P_2$  experiment can be calculated. This probability decreases as the sample size of  $P_2$  values increases within each rate of NSR simulated (fig. 4 illustrates this phenomenon). For instance, when NSR = 0%, the probability of having a distribution with an SD  $> 0.30$  decreases as  $nP_2$  is increased, being almost 0 for sample sizes over 30 (data not explicitly shown in fig. 4). However, the probability for obtaining SD  $> 0.30$  when NSR = 10% or 30% is always high, being  $> 0.5$  when NSR = 10% and between 0.9 and 1 when NSR = 30% for any sample size of  $P_2$  values (probabilities not shown in fig. 4). An SD value  $> 0.30$  can be considered roughly a threshold for the interpretation of sperm mixing, first- or last-male sperm precedence, or bimodality in the distribution of  $P_2$  values (see fig. 2.4 and table 2.3 in Simmons 2001).

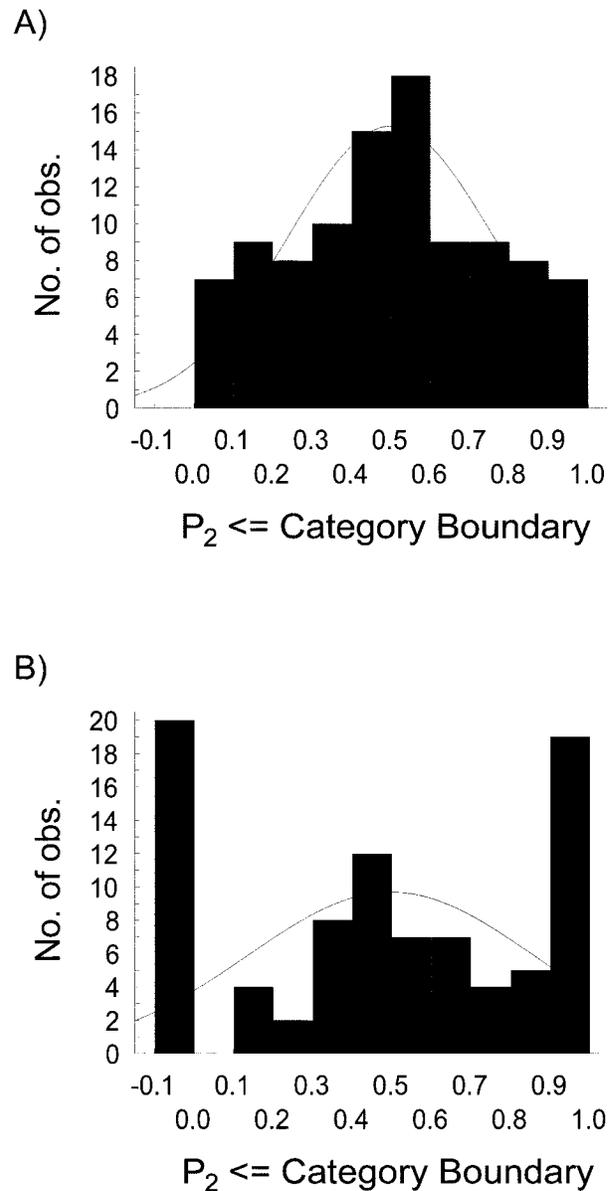
It is clear that NSR in a population leads to a great



**Figure 4:** Effect of varying the %NSR and the sample size of  $P_2$  values ( $nP_2$ ) over the standard deviations for the  $P_2$  values. The mean, SD (box), and minimum and maximum values (lines) for the standard deviations obtained in each set of simulations are shown.

increase in the variance at any  $P_2$  sample size. Any distribution of  $P_2$  obtained under NSR will present a very high variance, not as a result of numerical competition between the ejaculates but as a result of the random effects generated by NSR. In general, increasing the rate of NSR leads to mean  $P_2$  values that do not support the predictions of random sperm mixing and high SDs that do not support random sperm mixing. The use of small sample sizes of  $P_2$  values accentuates these negative effects.

The effect of %NSR and  $nP_2$  on the shape of  $P_2$  distributions can be further evaluated by inspecting some distributions that can serve as critical distributions. In this way, I have plotted, for the cases in which NSR = 0%, the distribution with an SD beyond which the probability of obtaining a distribution with a greater SD is 0.05. This critical distribution gives a general understanding of the shape of the majority of the distributions obtained when NSR = 0%. This general shape of the distributions when NSR = 0% can be compared with the general shape of the distributions when NSR > 0%. Because we want to examine whether unbiased distributions expected when NSR = 0% differ from the distributions obtained when NSR > 0%, I have conservatively plotted for this latter case the distribution with a SD beyond which the probability of obtaining a distribution with a greater SD is 0.95. This critical distribution gives us an idea of the flatness or bimodality of the majority of the distributions when NSR > 0%, which can be visually compared with the distribution shown for NSR = 0%. For instance, the distribution plotted for  $nP_2 = 100$  and NSR = 0% shows that at least 95% of the 300 distributions generated conform well to a normal distribution, whereas the distribution plotted for  $nP_2 = 100$  and NSR = 30% shows that at least 95% of the 300 distributions generated under these circumstances follow bimodal or flat patterns (fig. 5). In figure A1 in the online edition of the *American Naturalist*, I have plotted these critical distributions for every combination of %NSR and sample size of  $P_2$  values assessed. In short, figure A1 shows that for NSR = 0%, the majority of distributions conform well to a normal distribution, whereas for NSR > 0%, the majority of the distributions tend to be flat or bimodal with some cases of high skewness. The higher the %NSR, the more likely the distribution is to be bimodal. This trend is confirmed in figure A2 in the online edition of the *American Naturalist*, which shows the distribution with the highest SD within each set of simulations. To summarize what is shown in figures A1 and A2, first,  $P_2$  distributions better support random sperm mixing (when this is the sperm competition mechanism operating) when the sample size of the  $P_2$  values increases, and second,  $P_2$  distributions support random sperm mixing to a lesser extent when the %NSR in the population increases. The lower the  $nP_2$  and the higher the %NSR in



**Figure 5:** Example of the change in shape of the  $P_2$  distributions due to nonsperm representation when the sample size of  $P_2$  values is 100. *A*, NSR = 0%; this figure shows the distribution with a SD beyond which the probability of obtaining a distribution with a greater SD is .05 ( $n = 300$  simulations). *B*, NSR = 30%; this figure shows the distribution with a SD beyond which the probability of obtaining a distribution with a greater SD is .95 ( $n = 300$  simulations). The line indicates the normal curve.

the population, the more likely we are to obtain a distribution of  $P_2$  values that can lead to mistakenly reject sperm mixing when in fact this is the real sperm competition mechanism operating in the species.

## Discussion

Only recently has it been acknowledged that fertility should be more seriously considered in the study of sperm competition. Siva-Jothy (2000) points out that the idea that all the viable sperm (i.e., live sperm) in an ejaculate have fertilizing potential may not reflect reality, and Wedell et al. (2002) review the strength of evidence for female sperm limitation and its consequences for sperm competition. In the same line of reasoning, Mesterton-Gibbons (1999a) recently included the possibility of sperm limitation when revisiting Parker's (1990a) sperm competition game model. The present study illustrates the largely ignored confounding effects of infertility and sperm limitation on the pattern of sperm competition, supporting the view that both aspects are worthy of investigation. First, a review of the literature in which there is information about infertile matings shows that some degree of infertility may be widespread in natural populations, and second, simulation results presented here indicate that purely random effects derived from nonsperm representation can affect the probability of obtaining distributions of  $P_2$  that do not represent the underlying patterns of sperm use.

Probabilities of obtaining  $P_2$  distributions that deviate from those expected under random sperm mixing show that a moderate to high rate of NSR can lead to two main errors. First, the mean  $P_2$  can be biased and so not reflect the real sperm competition mechanism behind the sperm precedence patterns. This error can be avoided by using large sample sizes for estimating  $P_2$ . Such error is unlikely to be avoided in samples sizes  $<30$  for rates of NSR  $>10\%$ , where it appears with a probability  $>0.1$  (see fig. 3). Second, a biased or bimodal distribution suggesting an incorrect mechanism of sperm competition may be obtained. This is the most likely effect, and it is especially important when the rate of NSR is high (fig. 4).

Results shown here support Harvey and Parker's (2000) contention that unexplained variation in  $P_2$  experiments cannot be attributed in the first instance to phenotypic variables or cryptic female choice. Only after removing the variation due to random effects related to the degree of mixing or to NSR should adaptive explanations be invoked to explain the remaining variation. Random sperm mixing has been simulated in this study as the principal pattern-determining mechanism, but the confounding effects derived from NSR are likely to be present when other sperm precedence patterns determine the paternity of the eggs generated by polyandrous females. The effects of NSR on the sperm precedence patterns should typically be less acute in other mechanisms because it would be unlikely that strongly skewed distributions resulting from adaptations to achieve first- or last-male sperm precedence are

reversed or become bimodal as a result of NSR. However, further investigations are warranted, especially if the rate of NSR in the populations proves to be high.

A prediction that follows from this study is that under random sperm mixing and a high rate of NSR in a population, a bimodal pattern obtained for  $P_2$  (expected as an effect resulting from the rate of NSR) should change to a nonbimodal pattern when examining the proportion of offspring fathered by the  $n$ th male to mate with a female when the number of males is  $>2$  ( $P_n$ ). This is because under a rate of NSR  $>0$ , the probability of obtaining complete first-male or last-male sperm precedence decreases as the number of mating males increases.

The corrupting effects of NSR on  $P_2$  distributions can be inflated when using the sterile-male technique, one of the most widely used methods to assign paternity in  $P_2$  experiments (Boorman and Parker 1976; Simmons 2001). Under this technique, one of the two males in competition is exposed to sublethal doses of radiation that induce chromosomal mutations in sperm. An irradiation dose that provides embryonic death without affecting sperm viability and fertilizing capacity is used, and paternity is determined on the basis of egg viability following a double mating with both a normal male (N) and an irradiated one (I). However, the irradiated males usually have a reduced fertilizing capacity compared with normal males (Simmons 2001). The use of the sterile-male technique is therefore likely to increase  $P_2$  variance. Moreover, if some rate of NSR is present in the population, the number of  $P_2$  values equal to 0 and equal to 1 irrespective of the order of matings will be overestimated. As such, the sterile-male technique will further obfuscate the mechanisms responsible for differential male fertilization success. For example, under the sterile-male technique, complete last-male sperm precedence ( $P_2 = 1$ ) is assumed when the hatching success of the eggs laid by a female mated in the sequence N-I is 0%, because it would mean that the irradiated male has been successful in sperm competition (and no eggs hatch because irradiated males are not able to fertilize eggs). However, the same result could arise if both the normal and irradiated male were unable to transfer sperm to the female; that is, a result of 0% hatching success could arise in the absence of sperm competition. Table 1 shows that the errors that result from the use of the sterile-male technique when there is NSR lead to overestimating extreme values of  $P_2$ . Consequently, when a rate of NSR  $>0\%$  is present in the population, the sterile-male technique will offer distributions that tend to be bimodal. The higher the extent of NSR, the larger the difference will be between the distribution obtained with this technique and that expected from the mechanism of sperm competition that operates in the species. Therefore, although the sterile-male technique is a simple and affordable way to assign

**Table 1:** Possible errors in  $P_2$  values when the sterile-male technique is used and a rate of nonsperm representation (NSR) greater than 0% is present in the population of males

Mating order	Hatching success (%)	Assumed $P_2$ value	$P_2$ value does not reflect the possibility of NSR for male	Possible errors in $P_2$ values
N-I	100	0	I	Overestimation of 0's
N-I	0	1	I and N	Overestimation of 1's
I-N	100	1	I	Overestimation of 1's
I-N	0	0	I and N	Overestimation of 0's

Note: N = normal male, I = irradiated male. The cases in which there is NSR of both males would not be included in  $P_2$  values when using N-N matings because females would lay no fertile eggs.

paternity in  $P_2$  experiments, the use of other methods to determine paternity is advisable when NSR is suspected. In the last decades other methods to determine paternity in sperm competition studies have been used, including DNA markers such as microsatellites (Simmons and Achmann 2000) and amplified fragment length polymorphisms (García-González et al. 2003), which can overcome the drawbacks of the sterile-male technique.

A large number of species show ranges of  $P_2$  between 0 and 1, and for the species of lepidopterans for which data exist, almost all follow bimodal distributions with modes of 0 and 1 (Retnakaran 1974; LaMunyon and Eisner 1993a; Svárd and McNeil 1994; Cook et al. 1997; Simmons 2001). Bimodal patterns can be generated by different mechanisms. First, they are expected if the first males to copulate use mating plugs to prevent future inseminations. Values for  $P_2$  can then be very low if the plug remains intact or very high when the plug's protection is breached (Simmons and Siva-Jothy 1998), and the same is true if other sperm competition avoidance mechanisms are used. A mechanism of sperm displacement can also lead to first- or last-male sperm precedence depending on the mating interval (Retnakaran 1974). Drummond (1984) suggested that in lepidopterans the spermatophore of the second male may displace the spermatophore of the first male and that if females remate very rapidly, this would result in few sperm from the first male reaching the spermatheca. Second, apart from mechanisms to directly avoid sperm competition or to displace sperm, extreme  $P_2$  values can also result from very low levels of ejaculate mixing prior to fertilization (Harvey and Parker 2000). Third, results in this study have shown that strong modes of  $P_2$  near 0 and 1 may be explained by a complete absence of sperm competition as a result of NSR. Finally, extreme cryptic female choice that leads to the nonuse of spermatozoa of a given male may produce similar effects on  $P_2$  distributions.

Cryptic female choice has been suggested to occur in a vast number of species (Eberhard 1996). Among the possible mechanisms for cryptic female choice are sperm dis-

carding, prevention of complete intromission and ejaculation, failing to transport sperm to storage organs or fertilization sites, forcefully terminating copulation before sperm are transferred, failing to ovulate or to mature eggs, and moving previous ejaculates to a site where the current male can manipulate them (Eberhard 1996, 1997). For instance, female crickets may remove the attached spermatophore of a male before complete sperm transfer has occurred, which allows females to exert postcopulatory mate choice (Sakaluk 1984; Simmons 1986; Sakaluk and Eggert 1996), and in the kittiwake *Rissa tridactyla*, females eject sperm in around 20% of within-pair copulations apparently depending on the age of their mate's sperm (Helfenstein et al. 2003). All of these postcopulatory paternity-biasing mechanisms compromise the fertilizing abilities of the sperm from the nonselected males, therefore potentially leading to the same effects caused by NSR (Sakaluk and Eggert 1996). Female choice over ejaculates has been suggested as one explanation for the strong bimodal  $P_2$  patterns in the moth *Utetheisa ornatrix* because the females appear to control the movement of sperm from the bursa to the spermatheca in this (LaMunyon and Eisner 1993a, 1993b) and other species of Lepidoptera (Tschudi-Rein and Benz 1990). Ideally, in addition to controlling for the effects of NSR, experiments to partition variance in  $P_2$  attributable to sperm competition and cryptic female choice should be carried out to completely understand nonrandom patterns of sperm use (see Pitnick and Brown 2000). Sperm selection has recently been included in the theoretical models of sperm competition (Ball and Parker 2003), although it has not been used to predict variation in sperm precedence patterns. Future research in this direction would undoubtedly contribute to a better understanding of postcopulatory sexual selection.

Cook et al. (1997) suggested an approach to examine the intraspecific variation in  $P_2$  that consists of predicting beforehand the expected variance in  $P_2$  based on variation in sperm numbers delivered during mating. Guidelines for analyzing the variance in  $P_2$  are as follows: assume the mechanism of sperm competition; examine the variation

in sperm numbers delivered during mating by the population of males used for the sperm competition study; given the mechanism of sperm competition, use the variation in sperm numbers to predict the variation in  $P_2$ ; and compare the observed and predicted  $P_2$  distributions (Cook et al. 1997). Ideally, a combination of this method with one implementing the source of random variation due to NSR would offer accurate expectations for the variance in  $P_2$ . Unfortunately, the assessment of sperm numbers delivered during mating can often be difficult and present problems associated with sampling errors at various levels (see Cook et al. 1997).

NSR should be explored in experiments of sperm competition to completely rule out random variation around  $P_2$  values due to NSR. Some studies have attempted to control for infertility in sperm competition studies by either allowing second matings only to the females that lay eggs after the first mating (Lewis and Austad 1990; LaMunyon and Eisner 1993a; Lewis and Jutkiewitz 1998; Bernasconi and Keller 2001; LaMunyon 2001) or checking by postmortem dissection of the female for the presence of two spermatophores in the female tract following the double-mating trial (LaMunyon and Eisner 1993a; Svård and McNeil 1994; Cook et al. 1997). However, the first method can result in the female running out of sperm from the first male as it is used to fertilize eggs, which may affect sperm precedence values, whereas the second method assumes that transference of sperm equals fertilization. Moreover, in both methods the infertility of the first males is assessed and controlled in the experiment but not the infertility of the second males. Consequently, left-biased distributions can be obtained if the rate of NSR is high.

There are other methods to account for the rate of NSR. One method is to carry out single matings and calculate the rate of infertile matings. Another method is to obtain the rate of infertile double matings from the  $P_2$  experiment, being the proportion of females that do not lay fertile eggs following copulation with two males and representing the cases in which both males mated with a female attain no sperm representation. The square root of the rate of infertile double matings would offer a rough approximation of the rate of NSR in the population. However, these two methods present the problem that some of the infertile matings might be the consequence of female infertility. A method that mitigates the problem of female infertility is to mate each male with two females and calculate the rate of insemination failure only for those cases in which the two females mated with a given male do not produce fertile eggs. This rate would include male sterility and mechanical infertility due to males such as failures in sperm transfer and would control to some extent for exclusive female effects.

Once the rate of NSR is known, the probability of obtaining a mean  $P_2$  value, a given SD for the distribution, and a given shape for the distribution can be obtained by means of the simulations shown here and for any sample size of  $P_2$ . Depending on the rate of NSR in the population, the sample size for  $P_2$  values that should be used can be determined. This number should be high enough to obtain a probability of a deviation from random sperm mixing so low ( $<0.05$ ) as to be sure that if such a deviation is found it is not due to random effects derived from the NSR. If a correct NSR has been estimated and the proper  $P_2$  sample size is used, a deviation from random sperm mixing would be indicative of the sperm precedence mechanism inferred from the distribution. The method suggested here serves to reasonably rule out NSR effects when random sperm mixing is taken as the null hypothesis. With further investigation of the effects of NSR when other sperm precedence patterns different from random sperm mixing are operating in the species, a wide range of expectations could be contrasted.

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