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Note

An Analytical Framework for Estimating Fertilization Bias and the Fertilization Set from Multiple Sperm-Storage Organs

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abstract: How sperm from competing males are used to fertilize eggs is poorly understood yet has important implications for postcopulatory sexual selection. Sperm may be used in direct proportion to their numerical representation within the fertilization set or with a bias toward one male over another. Previous theoretical treatments have assumed a single sperm-storage organ, but many taxa possess multiple organs or store sperm within multiple regions of the reproductive tract. In *Drosophila*, females store sperm in two distinct storage organ types: the seminal receptacle (SR) and the paired spermathecae. Here, we expand previous "raffle" models to describe "fertilization bias" independently for sperm within the SR and the spermathecae and estimate the fertilization set based on the relative contribution of sperm from the different sperm-storage organ types. We apply this model to three closely related species to reveal rapid divergence in the fertilization set and the potential for female sperm choice.

Keywords: Drosophila, cryptic female choice, model, raffle, sperm competition, sperm use.

Introduction

Cryptic female choice arises from nonrandom paternity biases resulting from female morphology, physiology, biochemistry, or behavior that occur after coupling (Thornhill 1983; Eberhard 1996; Pitnick and Brown 2000), and it can play an important role in postcopulatory sexual selection. Studies of cryptic female choice in diverse taxa have convincingly shown that females are not passive vessels in which males or their ejaculates compete to fertilize eggs but instead can actively and substantively influence paternity. Sperm choice is a special case of cryptic female choice that involves differential treatment of ejaculates or sperm (Birkhead 1998). It is poorly understood due to empirical difficulties of observing events within the female reproductive tract and of distinguishing between sperm from different males, two components critical for studying mechanisms of sperm choice. Indeed, the closer we get to fertilization, the less we understand about the mechanisms of cryptic female choice. Thus, we know more about female-mediated processes influencing intromission (e.g., Brennan et al. 2007), sperm transfer (e.g., Sakaluk and Eggert 1996; Pilastro et al. 2004; Bussière et al. 2006; Hall et al. 2010), and retention of sperm (e.g., Bishop et al. 1996; Pizzari and Birkhead 2000; Dean et al. 2011; Lüpold et al. 2012) than we do about sperm storage and use for fertilization (e.g., Ward 1993; Otronen et al. 1997; Córdoba-Aguilar 1999; Fedina and Lewis 2004; Pattarini et al. 2006).

When considering postcopulatory sexual selection occurring in the context of double mating by females, it is useful to discriminate between two stages: (1) formation of the "fertilization set" (the population of sperm potentially competing to fertilize eggs; sensu Parker et al. 1990) and (2) how sperm in the fertilization set are used for fertilization. Although the fertilization set has rarely been empirically identified (e.g., Manier et al. 2010, 2013*a*; Lüpold et al. 2012), it is likely to constitute all sperm occupying the female's sperm-storage organs, or the sperm "reservoir" in the case of many mammals. On the other hand, the fertilization set could be more expansive, additionally including sperm retained in the deposition site (e.g., Siva-Jothy and Hooper 1995, 1996; Naud et al. 2005), or it could be more restrictive, as in the sperm occupying a subset of multiple sperm-storage organs (Pitnick et al. 1999; Manier et al. 2010) or even a specific region within an organ. Here we introduce the term "fertilization bias" to refer to nonrandom deviations from the null expectation that sperm are used in direct proportion to their numerical representation in the fertilization set. In other words, fertilization bias occurs when one male's sperm are disproportionately favored over another's above and beyond their relative abundance in the fertilization set. Fertilization bias should not be confused with the "fair/loaded

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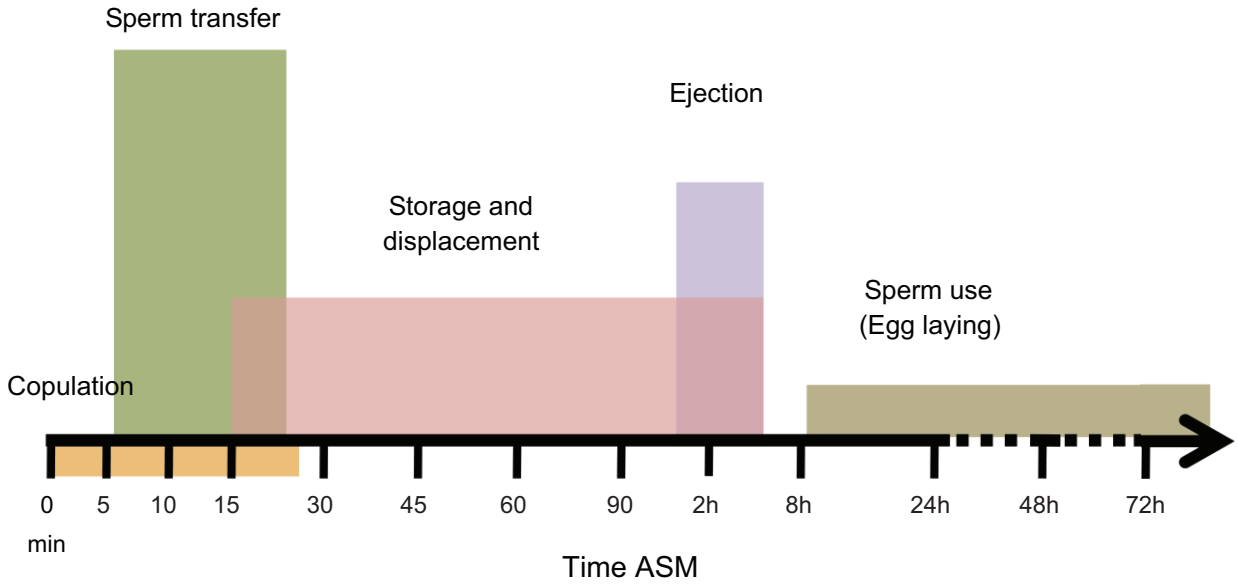

Figure 1: A schematic of the four stages of postcopulatory sexual selection in *Drosophila* at various time points after the start of remating (ASM): sperm transfer, storage and displacement, ejection, and sperm use. Height of the blocks roughly represents relative sperm numbers. Copulation duration is shown in orange. Formation of the fertilization set occurs during the first three stages, the fertilization set is established upon ejection, and fertilization bias occurs during the sperm use (or egg-laying) phase.

raffle" mechanisms as proposed by Parker et al. (1990) and addressed in numerous models and empirical studies (reviewed by Parker and Pizzari 2010), as raffles refer to the probability of each sperm from each male entering the fertilization set (Parker et al. 1990). For example, Parker et al. (1990) refer to differences between males in the number of sperm transferred or the timing of insemination as putative mechanisms determining the type of raffle and explicitly assume that sperm mixing removes any further bias in sperm use. It should be noted here that we assume that sperm use for fertilization can be directly inferred from offspring paternity and disregard any effect of differential paternity success due to mortality postfertilization. Fertilization bias should also not be confused with sperm precedence, which describes the proportion of progeny sired by a male depending on mating order. Second-male sperm precedence, for example, occurs when the majority of offspring are sired by the second of two males. This pattern alone, however, reveals nothing about fertilization bias, because displacement or other processes may establish a fertilization set consisting of a larger proportion of sperm belonging to the second male. If these sperm are then used in direct proportion to their relative abundance, then there is no fertilization bias, even though there is second-male sperm precedence.

Female sperm-storage organs enhance sperm survival and temporally separate insemination from fertilization, expanding the temporal, morphological, and biochemical arenas of postcopulatory sexual selection in general, and are predicted to extend the opportunity for female sperm choice in particular (Birkhead et al. 1993; Eberhard 1996; Pitnick et al. 2009). Sperm-storage organ morphology can be highly diverse (e.g., birds: Birkhead and Møller 1992; pulmonate snails: Baur 1998; spiders: Uhl 2002; insects: Theodor 1976; Puniamoorthy et al. 2010; Higginson et al. 2012*b*), rapidly divergent (e.g., Pitnick et al. 1999, 2003) and have been found to exhibit correlated evolution with sperm morphology in diverse taxa (reviewed by Pitnick et al. 2009; Higginson et al. 2012*b*), which in turn may promote reproductive isolation between species (Howard et al. 2009; Manier et al. 2013*a*). Relatively little is known, however, about female sperm-storage organ function (Schnakenberg et al. 2012), particularly with regard to sperm choice.

Our recent investigations of sperm precedence mechanisms in three closely related species of *Drosophila* (Manier et al. 2010, 2013*a*; Lüpold et al. 2012) revealed that postcopulatory sexual selection can involve four distinct phenomena: (1) sperm transfer, (2) sperm storage and displacement from storage, (3) ejection, and (4) sperm use for fertilizations during egg laying (fig. 1). The first three phenomena all contribute to the formation of the fertilization set, whereas any fertilization bias would occur later, during egg fertilization and oviposition (fig. 1). Sperm are transferred during copulation and begin to enter storage in the seminal receptacle (SR) or paired spermathecae (fig.

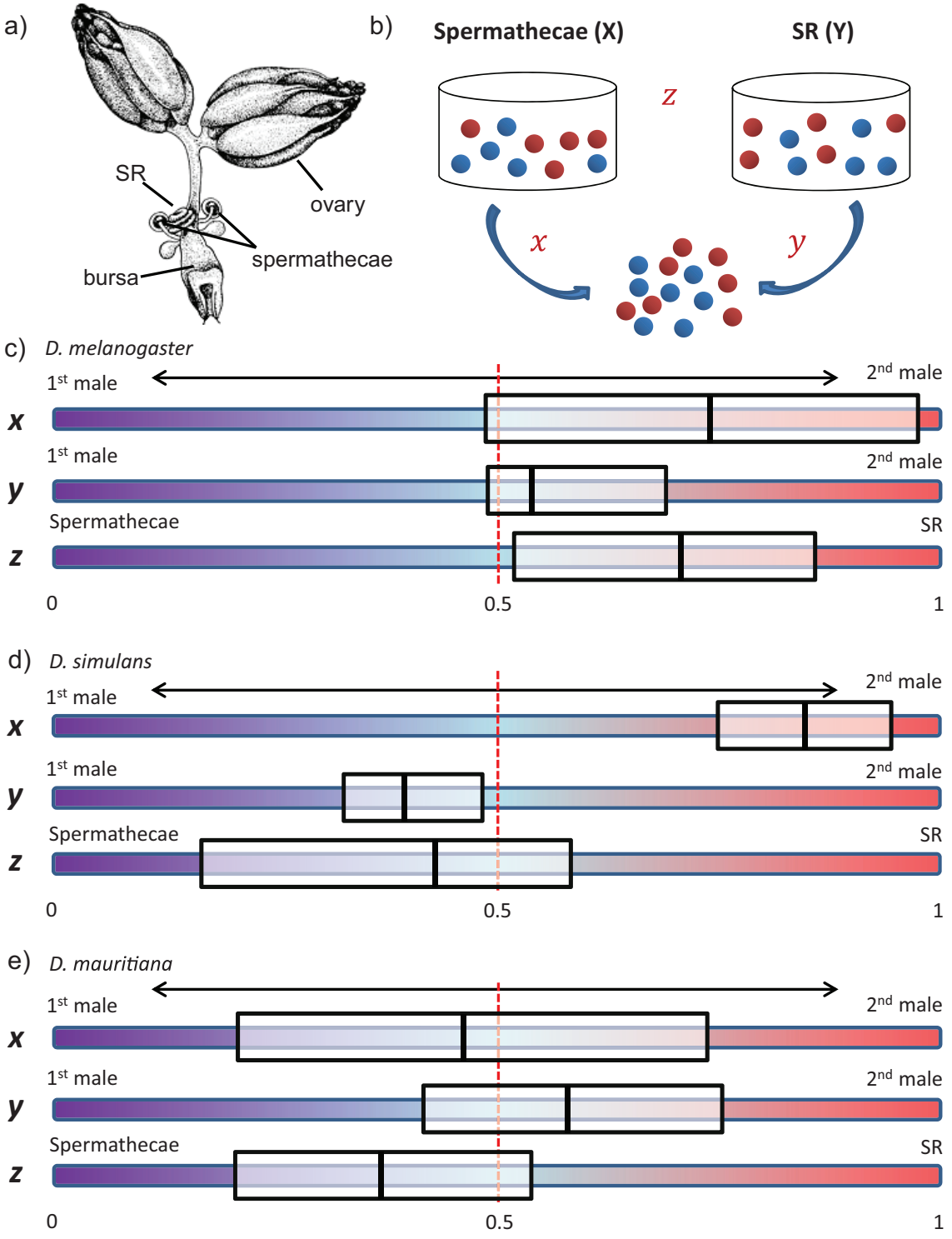

Figure 2: Schematic of the female reproductive tract of *Drosophila simulans* (*a*; from Patterson 1943), describing the three estimated sources of bias (*b*) from the spermathecae (x); seminal receptacle (SR; y) and between the two storage organ types, z; and the fertilization bias results from *Drosophila melanogaster* (*c*), *D. simulans* (*d*), and *Drosophila mauritiana* (*e*). Boxes (estimate mean and 95% confidence interval) overlapping with 0.5 indicate no significant bias; x or $y < 0.5$ indicates first-male bias, and x or $y > 0.5$ indicates second-male bias; $z < 0.5$ is biased toward the spermathecae, and $z > 0.5$ is biased toward the SR.

Table 1: Variables/parameters and their definitions

Variable	Definition	Original reference
x	Fertilization bias toward second-male sperm originating from the spermathecae (range 0 to 1)	This study
y	Fertilization bias toward second-male sperm originating from the seminal receptacle (SR) (range 0 to 1)	This study
z	Fertilization bias favoring the SR over the spermathecae (range 0 to 1)	This study
$X1$	Number of first-male sperm in the spermathecae	This study
$X2$	Number of second-male sperm in the spermathecae	This study
$Y1$	Number of first-male sperm in the SR	This study
$Y2$	Number of second-male sperm in the SR	This study
$N1$	Number of offspring sired by the first male to mate	Eggert et al. 2003[a]
$N2$	Number of offspring sired by the second male to mate	Eggert et al. 2003[a]
p_1	Probability that the first male's sperm enters the fertilization set	Parker et al. 1990
p_2	Probability that the second male's sperm enters the fertilization set	Parker et al. 1990
P_2	Proportion of progeny sired by the second male	Boorman and Parker 1976
r	p_1/p_2; bias of first-male sperm relative to second-male sperm in forming the fertilization set	Parker et al. 1990
$S1$	Number of sperm transferred by the first male to mate	Parker et al. 1990[a]
$S2$	Number of sperm transferred by the second male to mate	Parker et al. 1990[a]
S_2	Proportion of second-male sperm stored by the female	Hellriegel and Bernasconi 2000
T	Economy of scale, quantifying disproportionate returns to sperm number	Neff and Wahl 2004

[a] Number subscripted in original paper.

2*a*), where they displace resident first-male sperm from storage (primarily from the SR) back into the bursa (Manier et al. 2013*a*). The displacement process ends when the female forcibly ejects virtually all of the sperm in her bursa, consisting of excess second-male sperm and displaced resident sperm. At this point, second-male sperm are no longer entering storage and displacing resident sperm, and all sperm remaining in the SR and spermathacae potentially constitute the fertilization set.

Male- and female-mediated mechanisms influencing formation of the fertilization set, and their interspecific divergence among all three species, were directly quantifiable and are reported elsewhere (Manier et al. 2010, 2013*a*; Lüpold et al. 2012). Because of inherent difficulties in observing sperm use for fertilization directly or identifying the fertilization set at multiple time points for individual females, it is not possible to empirically identify the fertilization set or to directly quantify any fertilization bias, thus necessitating a modeling approach. In this note, we use an analytical model to identify the fertilization set and to estimate fertilization bias that occurs after the fertilization set is established. The model uses empirical data for *Drosophila melanogaster*, *Drosophila simulans*, and *Drosophila mauritiana* on the numbers of progeny sired by the first and second male to mate with each female and on the number of each male's sperm remaining in the SR and spermathecae, respectively, following progeny production (Manier et al. 2013*a*). Our data are available from the Dryad Data Depository, http://dx.doi.org/10.5061/dryad.jd87f (Manier et al. 2013*c*). For each species, we estimate two independent parameters of fertilization bias within each sperm-storage organ type (the SR and spermathecae) as well as a third parameter that quantifies any biased use of sperm from one storage organ type over the other and thus identifies the fertilization set. Our analysis builds on a model originally developed by Parker et al. (1990), which was then embellished by Eggert et al. (2003) and Neff and Wahl (2004).

The original raffle model from Parker et al. (1990; henceforth "Parker model") defines P_2, the proportion of second-male progeny (Boorman and Parker 1976), as

$$P_2 = \frac{S2}{rS1 + S2}, \tag{1}$$

where $r = p_1/p_2$, the probabilities of first- or second-male sperm entering the fertilization set, and $S1$ and $S2$ are numbers of first- and second-male sperm transferred, respectively. For clarity, we will refer here to proportions using a subscript (e.g., S_2 as the proportion of second-male sperm) and numbers or counts without a subscript (e.g., $S2$ as number of second-male sperm), even if the original model was published with the subscripts (table 1). Inverting both sides of the Parker model creates a linear regression of $S1/S2$ on $1/P_2$ with a slope of r:

$$\frac{1}{P_2} = r\left(\frac{S1}{S2}\right) + 1. \quad (2)$$

However, the linear model produces nonnormal data due to its dependency on ratios and was revised by Eggert et al. (2003):

$$\log\frac{N2}{N1} = \log\frac{S2}{S1} + \log\frac{p_2}{p_1}, \quad (3)$$

with $N1$ and $N2$ being numbers of progeny sired by the first and second male, respectively. The log transformations of the Eggert et al. (2003) model can help restore normality to the regression of $S1/S2$ on $1/P_2$, but they necessitate nonzero values for $N1$ and $N2$. Using maximum likelihood methods, Neff and Wahl (2004) proposed a nonlinear solution that additionally incorporates a measure of economy of scale, t, quantifying disproportionate returns to number of sperm, such as in cases of sperm conjugation (Higginson and Pitnick 2011). They also redefine r as a bias relative to the second male's sperm, to be consistent with the focus on P_2 (Neff and Wahl 2004; also see Eggert et al. 2003):

$$\frac{N1}{N1 + N2} = \frac{S1^t}{S1^t + rS2^t}. \quad (4)$$

When $r > 1$, fertilization is biased in favor of the second male, and when $r < 1$, fertilization bias favors the first male.

We build on this conceptual framework with a model that tests for fertilization bias within each of two sperm storage sites and relative sperm use between sperm storage sites. We have previously performed a rudimentary analysis in which we estimated the slope of the regression of S_2, in this case referring to the proportion of second-male sperm in storage (Hellriegel and Bernasconi 2000), on P_2 (Manier et al. 2010; also see Holman et al. 2011). We expected that in the absence of any fertilization bias, the slope should equal 1, while a bias favoring the first male should yield a slope less than 1 and a second-male bias a slope greater than 1. We now consider this approach to be inadequate due to (1) nonlinear relationships between S_2 and P_2 that may arise under biased fertilization and (2) nonindependence of separate analyses on each storage organ type. Specifically, regressions of S_2 on P_2 using sperm storage data in the SR and spermathecae separately cannot be independent, because both analyses include the same P_2 data. Here, we reanalyze the *D. melanogaster* data using a binomial mixture model (McLachlan and Peel 2000; Royle 2004) specifically applied to a two-organ model (TOM) that is estimated using nonlinear maximum likelihood methods after Neff and Wahl (2004). We compare results from the TOM among three closely related species, *D. melanogaster*, *D. simulans*, and *D. mauritiana*.

Methods

The Model

For our specific model, we began with a modified version of the Parker model,

$$P_2 = \frac{rS2}{S1 + rS2}, \quad (5)$$

with r quantifying bias relative to the second male's sperm, following Neff and Wahl (2004). Using the same general structure, we then modified this model to a two-organ model (TOM) that estimates fertilization biases toward the second male in the spermathecae (X) and SR (Y) independently by the two fertilization bias parameters x and y, respectively:

$$P_2 = \frac{X2(x)(1 - z) + Y2(y)(z)}{(1 - z)[X1(1 - x) + X2(x)] + z[Y1(1 - y) + Y2(y)]}, \quad (6)$$

where $X1$, $X2$, $Y1$, and $Y2$ are the numbers of first- and second-male sperm in each of two separate storage organs, with X and Y obtained from actual sperm counts (Manier et al. 2010, 2013*a*). The parameter z represents disproportionate sperm use from storage organ Y (fig. 2*b*) and thus identifies the fertilization set as including primarily X, primarily Y, or both. This equation is a type of mixture model, which is a class of probabilistic models that allow subpopulations within an overall population to be identified. They have been extensively used in a wide range of disciplines, including statistics, economics, forensics, chemistry, and physics, as well as numerous fields within biology (e.g., ecology, evolution, behavior, genomics, biochemistry, microbiology) including studies of sperm competition (Pattarini et al. 2006). An extensive mathematical treatment and examples of applications are provided in McLachlan and Peel (2000). Here, we specifically use a binomial mixture model with binomial parameters x, y, and z. We treat the paired spermathecae as a single unit, since, unlike with the yellow dung fly *Scathophaga stercoraria* (Ward 1993; Otronen et al. 1997; Hellriegel and Bernasconi 2000), we have no evidence to suggest that they store or use sperm differently in *Drosophila*. Parameters x, y, and z are all bounded by 0 and 1; when $z = 0.5$, sperm are equally likely to be selected from either storage organ X or Y, and x or $y = 0.5$ represent equal probabilities for first- or second-male sperm to be used for fertilization (i.e., no fertilization bias, meaning sperm

from competing males used in proportion to their representation).

We examined variation in fertilization bias within sperm-storage organs and identified the fertilization set for three closely related *Drosophila* species (*D. melanogaster*, *D. simulans*, and *D. mauritiana*). Full details on experimental methods for the data sets of these species can be found in Manier et al. (2010) and Manier et al. (2013*a*). For all three species, females were initially mated to a male with sperm heads expressing green or red fluorescent protein (GFP or RFP) and subsequently mated to a male of the reciprocal color. Females were allowed to oviposit in fresh food vials up to 72 h after remating and were then flash-frozen in liquid nitrogen. Female reproductive tracts were dissected, and GFP and RFP sperm in the SR and spermathecae counted. Adult progeny were scored for paternity using labeled sperm in the testes of sons (*D. melanogaster* and *D. mauritiana*) or a fluorescent eye marker (*D. simulans*). All models were estimated using maximum likelihood following Neff and Wahl (2004), implemented in SAS using PROC NLMIXED (SAS Institute 2008).

Model Testing

Our model aims to predict how sperm in storage will be used for future fertilizations, but empirical estimates for sperm numbers in storage reflect sperm remaining in storage after some fertilization and progeny production have already occurred. In order to examine the effect of this time lag between sperm used (i.e., progeny produced) and sperm remaining, we ran a series of simulations modeling sperm use. The simulation conditions were obtained by permuting two values for the distribution of sperm in storage over three values for each of x, y, and z for a total of $2 \times 3^3 = 54$ simulated data sets. For each data set, 500 sperm were allocated to the SR or spermathecae according to a random binomial variate (representing first- or second-male sperm) with a probability of 0.5 or 0.75, representing the proportion of sperm in the SR. From the resulting numbers of first- and second-male sperm in each storage organ ($X1$, $X2$, $Y1$, and $Y2$), a subset of 50 sperm were then allocated for "fertilization" using a randomly generated uniform variate between 0 and 1, defined by the model parameters (x, y, and z), which were set to a value of 0.25, 0.5, or 0.75 in all possible combinations. This sampling process was repeated 100 times to represent 100 simulated females for each condition. The two proportions of sperm distributed among storage organ types (0.5 and 0.75), sperm numbers originally in storage (500) and sperm numbers sampled for fertilization (50) were based on empirically determined typical values for these *Drosophila* species (Manier et al. 2010, 2013*a*; Lüpold et al. 2012). We used both nonlinear regression implemented in SAS PROC NLIN and mixed-model nonlinear regression implemented in SAS PROC NLMIXED to compare parameter estimates obtained from the original storage numbers with those obtained from the storage numbers after sampling without replacement. Both approaches yielded similar results; only those from PROC NLIN are reported here.

The simulation served two purposes. First, we asked whether the parameter estimates for each of the 54 data sets were significantly different from the starting parameters designated for that data set. Second, we asked whether the parameter estimates obtained by sperm counts after sampling for fertilization were significantly different from those using the sperm counts in storage before fertilization. These two sets of comparisons were carried out using paired t-tests for each parameter (x, y, and z), and results are expressed in terms of the mean absolute difference in parameter ($|\text{diff}|$), t-statistic, and P value.

Model Implementation

We applied the TOM to our empirical data using PROC NLMIXED. Parameters were bounded by their defined limits and initial values set at their null model values of $x = y = z = 0.5$. Because maximum likelihood calculations in SAS take the logarithm of the likelihood function, all observed values of P_2 that were 0 or 1 were replaced by 0.0001 or 0.9999, respectively. In PROC NLMIXED, we used the model statement "Model P2~B(N1 + N2, P2);" where B represents binomial, $N1$ is the number of offspring sired by the first male, $N2$ is the number of offspring sired by the second male, and P_2 is the proportion of offspring from the second male ($P_2 = N2/(N1 + N2)$). Parameter significance was determined by t-statistics.

Results

We found no difference for x, y, or z between the parameter estimates and the original starting parameters ($|\text{diff}| < 0.004$, $t < 1.09$, $P > .28$). We also found no difference in parameter estimates obtained from sperm counts in storage before versus after sampling for fertilization ($|\text{diff}| < 0.002$, $t < 1.19$, $P > .24$). We conclude that the model is effective at obtaining accurate estimates of fertilization bias parameters from two sperm-storage organs and that using the number of sperm left in storage is a reasonable approach to estimating the parameters of the model.

When applied to the *Drosophila melanogaster* data set, the TOM showed no significant fertilization bias (i.e., nonsignificant difference from the null of 0.5) in both the spermathecae ($x = 0.74 \pm 0.12$, $t_{76} = 1.96$, $P = .054$; fig.

2*c*) and the SR ($y = 0.55 \pm 0.028$, $t_{76} = 1.87$, $P = .066$; fig. 2*c*), though both types of storage organ trended toward the second male. Sperm use from the different storage organs was significantly biased toward the SR ($z = 0.69 \pm 0.090$, $t_{76} = 2.09$, $P = .040$; fig. 2*c*). The pattern of sperm use for *Drosophila simulans* was more complex, with a second-male fertilization bias in the spermathecae ($x = 0.83 \pm 0.052$, $t_{64} = 6.37$, $P < .0001$; fig. 2*d*), a first-male fertilization bias in the SR ($y = 0.37 \pm 0.053$, $t_{64} = 2.35$, $P = .022$; fig. 2*d*), and no fertilization bias between storage organs ($z = 0.44 \pm 0.077$, $t_{64} = 0.84$, $P = .41$; fig. 2*d*). Our results for *Drosophila mauritiana* were different still, with unbiased fertilization both within and between storage organs ($x = 0.46 \pm 0.13$, $t_{79} = 0.27$, $P = .79$; $y = 0.58 \pm 0.08$, $t_{79} = 1.01$, $P = .32$; $z = 0.38 \pm 0.08$, $t_{79} = 1.47$, $P = .15$; fig. 2*e*).

Discussion

In this study, we used an analytical model to simultaneously estimate three parameters representing fertilization bias within two types of female sperm-storage organ (x and y) and the fertilization set (z) for three closely related species. There were significant differences among species for all three parameter estimates (fig. 2), revealing that these traits are evolutionarily labile, despite general similarities in female reproductive tract morphology and the intensity of postcopulatory sexual selection (Manier et al. 2013*a*). The identification of the sources of sperm for fertilization revealed that the fertilization set in *Drosophila melanogaster* comprises primarily sperm in the SR, consistent with conclusions from previous studies using less rigorous methods (e.g., Manier et al. 2010). In contrast, sperm from both storage organ types statistically comprise the fertilization set in *Drosophila simulans* and *Drosophila mauritiana*, with the spermathecae playing an equal-to-larger role than the SR in providing sperm for fertilization. At the same time, no significant fertilization bias was detected within either the spermathecae or the SR in *D. melanogaster* and *D. mauritiana*. In contrast, *D. simulans* exhibited significant and opposing patterns of fertilization bias in the SR (first-male bias) and the spermathecae (second-male bias).

Species differences in fertilization bias and the fertilization set have implications for patterns of sperm precedence and for female-generated sexual selection on male ejaculate characters. Because displacement of resident sperm from storage by sperm from the second male is largely restricted to the SR in all three species, it is not uncommon for the ratios of competing sperm to differ between the SR and the spermathecae (Manier et al. 2013*a*; also see cover for *Reproduction* 145(5), http://www.reproduction-online.org/). Differences in the relative contribution of sperm from the two organ types can therefore directly impact sperm precedence. In addition, only the SR is believed to generate selection favoring the production of costly, longer sperm (Miller and Pitnick 2002, 2003; Pattarini et al. 2006; Lüpold et al. 2012). Consequently, the greater contribution of the spermathecae to the fertilization set in *D. simulans* and *D. mauritiana* may explain the considerably shorter sperm of these species relative to *D. melanogaster* (Manier et al. 2013*a*). For any taxon, resolving functional aspects of female sperm handling, including the fertilization set and fertilization bias, would likely advance our understanding of diversification in male adaptations to postcopulatory sexual selection in any taxon.

Based on the within-organ fertilization bias estimates, we predict *D. simulans* to have a greater potential for female sperm choice than *D. melanogaster* or *D. mauritiana*. Had these biases been consistent in direction across organ types, then it would not be possible to interpret the patterns as being attributable to male- versus female-mediated mechanisms. For example, hypothetical second-male sperm advantage in both organ types could be explained by females somehow exerting a bias in favor of sperm from her more recent mate and/or by detrimental effects of sperm aging while in storage rendering first-male sperm less competitive, the second-male ejaculate impairing first-male sperm performance, and so forth. However, the observed diametrically opposed biases in *D. simulans* can be explained only by female-mediated differences in action or condition between the spermathecae and the SR. Mechanisms that could maintain consistent and opposing fertilization biases are unknown but may involve differential patterns of gene expression underlying organ-specific biochemical environments (e.g., as shown in *D. melanogaster*: Prokupek et al. 2008, 2009, 2010).

It is important to note that female-mediated fertilization bias does not constitute evidence for female sperm choice. Our experimental design only allowed detection of fertilization bias with respect to mating order (i.e., toward first-male or second-male sperm). Because we did not systematically vary male quality, we cannot infer from these results alone that *D. simulans* females use the demonstrated fertilization biases for cryptic female choice nor can we rule out the possibility of fertilization biases in *D. melanogaster* and *D. mauritiana* when a disparity in male quality is present.

For the demonstrated within-organ fertilization biases of *D. simulans* to function as a mechanism of sperm choice, females would have to manifest plasticity in the among-organ fertilization bias (z), adaptively shifting the source of sperm for fertilization toward whichever organ matches the directional disparity in male quality. Although we have not performed the critical test of this prediction using

within-species variation in male quality, we have demonstrated precisely this mechanism of sperm choice using an extreme case of differential male quality: species identity. When *D. simulans* females were inseminated by both a *D. simulans* and a *D. mauritiana* male, females significantly switched between using sperm predominantly from the SR when the conspecific male mated first and from the spermathecae when the conspecific male mated second, thus biasing fertilization toward conspecific sperm (i.e., conspecific sperm precedence; Price 1997; Howard et al. 2009) irrespective of mating order (Manier et al. 2013*b*). Although this may seem unexpectedly sophisticated, biased use of sperm from different storage sites has also been demonstrated for the giant cuttlefish *Sepia apama* (Naud et al. 2005), and there is evidence that females of the damselfly *Calopteryx splendens xanthostoma* similarly shift between different storage locations differing in composition of competing sperm dependent on the context of oviposition (Siva-Jothy and Hooper 1995, 1996).

In this study, we analyzed the composition of the fertilization set empirically remaining after some fertilization had already occurred. Based on comparisons with simulated data sets, we concluded that this approach is appropriate for our system. However, this conclusion may not be appropriate for cases where a larger percentage of sperm is used for fertilization, and thus, the numbers left in storage may not be representative of those used for fertilization. Nevertheless, most species transfer and store far more sperm than our study organisms while using fewer sperm for fertilization. Males within the *Drosophila* lineage produce some of the longest sperm recorded (Pitnick et al. 1995), and sperm length trades off with sperm number across species (Immler et al. 2011). Sperm use efficiency in these species also tends to be extremely high, with up to only a few sperm dedicated to fertilizing each egg (Lefevre and Johnsson 1962; Snook and Markow 2002; Manier et al. 2013*a*). In other species with smaller sperm and larger sperm numbers, hundreds or thousands of sperm (out of millions transferred) reach storage or the site of fertilization (Pitnick et al. 2009), while only a small proportion of these may be used for fertilization. Because our model was effective using the much smaller numbers of sperm stored in *Drosophila*, it should be applicable in most other cases in which many more sperm are stored than are used.

Females of many species have multiple sperm-storage organs and organ types (Pitnick et al. 2009), and our model can be applied to any of these species for which first- and second-male sperm can be counted (e.g., Otronen et al. 1999). In some instances, proportions of second-male sperm (i.e., S_2) are more readily obtained, such as by competitive polymerase chain reaction (Bussiere et al. 2010), in which case the relationship between S_2 and P_2 can be applied to confirm or rule out fair raffle within a single sperm-storage organ (Holman et al. 2011). Our model could also be modified to account for multiple sperm-storage organs using second-male sperm and paternity proportions, but this approach necessarily uses less information (i.e., a single proportion rather than numbers of first- and second-male sperm), and we therefore expect it would be less accurate than our model.

Despite its importance to our understanding of evolutionary diversification and speciation, female reproductive tract design and function have received relatively little empirical attention. Female reproductive tract morphology, especially of the sperm-storage organs and their associated traits (e.g., ducts and secretions), tend to evolve rapidly, giving rise to dramatic differences between species (reviewed by Pitnick et al. 2009) and even populations (Pitnick et al. 2003). There is a taxonomically widespread pattern of correlated evolution between female reproductive tract morphology and sperm structure (reviewed by Pitnick et al. 2009). Seminal fluid proteins and female secretions and receptors that interact with these proteins are also predicted to exhibit rapid, correlated evolution, but there is not sufficient knowledge of the female side of the equation to test this prediction (Ravi Ram and Wolfner 2007; Pitnick et al. 2009). Female reproductive tract innervation and secretory biology are also known to interact with the ejaculate in ways critical for sperm storage and fertilization (e.g., Adams and Wolfner 2007; Avila and Wolfner 2009; Schnakenberg et al. 2011) and hence may also play a role in postcopulatory sexual selection. Our limited knowledge suggests that female reproductive tracts undergo rapid evolutionary diversification, with important implications for the intensity of sexual selection generated on males in general and on the form and function of numerous ejaculate traits in particular (Swanson et al. 2001; Miller and Pitnick 2002; Higginson et al. 2012*a*, 2012*b*). Important advances in postcopulatory sexual selection theory are likely to come from research into the selective causes of female reproductive tract divergence and addressing whether the same models for the evolution of premating female preferences (Andersson 1994) are sufficient to explain divergence in female tract traits functioning as the proximate basis of sperm choice (e.g., Curtsinger 1991; Keller and Reeve 1995; Simmons and Kotiaho 2007).

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