RAPID DIVERSIFICATION OF SPERM PRECEDENCE TRAITS AND PROCESSES AMONG THREE SIBLING DROSOPHILA SPECIES

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Postcopulatory sexual selection is credited with driving rapid evolutionary diversification of reproductive traits and the formation of reproductive isolating barriers between species. This judgment, however, has largely been inferred rather than demonstrated due to general lack of knowledge about processes and traits underlying variation in competitive fertilization success. Here, we resolved processes determining sperm fate in twice-mated females, using transgenic Drosophila simulans and Drosophila mauritiana populations with fluorescently labeled sperm heads. Comparisons among these two species and Drosophila melanogaster revealed a shared motif in the mechanisms of sperm precedence, with postcopulatory sexual selection potentially occurring during any of the three discrete stages: (1) insemination; (2) sperm storage; and (3) sperm use for fertilization, and involving four distinct phenomena: (1) sperm transfer; (2) sperm displacement; (3) sperm ejection; and (4) sperm selection for fertilizations. Yet, underlying the qualitative similarities were significant quantitative differences in nearly every relevant character and process. We evaluate these species differences in light of concurrent investigations of within-population variation in competitive fertilization success and postmating/prezygotic reproductive isolation in hybrid matings between species to forge an understanding of the relationship between microevolutionary processes and macroevolutionary patterns as pertains to postcopulatory sexual selection in this group.

KEY WORDS: Cryptic female choice, fertilization, postcopulatory sexual selection, reproductive isolation, sperm competition, sperm storage.

When a female mates with multiple males, their ejaculates may temporally overlap (Parker 1970), generating potential conflict between the sexes over paternity (Parker 1979; Arnaqvist and Rowe 2005), selection on male traits that enhance competitive fertilization success (Parker 1970, 1998; Simmons 2001; Snook 2005; Pizzari and Parker 2009) and selection on female traits that mediate cryptic female choice (Birkhead et al. 1993; Eberhard 1996). These selective pressures collectively constitute postcopulatory sexual selection (PSS), which is amplified by specialized long-term sperm-storage organs within the female reproductive tract of most internally fertilizing species (Birkhead and Møller 1993; Eberhard 1996; Neubaum and Wolfner 1999; Pitnick et al. 2009a). As with premating competition and choice, PSS is credited with driving rapid diversification of reproductive characters among isolated populations and, hence, playing an important role in formation and maintenance of species boundaries (Markow 1997; Parker and Partridge 1998; Eady 2001; Coyne and Orr 2004; Ritchie 2007; Howard et al. 2009; Kraaijeveld et al. 2011; Maan and Seehausen 2011; Butlin et al. 2012).

A causal relationship between PSS and reproductive isolation, however, has not been empirically established (Howard et al. 2009), and the role of PSS in diversification remains largely
inferential. The strongest inference derives from observations of rapid divergence in four classes of traits intuitively expected to be the principal mediators of competitive fertilization success: (1) male genitalia (size, shape, and complexity of accessory structures; Eberhard 1985; Leonard and Córdoba-Aguilar 2010); (2) seminal plasma composition (Poiani 2006); (3) sperm characters (size, shape, number; Snook 2005; Pitnick et al. 2009b); and (4) female reproductive tract characters (e.g., duct and sperm-storage organ morphology, secretory biochemistry; Pitnick et al. 2009a). Nevertheless, structure–function relationships for these traits, and thus selective process, are poorly understood.

For example, seminal fluid is complex in most internally fertilizing species (Poiani 2006), with about 150 seminal fluid proteins [Sfps] in Drosophila melanogaster alone (Findlay et al. 2008, 2009). Many Sfps are widely regarded as important mediators of competitive fertilization success and likely targets of sexual selection (Chapman 2001; Ravi Ram and Wolfner 2007). However, only a few studies have demonstrated a direct association between within-population Sfp variation (i.e., sequence variation) and sperm competition outcomes (Clark et al. 1995; Fuimera et al. 2005, 2007; Zhang et al. 2012), and only a single study directly implicates PSS in the evolutionary divergence of an Sfp (Dorus et al. 2004).

Direct demonstrations of trait divergence due to PSS are rare due to three major empirical challenges: (1) observing events within the female reproductive tract; (2) discriminating among the sperm of competing males and, hence; and (3) quantifying trait variation in the appropriate context of in vivo competition. Despite these challenges, several studies have successfully associated variation in male genitalia, sperm, or female reproductive tract traits with variation in sperm competition success in a variety of internally fertilizing species (e.g., Radwan 1996; Malo et al. 2005; Dziminski et al. 2009; Chow et al. 2010; Gasparini et al. 2010; Boschetto et al. 2011; see reviews by Simmons 2001; Snook 2005; Pizzari and Parker 2009). Other studies have used clever approaches such as phenotypic “engineering” of putative sperm competition traits using laser ablation (Hotzy et al. 2012) or experimental evolution (Hosken and Ward 2001; Morrow and Gage 2001; Pitnick et al. 2001; Miller and Pitnick 2002, 2003; Martin and Hosken 2004; Pattarini et al. 2006; Hotzy et al. 2012), in vitro assays of sperm performance (e.g., Birkhead et al. 1999; Froman et al. 2002), or exploitation of among-population variation (e.g., Hotzy and Arnqvist 2009). Numerous comparative studies have also found significant relationships between putative targets of PSS (e.g., relative testis mass and sperm length) and sperm competition risk or female reproductive tract morphology (reviewed by Snook 2005; Pitnick et al. 2009a, b; Pizzari and Parker 2009). What is largely lacking even in the majority of these studies, however, is an understanding of how the phenotypic variation confers a reproductive advantage.

Our best understanding of functional diversification by PSS comes from LaMunyon and Ward’s (1998, 1999, 2002) elegant experiments and direct observation of competitive fertilization processes in Caenorhabditis elegans, made possible by the nematode’s transparency. They used (1) intraspecific comparisons of hermaphrodite and male amoeboid sperm size, performance, and fertilization success; (2) experimental enhancement of sperm competition intensity and evolutionary response of sperm size; and (3) phylogenetic analysis of sperm size and sperm competition risk codiversification to convincingly establish the relationship between microevolutionary process and macroevolutionary pattern attributable to PSS.

Our goals here were to resolve the detailed processes and interacting sex-specific traits that underlie patterns of competitive fertilization success and their evolutionary diversification among three closely related species of Drosophila (Drosophila melanogaster, Drosophila simulans, and Drosophila mauritiana). We created transgenic lines of D. simulans and D. mauritiana with sperm heads expressing green- (GFP) or red-fluorescent protein (RFP), allowing unambiguous discrimination among sperm from competing males and detailed resolution of in vivo processes contributing to last-male sperm precedence (see Movies S1, S2). These three species diverged relatively recently, with the split between D. melanogaster and D. simulans occurring 2–3 million years ago, whereas D. mauritiana arose from a cosmopolitan D. simulans-like ancestor approximately 260,000 years ago (Throckmorton 1975; Hey and Kliman 1993; Kliman et al. 2000; McDermott and Kliman 2008). We discuss diversification in sperm precedence traits and processes across the three species in the context of (1) traits contributing to competitive fertilization success within species (e.g., Miller and Pitnick 2002, 2003; Pattarini et al. 2006; Lüpold et al. 2012) and (2) mechanisms of conspecific sperm precedence between species (Howard 1999; Howard et al. 2009; Manier et al., 2013). Our overarching goals are to establish the role of PSS in reproductive trait diversification and, as a consequence, speciation.

Methods

GENERAL EXPERIMENTAL PROTOCOLS

We used D. simulans and D. mauritiana with GFP- or RFP-labeled sperm heads (henceforth “GFP” or “RFP”) to quantify species-specific (1) numbers of sperm transferred, stored, and ejected in first matings to virgin females; (2) spatiotemporal patterns of second-male sperm transfer, storage, and displacement of first-male sperm; (3) first-male sperm velocity in storage during displacement; (4) female ejection of excess second-male and displaced first-male sperm; and (5) bias in competitive sperm use for fertilizations over the course of 72 h following
remating. All patterns of sperm storage, displacement, and use were assessed and analyzed separately for the two types of female sperm-storage organs: the paired spermathecae (ST) and the seminal receptacle (SR). For each species, all females derived from the same wild-type population into which the original transgenic lines were backcrossed. Within each experiment, females, GFP males, and RFP males were randomly assigned to treatments. In the sperm velocity experiments, first males were always from the GFP lines; in all other experiments, females were randomly assigned to a GFP or RFP first male in a balanced design, with the second male of the alternate color in the female remating treatments. The experimental treatments were identical to Manier et al. (2010), thereby allowing direct comparisons with previous results for *D. melanogaster* (when necessary, those data were reanalyzed), except for data on first matings and the number of sperm ejected, as these variables were not assayed in the previous study. Sample sizes and means of all variables are presented in Table 1. Each value presented in Results represents the mean ± 1 SE. See online Supporting Information for details of transgenic line generation and within-species analyses of GFP versus RFP population differences.

All stocks were maintained at ambient room temperature (23–25°C) and light regime in half-pint milk bottles on standard corn meal–agar–yeast–molasses medium sprinkled with live yeast grains. Starting density was 100 flies, and adults were transferred to new bottles every 2–3 days. Experimental males and females

### Table 1. Mean ± SE, *N*, and letters indicating significant differences (post hoc comparisons by Tukey's studentized range test at *α* = 0.05) for reproductive and sperm traits for first and second matings of *Drosophila simulans* and *Drosophila mauritiana* and second matings from *Drosophila melanogaster*.

<table>
<thead>
<tr>
<th>Mating</th>
<th><em>Drosophila simulans</em></th>
<th><em>Drosophila mauritiana</em></th>
<th><em>Drosophila melanogaster</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
<td>First</td>
</tr>
<tr>
<td>Remating interval (days)</td>
<td>2.7±0.04</td>
<td>3.46±0.07</td>
<td>3.5±0.03</td>
</tr>
<tr>
<td>Copulation duration (min)</td>
<td>23.3±0.30</td>
<td>15.8±0.24</td>
<td>23.7±0.69</td>
</tr>
<tr>
<td>Num. sperm transferred</td>
<td>1340±96.74</td>
<td>1532±103.3</td>
<td>2348±93.3</td>
</tr>
<tr>
<td>Ejection time (min ASM)</td>
<td>183±5.2</td>
<td>125±4.5</td>
<td>101±6.0</td>
</tr>
<tr>
<td>Sperm stored (SR)</td>
<td>316±17.3</td>
<td>228±9.58</td>
<td>309±12.6</td>
</tr>
<tr>
<td>Sperm stored (ST)</td>
<td>200±15.1</td>
<td>201±12.8</td>
<td>237±12.5</td>
</tr>
<tr>
<td>Sperm stored (total)</td>
<td>516±25.7</td>
<td>430±16.9</td>
<td>545±21.4</td>
</tr>
<tr>
<td>Sperm stored (second male)</td>
<td>522±18.4</td>
<td>425±23.1</td>
<td>309±15.0</td>
</tr>
<tr>
<td>Residual sperm at remating</td>
<td>134±10.0</td>
<td>252±8.83</td>
<td>214±6.54</td>
</tr>
<tr>
<td>Proportion sperm displaced</td>
<td>0.38±0.04</td>
<td>0.48±0.04</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>S2 (SR)</td>
<td>0.80±0.03</td>
<td>0.88±0.02</td>
<td>0.79±0.03</td>
</tr>
<tr>
<td>S2 (ST)</td>
<td>0.82±0.02</td>
<td>0.77±0.02</td>
<td></td>
</tr>
<tr>
<td>Sperm length (mm)</td>
<td>1.10±0.007</td>
<td>0.98±0.006</td>
<td>1.85±0.008</td>
</tr>
<tr>
<td>SR length (mm)</td>
<td>1.22±0.02</td>
<td>1.03±0.03</td>
<td>2.22±0.03</td>
</tr>
</tbody>
</table>

1 Data arcsine square root transformed before statistical analysis.

2 Data from Amitin and Pitnick (2006).
were collected as virgins, maintained in plastic vials with 1.5 cm³ medium supplemented with live yeast and used at 3–4 days posteclosion (with the exception of the D. mauritiana spatiotemporal experiment, in which flies were 3–9-day-old virgins, with all ages equally distributed across all treatments; otherwise all males and females were randomly assigned among treatments). During experiments, females and males were paired in individual vials, the start and end times of all copulations were recorded to the nearest minute, and males were removed from vials shortly after the end of copulation.

Reproductive tracts were dissected from CO₂-anesthetized or frozen females and slide mounted as described in Manier et al. (2010). Slides were stored at 4 °C until counting, and all slides were counted within a week of dissection at 400× on an Olympus BX60 compound microscope with an X-Cite Series 120 fluorescent lamp (EXFO Life Sciences, Mississauga, Ontario, Canada) and multiband GFP-DsRed-A filter set (Semrock, Rochester, NY). Individual GFP and RFP fluorescent sperm were counted in the bursa, SR, and ST. Statistical analyses were performed in R version 2.12.1 (R Core Team 2010). Sperm and SR length was determined for each species using methods of Amitin and Pitnick (2007), N = 10 males and N = 20 females assayed per species for D. simulans and D. mauritiana. For D. melanogaster, sperm length derives from the mean measurements among 32 isogenic lines, with N = 5 males sampled per line. For generation and maintenance of these lines, see Lüpold et al. (2012). Seminal receptacle measurements are from F₁ females generated by diallel crosses among 10 of the above isogenic lines, with N = 6 females measured for each of 90 nondiagonal, reciprocal line crosses (Lüpold et al. 2013).

**SPERM TRANSFER AND STORAGE AFTER VIRGIN MATING**

We examined the number of sperm transferred and stored after a single mating with either a GFP or an RFP male by flash-freezing females and males in liquid nitrogen, either immediately after copulation (for sperm transferred) or 2 h after the start of mating (for sperm stored). The 2 h time point was chosen based on D. melanogaster data, showing that the total number of sperm stored after a second mating reached a maximum after 1 hour.

**SPATIOTEMPORAL PATTERNS OF SPERM STORAGE AND USE AFTER REMATING**

Females were mated to a GFP or an RFP male and provided the opportunity to remate 2 days later to a male of the reciprocal sperm-tag color, with additional 6-h remating opportunities on days 3–4 (D. simulans) or 3–5 (D. mauritiana) for any refractory females. Pairs in copula or single, post copula females were flash-frozen at different time-points after the start of mating (ASM): 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 2 h, 4 h, 24 h, 48 h and 72 h. Females in the 24, 48, and 72 h treatments were transferred to fresh vials daily, and paternity of eclosing offspring was quantified. For D. simulans, paternity was determined for all eclosed progeny using a GFP eye marker present in that species’ GFP-labeled sperm line. Because neither of the D. mauritiana populations has an eye marker, paternity was determined for eclosed sons only, through dissection and examination of the testes for GFP or RFP sperm. For D. mauritiana, the 24, 48, and 72-h treatments were replicated to increase sample size in a separate experiment. Because P₂ (i.e., the proportion of progeny produced after remating that were sired by the second male) was not significantly different between these replicates (0.83 ± 0.02 and 0.90 ± 0.03, respectively; arcsine square-root transformed, t_{26} = 1.73, P = 0.09), the data were combined for subsequent statistical analyses. Females who produced no progeny before remating and had no first-male sperm in storage, and those producing no second-male progeny and having no second-male sperm in storage, were excluded from all statistical analyses. We found few differences between GFP and RFP sperm in D. simulans and D. mauritiana (see online Supporting Information); to compare general patterns among species, we combined data from both mating orders (i.e., GFP or RFP as first male).

**SPERM VELOCITY**

We examined sperm velocity in two ways. First, we determined if the first male’s sperm velocity in the SR changed in the presence of second male sperm 60 min after the start of the focal mating. This time point was determined in the spatiotemporal pattern experiments to be a time of peak sperm displacement for both species, with substantial numbers of first-male sperm reentering the bursa. Females were randomly assigned to one of three experimental treatments: (A) dissection 60 min after the start of first mating, (B) dissection 60 min after the start of second mating, and (C) females not given the opportunity to remate but dissected on the same timescale as treatment B. All second males were RFP. Females remated 2–4 days (D. simulans) or 3–5 days (D. mauritiana) after the first mating. Second, we quantified velocity of first- and second-male sperm temporally co-occurring in the SR.

Following female anesthesia with CO₂, each reproductive tract was dissected into 20 μL of Grace’s Supplemented Insect Medium (Invitrogen) at room temperature. A 10-second long movie (AVI) was recorded immediately upon subjecting the slide to epifluorescence (and within 5 min of anesthesia) at 400× magnification using a multiband GFP-DsRed-A filter set and an Olympus DP71 digital camera with DPController Software version 3.3.1.292 (Olympus America Inc.). For each movie, instantaneous linear velocities for up to 10 sperm were traced in ImageJ version 1.44p, using the Manual Tracking plugin (available at http://rsbweb.nih.gov/ij/plugins/index.html). Following log
transformation ($X' = \ln[X + 0.0001]$), mean log velocities were calculated for GFP and RFP sperm in the SR, and these values were used to calculate a grand-mean velocity for each male. Numbers of first- and second-male sperm in the SR were counted to statistically account for effects of sperm density on velocity.

**FEMALE SPERM EJECTION**

We quantified the number of first- and second-male sperm that females ejected after remating and the timing of ejection. Females from the 4 h time treatment of the spatiotemporal pattern experiments (above) were gently aspirated immediately after copulation into individual wells of glass 3-well spot plates (Pyrex) and covered with glass coverslips secured with spots of clay. Females were monitored for ejection every 10–15 min, until either an ejection was observed or the freeze time (i.e., 4 h) was reached. Each ejected mass was collected and slide mounted in PBS, and sperm from both males were counted. Ejection time for *D. melanogaster* is derived from females doubly mated to genetically standardized males (Lüpold et al. 2013).

**Results**

**SPERM AND FEMALE SR LENGTH**

Consistent with previous reports (Joly 1987; Joly and Bressac 1994; Pitnick et al. 1999), sperm and SRs lengths differ significantly among all three species. *Drosophila mauritiana* sperm and SRs were approximately 10% and 16% shorter, respectively, than those of *D. simulans*, and 49% and 51% shorter, respectively, than those of *D. melanogaster*. *Drosophila simulans* sperm and SRs were approximately 43% and 41% shorter, respectively, than those of *D. melanogaster* (Table 1).

**COPULATION DURATION AND NUMERICAL ASPECTS OF SPERM TRANSFER, EJECTION, AND STORAGE**

Copulations with virgin females were 45% longer in *D. simulans* (23.2 ± 0.25 min) than *D. mauritiana* (16.0 ± 0.24 min; $t_{291} = 20.5, P < 0.0001$). In contrast, there was no difference between species (including *D. melanogaster*) in copulation duration with nonvirgin females ($F_{2,767} = 2.13, P = 0.12$). This pattern is attributable to significantly longer copulations with virgin than with nonvirgin females in both *D. simulans* and *D. mauritiana* (note that males were virgins in both types of matings), but the difference was only 2 min in *D. simulans* (Table 1; paired $t$-test: $t_{201} = -3.21, P < 0.0016$), whereas it was 8 min in *D. mauritiana* (Table 1; paired $t$-test: $t_{39} = -10.15, P < 0.0001$).

Despite interspecific differences in virgin copulation duration, we found no differences in sperm numbers transferred to virgin females (Table 1; $t_{0.3} = 1.34, P = 0.19$). Comparing virgin and nonvirgin matings, however, males of both *D. simulans* and *D. mauritiana* transferred significantly more sperm to previously mated than to virgin females: over twice as many in *D. simulans* ($t_{55} = 8.18, P < 0.0001$) and about 50% more in *D. mauritiana* ($t_{58} = 5.18, P < 0.0001$). These sperm numbers are 98% and 65% higher, respectively, than those transferred by *D. melanogaster* males to previously mated females (Table 1). A previous study has also found that *D. melanogaster* males tailor their ejaculates based on female mating status by transferring about 15% more sperm to nonvirgin females (Lüpold et al. 2011).

We found significant differences among species and treatments in the timing of female sperm ejection of excess and displaced sperm, with means varying from about 1.7 to 3 h ASM (Table 1). Virgin *D. mauritiana* females ejected sperm significantly sooner than did virgin *D. simulans* females ($t_{58.9} = -8.66, P < 0.0001$; Table 1). Similarly, previously mated *D. mauritiana* females ejected significantly sooner than *D. simulans* and *D. melanogaster* ($F_{2,580} = 8.23, P = 0.0003$; Table 1). Virgin females ejected sperm significantly later than previously mated females in both *D. simulans* (50 min longer; paired $t$-test: $t_{56} = 6.30, P < 0.0001$) and *D. mauritiana* (24 min longer; $t_{12} = 2.24, P = 0.045$). Although ejection times differed between virgin *D. simulans* and *D. mauritiana* females, there was no significant difference in either the number of sperm ejected by single-mated (Table 1; *D. simulans*: 518 ± 60; *D. mauritiana*: 464 ± 72; $t_{71.1} = -0.57, P = 0.57$) or by twice-mated females (*D. simulans*: 1315 ± 110; *D. mauritiana*: 1235 ± 199; $t_{21.3} = -0.36, P = 0.72$). We caution, however, that timing of ejection may be a more reliable metric (see Discussion) than the number of sperm ejected, as measurement error in the latter variable is expected to be high; females can eject multiple small masses and it is impossible to know if the entirety of ejected sperm was collected. There also was no relationship between timing of ejection and proportion of first-male sperm displaced (arcsine square-root transformed) within either *D. simulans* ($F_{1,18} = 0.07, R^2 = 0.004, P = 0.79$) or *D. mauritiana* ($F_{1,11} = 1.74, R^2 = 0.14, P = 0.21$).

Both *D. simulans* and *D. mauritiana* females stored significantly more sperm after the second mating than after the first mating (*D. simulans*: $t_{77.6} = -2.09, P = 0.040$; *D. mauritiana*: $t_{67.7} = -4.24, P < 0.0001$). This increase was statistically accounted for entirely by a greater number of sperm in the ST in *D. simulans*, whereas the increase was predominantly attributable to more sperm in the SR in *D. mauritiana* (Table 1). Female *D. melanogaster* stored fewer total sperm after remating than did female *D. simulans* and *D. mauritiana* (analysis of variance: $F_{2,184} = 9.98, P < 0.0001$; Table 1). The number of first-male sperm remaining in storage upon remating also differed significantly among all three species ($F_{2,735} = 39.39, P < 0.0001$), being lowest in *D. simulans* and greatest in *D. mauritiana* (Table 1). Much of this pattern may be attributable to species differences in sperm-use efficiency (Table S1), given the trend for *D. simulans* to store the most sperm after the initial mating (Table 1).
DIVERGENT SPERM PRECEDENCE MECHANISMS

Figure 1. Mean and SEM for numbers of first-male (dark gray) and second-male (light gray) sperm for *Drosophila simulans* (left column), *Drosophila mauritiana* (middle column), and *Drosophila melanogaster* (right column) in the bursa (first row), seminal receptacle (SR; second row), spermathecae (ST; third row), and overall storage (fourth row) for various time points after the start of mating (ASM) averaged over both reciprocal mating orders (first male as green- [GFP] or red-fluorescent protein [RFP]). The gray bar represents copulation duration. Plots for *D. melanogaster* are reproduced from Manier et al. (2010).

SPATIOTEMPORAL PATTERNS OF SPERM TRANSFER, STORAGE, AND DISPLACEMENT

Patterns of sperm storage and use were, in a very general sense, similar among *D. simulans*, *D. mauritiana*, and *D. melanogaster*, yet we found several important differences. For example, *D. simulans* and *D. mauritiana* do not exhibit early release of first-male sperm from storage during female remating, as was observed in *D. melanogaster* (Manier et al. 2010). In addition, *D. simulans* males transferred sperm later during copulation (Fig. 1A), with numbers of second-male sperm in the bursa peaking closer to the end of copulation (i.e., 30 min ASM) as compared with 15 min ASM in *D. mauritiana* (Fig. 1B) and *D. melanogaster* (Fig. 1C). Regarding the timing of sperm storage, the earliest study of reproduction in *D. melanogaster* found
sperm entering the SR well before the ST (Nonidez 1920), a pattern confirmed by Adams and Wolfner (2007) and Manier et al. (2010). In contrast, sperm transferred to D. simulans and D. mauritiana enter the SR and ST at approximately the same time (Fig. 1A, B).

We observed the greatest interspecific differences in sperm storage and displacement in the two sperm-storage organ types, including evidence for movement of sperm between the SR and ST. In D. simulans, the number of second-male sperm in storage peaked in the SR at 368 ± 24 sperm around 60 min ASM, but numbers immediately decreased, and only 65 ± 16 sperm remained after 72 h (Fig. 1D). In the ST, sperm storage continued until 4 h ASM, peaked at 271 ± 26 sperm and was followed by an immediate and precipitous decline in sperm numbers, with only 28 ± 5 sperm remaining by 72 h (Fig. 1G). Because D. simulans females typically eject sperm from the bursa around 2 h ASM (Table 1), sperm entering the ST after this point would necessarily have come from the SR. Indeed, the decrease in sperm number in the SR (Fig. 1D) coincided with the increase in the ST (Fig. 1G) from 60 min to 4 h ASM, and accounted for a mean loss of 112 sperm from the SR and gain of 129 sperm in the ST. Total numbers of second-male sperm in storage therefore did not change over this time period (Fig. 1J), lending further support to the interpretation that second-male sperm move from the SR to the ST. Although second-male sperm continued entering the ST until 4 h ASM, first-male sperm appeared not to be displaced from this storage organ up to this time-point (i.e., sperm numbers did not change from 0 min to 4 h ASM; $F_{7,208} = 1.28, P = 0.26$). Resident sperm numbers showed significant decreases in the ST only after 4 h ASM when sperm started to be used for fertilization (Fig. 1G).

In D. mauritiana, second-male sperm stored in the SR peaked at the same time as in D. simulans (despite earlier sperm transfer) at 289 ± 21 sperm, but more sperm were retained by 48 h than in D. simulans in the SR (Fig. 1E), ST (Fig. 1H), and thus in storage overall (Fig. 1K). In the SR, first-male sperm were almost completely lost, with only 12 ± 6 remaining at 4 h ASM (Fig. 1E), representing a 92% decrease from the 156 ± 13 resident sperm present in the SR at the start of the second copulation. This loss of resident sperm from the SR is much higher than in D. melanogaster (61%) but comparable to D. simulans (89%). In the ST, however, resident sperm experienced no significant decrease in D. simulans or D. mauritiana until after 4 h ASM (D. simulans: $F_{7,208} = 1.28, P = 0.26$; D. mauritiana: $F_{7,171} = 1.11, P = 0.36$), when they presumably begin to be used for fertilization (Fig. 1G, H). This also is in contrast with D. melanogaster, which does experience a significant decline in numbers of first-male sperm in the ST ($F_{6,422} = 3.12, P = 0.002$; Fig. 1I).

SPERM VELOCITY

Sperm velocity was significantly negatively associated with sperm density in the SR in D. simulans ($F_{1,54} = 15.15, R^2 = 0.22$, $P = 0.0003$), D. mauritiana ($F_{1,72} = 18.11, R^2 = 0.20, P < 0.0001$), and D. melanogaster ($F_{1,84} = 6.47, R^2 = 0.07, P = 0.013$) and was included in all initial analyses. However, if sperm density did not remain a significant covariate, it was removed from the final analyses.

The most direct test of the effect of female remating (e.g., effect of the second male’s ejaculate) on first-male sperm velocity is the comparison between first-male sperm in females 60 min after remating (treatment B) with those in females that were not given the opportunity to remate (treatment C), as this comparison controls for both effects of duration of sperm storage and use of first-male sperm to fertilize eggs over the preceding days. We found highly consistent results across all three species, with first-male sperm swimming significantly more slowly following remating (D. simulans: $F_{1,31} = 6.16, P = 0.019$; D. mauritiana: $F_{1,51} = 9.77, P = 0.003$; D. melanogaster: $F_{1,36} = 7.68, P = 0.009$; Fig. 2). The interpretation that the above effect represents the slowing of first-male sperm by the presence of the second-male ejaculate (or otherwise by female remating) is also supported by the comparison of first-male sperm velocity after the first mating (treatment A) and several days later but without remating (treatment C). This analysis revealed either a significant increase in velocity over this time interval in storage (D. simulans: $F_{1,24} = 35.68, P < 0.0001$; D. mauritiana: $F_{1,42} = 13.06, P = 0.0008$; Fig. 2A, B) or no significant change in velocity (D. melanogaster: $F_{1,39} = 0.29, P = 0.60$; Fig. 2C).

As previously reported for D. melanogaster (Manier et al. 2010), we found no evidence for sperm incapacitation in D. simulans or D. mauritiana. Some first- and second-male sperm in the bursa were frequently immotile, but sperm in the SR were observed to almost always be completely motile, irrespective of whether a female had remated (see Movies S1–S3).

PATERNITY AND COMPETITIVE FERTILIZATION SUCCESS

Mean $P_2$ differed significantly across species ($F_{2,233} = 4.16, P = 0.017$; Table 1) due to significantly higher $P_2$ in D. mauritiana (0.88 ± 0.02) than in D. simulans (0.80 ± 0.03) or D. melanogaster (0.79 ± 0.027). No species exhibited significant changes in $P_2$ over the first 72 h (D. simulans: $F_{2,136} = 0.967, P = 0.89$; D. mauritiana: $F_{2,122} = 0.13, P = 0.88$; D. melanogaster: $F_{2,116} = 1.04, P = 0.36$). The proportion of total sperm comprised by second-male sperm ($S_2$) also did not change over time in the SR (D. simulans: $F_{2,61} = 0.78, P = 0.46$; D. mauritiana: $F_{2,86} = 1.78, P = 0.17$; D. melanogaster: $F_{2,78} = 0.49, P = 0.61$) or
DIVERGENT SPERM PRECEDENCE MECHANISMS

Figure 2. Log-transformed velocity (μm/s) of the first male’s sperm in the seminal receptacle (SR) measured 60 min after the start of the first mating, 60 min after the start of the second mating, and in females not given the opportunity to remate but dissected on the same timescale as the remating treatment for Drosophila simulans (A), Drosophila mauritiana (B), and Drosophila melanogaster (C). *P < 0.05, **P < 0.01, ****P < 0.0001.

Discussion
We compared the events occurring within the female reproductive tract following remating across D. melanogaster, D. simulans, and D. mauritiana and found a shared motif in the processes that determine the pattern of sperm precedence. In this group, PSS can occur during any of three discrete stages: (1) insemination; (2) sperm storage; and (3) sperm use for fertilization, and may involve four distinct phenomena: (1) sperm transfer; (2) displacement; (3) ejection; and (4) selection for fertilizations. Yet, underlying these broad similarities were significant quantitative inter-specific differences in nearly every trait or event (Table 1; Fig. 1) and qualitative differences in female sperm use bias. These differences illustrate two important points. First, despite similar intensities of PSS (i.e., female remating rate; Table 1) acting upon very closely related species, there has been substantive and rapid diversification of sex-specific traits that interact or otherwise influence how competitive reproductive outcomes are achieved. Second, similar patterns of sperm precedence (i.e., P2; Table 1) in even very closely related species should not be used to infer identical sex-specific traits or mechanisms giving rise to those patterns.

Here we discuss the notable interspecific variation in sperm transfer, displacement, ejection and fertilization bias, and consider their potential adaptive significance. Of course, it is not possible to fully reconcile the extent to which demonstrated trait divergence is attributable to PSS, natural selection, and/or genetic drift. However, we have used related isogenic populations of D. melanogaster to reveal the extent to which heritable, within-population variation in these traits contributes to differential fertilization success, and hence the degree to which they are subject to extant PSS (Lüpold et al. 2012, 2013). We address the species differences in light of these investigations. Finally, we discuss how knowledge of species divergence in sperm precedence mechanisms can predict mechanisms and asymmetry of gametic isolation (e.g., conspecific sperm precedence) in hybrid matings between species.

SPERM TRANSFER
Males of all three species tailored ejaculate size, transferring significantly more sperm to nonvirgin than to virgin females (Table 1; Lüpold et al. 2011). This pattern supports classic risk models that predict ejaculate investment will increase with risk of sperm competition (Parker et al. 1997; Ball and Parker 1998; see Wedell et al. 2002 for a review of empirical data), but contrast with a recent meta-analysis across a wide range of species, which found that males typically transfer more sperm to virgins (Kelly and Jenning 2011). Nevertheless, our observed pattern is predicted by the established benefit of increased second-male sperm numbers on first-male sperm displacement and timing of female ejection in D. melanogaster (Manier et al. 2010; Lüpold et al. 2012, 2013). We have no compelling adaptive explanation for species divergence in the degree of tailoring. Theory predicts that selection for prudence in ejaculate allocation should intensify with the per sperm energetic investment (Wedell et al. 2002). However, this prediction is not supported, as D. melanogaster males have the costliest (i.e., longest) sperm (Pitnick and Markow 1994; Pitnick et al., 1995; Pitnick 1996) yet exhibit the least extent of ejaculate tailoring (Table 1; Lüpold et al. 2011). See online Supporting Information for discussion of among-species relationships between the duration of copulation and sperm transfer.
SPERM STORAGE, DISPLACEMENT, AND EJECTION

Physical displacement of resident sperm is significantly lower in D. melanogaster than in the other two species, a difference that is not explained by interspecific variation in the number of resident sperm remaining in storage at the time of female remating (Table 1). Sperm displacement has been demonstrated to be a consequence of sperm numbers (Manier et al. 2010; Lüpold et al. 2012), the relative velocity (Lüpold et al. 2012) and length (Miller and Pitnick 2002; Pattarini et al. 2006; Lüpold et al. 2012) of competing males’ sperm, the length of each male’s sperm relative to SR length (Miller and Pitnick 2002, 2003), and the timing of female sperm ejection (Manier et al. 2010; Lüpold et al. 2013). Fewer sperm are transferred by D. melanogaster males to remating females than are transferred by D. simulans and D. mauritiana males (due to the lower extent of ejaculate tailoring), which may contribute to the displacement difference. Furthermore, given that sperm length, SR length, and an interaction between them all contribute to the displacement process, it is notable that each of these traits has diverged dramatically among D. melanogaster, D. simulans, and D. mauritiana (Table 1). Yet, because these interacting sex-specific traits are coevolving (Pitnick et al. 1999, 2003; Pitnick and Miller 2002), such divergence may be neutral in terms of sperm displacement.

There is strong evidence that PSS mediated by the contribution of sperm length to sperm displacement drives divergence of sperm length (Miller and Pitnick 2002; Pattarini et al. 2006; Lüpold et al. 2012). However, such evidence raises the question of how sperm-SR coevolutionary divergence can result in “displacement neutrality.” With PSS on sperm and SR length held hypothetically constant, these traits may have diverged due to differential costs. Energetic costs of producing long sperm and SRs have been postulated to explain experimentally demonstrated trade-offs between sperm length and age at first reproduction (Pitnick et al. 1995) and sperm number (Pitnick 1996; Immler et al. 2011) and between SR length and egg-to-adult development time (Miller and Pitnick 2003). Longer SRs also have a longevity cost for mated females (Miller and Pitnick 2003). Thus, divergence in these traits could be driven by divergence in resource allocation (i.e., differential costs) rather than by PSS (Reznick 1985; van Noordwijk and de Jong 1986; Gomendio et al. 2011; Tourmente et al. 2011). Alternatively, our results reveal significant divergence in the source of sperm for fertilization, with sperm coming predominantly from the SR in D. melanogaster, yet no bias between the SR and ST in both D. simulans and D. mauritiana (see below; Manier et al. 2013). Because the longer-sperm advantage in sperm displacement is believed to derive only from competitive interactions within the elongate SR (Pattarini et al. 2006; Higginson et al. 2012), the evolutionary loss of organ bias in sperm for fertilization in the common ancestor of D. simulans and D. mauritiana would weaken the strength of selection for longer sperm in these species.

As our understanding of female sperm use increases, it is becoming clear that the SR and ST serve at least partially different roles. This has long been suspected for D. melanogaster based on studies demonstrating that (1) sperm fill the SR first (Nonidez 1920; Adams and Wolfer 2007; Manier et al. 2010); (2) greater numbers of sperm are stored in the SR versus the ST (Leefvre and Johnsson 1962; Fowler et al. 1968; Fowler 1973; Manier et al. 2010); (3) sperm are depleted from the SR before they are fully used from the ST (Nonidez 1920; Fowler 1973); and (4) the egg is positioned for fertilization with the micropyle oriented ventrally near the entrance to (and exit from) the SR and hence relatively distant from the entrance to the spermathecal ducts (Nonidez 1920; King 1970). A comparative analysis showed numerous independent losses of ST storage function (but only a single evolutionary loss of use of the SR), indicating that roles of these organs are evolutionarily dynamic throughout the Drosophila lineage (Pitnick et al. 1999; for more information on female sperm-storage organ diversification, see Pitnick et al. 2009a; Puniamoorthy et al. 2010; Higginson et al. 2012). Results of this study further reveal how rapidly divergent female sperm-storage organ structure and function can be. Resident sperm were significantly displaced from both storage organs in D. melanogaster (albeit more extensively from the SR) but only from the SR in both D. simulans and D. mauritiana (Fig. 1; Movie S3). In addition, sperm entered both storage organs simultaneously in D. simulans and D. mauritiana, in contrast with D. melanogaster (Fig. 1). This result points to species-level differences in conformational changes to the bursa that occur during and after copulation (Adams and Wolfer 2007) as well as Sfps influencing those changes (Avila and Wolfer 2009; Avila et al. 2010).

We postulate that displacement in the SR approximates a continual “counter-current” of sperm mixing that is established between the storage organ and bursa (Nonidez 1920), such that sperm from both males mix in the SR, are displaced into the bursa, mix in the bursa, and then potentially re-enter the SR (see Parker and Simmonds 1991). As this process continues, we can expect a displacement equilibrium to be reached that is characterized by a convergence of S₁ in the SR with S₂ in the bursa. In the ST, however, S₁ never approximates that in the bursa, suggesting that a counter-current of sperm mixing is never established. Here, the process may better be described as topping off (Jones et al. 2002).

The sperm displacement process abruptly ends when the female ejects all sperm in the bursa, which include excess second-male sperm and displaced resident sperm (Manier et al. 2010; Lüpold et al. 2012). Sperm ejection was first observed in Drosophila by Patterson (1946) and is likely to be widespread among animal taxa (Dean et al. 2011; see online Supporting
Information for an expanded discussion of sperm ejection and its relationship to “sperm dumping”). Within species, the timing of ejection can significantly influence $S_2$ (Lümpold et al. 2013), but only for ejections occurring before a displacement equilibrium is reached in the SR and while $S_2$ is still rising in either storage organ type. In all three species, $S_2$ was significantly associated with paternity success (Manier et al. 2013; also see Manier et al. 2010; Lümpold et al. 2012, 2013), so manipulation of $S_2$ may have important consequences for reproductive fitness. Our results indicate that ejections before 60 min ASM would have the greatest impact on $S_2$ in $D. simulans$ and $D. mauritiana$ and before 30 min ASM in $D. melanogaster$ (Fig. 3). Ejection times in this range were not observed for any of these species (this study). Nevertheless, the average time of ejection in $D. mauritiana$ and $D. melanogaster$ occurred around the time displacement equilibrium is reached (Fig. 3B, C), suggesting that in these species, females (and perhaps males by influencing female ejection time, potentially via Sfps) have more latitude to use ejection as a mechanism to influence $S_2$ in the SR. In $D. simulans$, however, ejection on average occurred over an hour after displacement equilibrium was reached in the SR (Fig. 3A), reducing the potential impact of ejection time on paternity success (but see below and Manier et al. 2013 for evidence that $D. simulans$ females use ejection to discriminate against ejaculates from heterospecific males).

SPERM SELECTION FOR FERTILIZATIONS

The impact of female ejection on $P_2$ will depend on how sperm from the SR and ST are used for fertilizations due to interorgan differences in resident sperm displacement. If sperm competing for fertilization mostly derive from the SR, then ejection time may strongly influence $P_2$. If fertilizing sperm mostly derive from the ST, then ejection time may have little influence on $P_2$. In addition, one or both storage organs may exhibit sperm-use bias in favor of first- or second-male sperm. Because it was not possible to directly observe either release of sperm from the SR or ST or fertilization of eggs in the bursa, we developed a binomial mixture model (Royle 2004) that builds on earlier approaches (Parker 1990; Eggert et al. 2003; Neff and Wahl 2004) to simultaneously estimate bias (i.e., deviations from proportional representation) between first- and second-male sperm (i) within the SR, (ii) within the ST, and (iii) sperm-use bias between the two storage organ types in $D. melanogaster$, $D. simulans$, and $D. mauritiana$. Details of the model, its application and conclusions drawn from it are reported by Manier et al. (2013); here we briefly summarize major conclusions.

Our model revealed significant differences among the three species in all three sources of fertilization bias. We found that $D. melanogaster$ supplies the preponderance of sperm for fertilization from the SR (supporting previous studies), and competing sperm from both the SR and ST are used in proportion to their numerical representation. In contrast, sperm for fertilization were supplied equally from the SR and ST in both $D. simulans$ and $D. mauritiana$. However, although both organs supplied sperm according to their relative abundance in $D. mauritiana$ (similar to $D. melanogaster$), $D. simulans$ exhibited a significant first-male
sperm bias in the SR and a significant second-male bias in the ST. This unexpected discovery of qualitatively different fertilization biases between organ types suggests that *D. simulans* females possess a physiological mechanism of putative “sperm choice”—a particularly nuanced form of cryptic female choice (Birkhead 1998; Pitnick and Brown 2000). Experiments have not yet been conducted to determine whether *D. simulans* females, with regard to within-population variation in male quality, alter the respective source of sperm for fertilization contingent upon whether first or second males are of superior quality. However, we have demonstrated that *D. simulans* females, mated to both a *D. simulans* and a *D. mauritiana* male, employ this mechanism to discriminate in favor of conspecific over heterospecific male sperm (Manier et al., 2013). Such sophisticated sperm choice is facilitated by the possession of two distinct types of female sperm-storage organs, which is uncommon outside of the Diptera (Pitnick et al. 1999). Perhaps the more important question is why *D. mauritiana* and *D. melanogaster* do not exhibit similar organ-specific sperm use biases.

See online Supporting Information for a discussion of species differences in sperm-use efficiency and for a speculative discussion of temporal variation in sperm motility and its contribution to fertilization bias.

**SPECIATION CONSEQUENCES OF PSS-DRIVEN DIVERSIFICATION**

Diversification of traits between allopatric populations is predicted to generate speciation phenotypes that arise upon secondary contact and act to maintain species boundaries, although few studies have used patterns of diversification to predict potential speciation phenotypes (Shaw and Mullen 2011). Here, we discuss how patterns of interspecific divergence among these three species may be used to test predictions about mechanisms of reproductive isolation in the model speciation system: the sister species *D. simulans* and *D. mauritiana* (Price 1997; Price et al. 2000, 2001).

The potential speciation consequences of PSS has been widely discussed (Markow 1997; Parker and Partridge 1998; Howard 1999; Eady 2001; Simmons 2001; Coyne and Orr 2004; Lorch and Servedio 2007; Howard et al. 2009; Butlin et al. 2012), but direct evidence remains elusive. Intuitively, rapid coevolutionary diversification of interacting ejaculate and female reproductive tract traits (Pitnick et al. 2009a) in isolated populations may generate barriers to gene flow mediated by ejaculate–female incompatibilities upon secondary contact (e.g., Brown and Eady 2001; Fricke and Arnqvist 2003; Ludlow and Magurran 2006), a phenomenon generally referred to as “gametic isolation” (Dobzhansky 1937; Howard et al. 2009). Competitive gametic isolation or “conspecific sperm precedence” (CSP; Howard and Gregory 1993) is taxonomically widespread (Coyne and Orr 2004; Howard et al. 2009) and refers to the phenomenon of females produc-
and *D. mauritiana* in sperm precedence traits and mechanisms enabled accurate predictions of both mechanisms of CSP and asymmetry in the degree of CSP in this model system (Manier et al., 2013).

**Conclusions**

This investigations resolved many of the traits and processes contributing to patterns of sperm precedence in sibling species. Because (1) these species recently diverged from common ancestors and (2) investigations of intraspecific variation have shown that these divergent traits and processes contribute to postcopulatory fitness (Miller and Pitnick 2002, 2003; Pattarini et al. 2006; Lipold et al. 2011, 2012, 2013), we conclude that PSS has driven the rapid evolution of reproductive characters in this lineage. These results highlight the importance of studying the mechanisms underlying paternity success to understand the nature of, and species-specific adaptations to, PSS. In addition, they provide critical baseline information for predictive studies of CSP and other aspects of the speciation process.

We suggest that the experimental methods employed here can be fruitfully applied to other experimental systems to resolve the processes and targets of PSS and thus to better discern the role of PSS in evolutionary diversification. We are quick to point out that there are always “mechanisms underlying mechanisms,” and there are many kinds of traits that could influence the reproductive processes described here, yet were not included in this investigation. Future investigations should explore within-population variance and among-species divergence in genitalic morphology, copulatory courtship behavior, and Sfp and female secretory biochemistry, for example, and their relationship to sperm performance and fate.

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**LITERATURE CITED**


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Sperm use efficiency from 24 to 72 h by species.

Movie S1. Typical female seminal receptacle (SR) of *D. simulans* showing first-male sperm (GFP-tagged sperm heads) motility in the presence of second-male sperm (RFP-tagged sperm heads).

Movie S2. Typical female seminal receptacle (SR) of *D. mauritiana* showing first-male sperm (green-fluorescent protein [GFP]-tagged sperm heads) motility in the presence of second-male sperm (red-fluorescent protein [RFP]-tagged sperm heads).

Movie S3. Common pattern of differential displacement between the two different types of female sperm-storage organ.