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Male mate choice via cuticular hydrocarbon pheromones drives reproductive isolation between *Drosophila* species

Michael P. Shahandeh[*,1], **Alison Pischedda**[2], and **Thomas L. Turner**[1]

[1]Department of Ecology, Evolution, and Marine Biology, University of California Santa Barbara

[2]Department of Biology, Barnard College, Columbia University, New York NY 10027

Abstract

Mate discrimination is a key mechanism restricting gene flow between species. While studied extensively with respect to female mate choice, mechanisms of male mate choice between species are far less studied. Thus, we have little knowledge of the relative frequency, importance, or overall contribution of male mate discrimination to reproductive isolation. In the present study, we estimated the relative contributions of male and female choice to reproductive isolation between *Drosophila simulans* and *D. sechellia*, and show that male mate discrimination accounts for the majority of the current isolation between these species. We further demonstrate that males discriminate based on female cuticular hydrocarbon pheromones, and collect evidence supporting the hypothesis that male mate discrimination may alleviate the costs associated with heterospecific courtship and mating. Our findings highlight the potentially significant contribution of male mate choice to the formation of reproductive isolating barriers, and thus the speciation process.

Keywords

male mate choice; reproductive isolation; courtship; behavior; speciation; pheromones

Introduction

The biological species concept posits that lineages are considered separate species when they can no longer interbreed – that is, there are sufficient reproductive barriers in place to severely limit gene flow between two populations (Mayr, 1940). These barriers can act postzygotically, via incompatibilities that make hybrids sterile or inviable (Dobzhansky, 1940). They can also act prezygotically, such that temporal, ecological, or behavioral isolating mechanisms prevent mating/fertilization (Hodges et al., 1994; Quinn et al., 2000). There are a multitude of studies examining postzygotic reproductive barriers, as these are easily studied in the lab (Brideau et al., 2006; Orr, 2005; Phadnis & Orr, 2009; Tang &

[*]Corresponding author: michael.shahandeh@lifesci.ucsb.edu, Life Sciences Building, University of California Santa Barbara, Santa Barbara, California 93106.

Author contributions

MPS, AP and TLT conceived and designed the study. MPS and AP collected the data. MPS analyzed and interpreted the data and wrote the manuscript. AP and TLT edited the manuscript.

Data will be archived at Dryad

Presgraves, 2009). However, prezygotic barriers are often stronger when species occur in sympatry (Coyne & Orr, 1997, 2004). For species that separate in allopatry and then come into secondary contact, prezygotic barriers can be essential to preventing costly hybrid mating, a process termed reinforcement (Dobzhansky, 1940). Such prezygotic barriers are often behavioral, like divergent preferences for elaborate sexual signals, resulting in increased mate discrimination.

Mate discrimination often evolves when there is a cost to mating, either through parental investment or direct fitness costs from courtship or mating (Partridge & Fowler, 1990). Historically, mate choice has been predominantly studied through the lens of females being the “choosy” sex, and males being “flashy” – having to compete for female attention (Kirkpatrick, 1987). This viewpoint stems from the fact that, in most systems, there is a greater cost to mating for females because of their increased investment in offspring compared to males (Trivers, 1972). Following this line of inquiry, male mate choice has been studied most extensively in systems with sex role reversal, like pipefish, in which males invest more in offspring than females (Rosenqvist, 1990). However, male mate choice can evolve whenever males stand to benefit from being choosy, like when there is a significant cost to courtship and mating, or there is variation in female quality that males can detect (Byrne & Rice, 2006; Edward & Chapman, 2011; Servedio & Lande, 2006). There is increasing evidence showing male mate choice acting in both invertebrate and vertebrate species with a variety of mating systems, including polygyny (Sargent et al., 1986; R. Shine et al., 2001), polyandry (Assis et al., 2017), social monogamy (Hill, 1993; Liu et al., 2017), and promiscuity (Long et al., 2009). Comparable studies between species are lacking, however, despite the potentially important role of male mate discrimination in the formation of reproductive isolation (Albert & Schluter, 2004; Ratcliffe & Grant, 1983; Shine et al., 2004; Zhang et al., 2014). In fact, simulations show that male mate choice can feasibly act as the sole driver of species recognition during reinforcement, completing the speciation process (Servedio, 2007). Still, the dearth of studies focusing on the male contribution to this process means we know little about how often male choice plays a role in reproductive isolation, and how important male discrimination is relative to female discrimination.

With the present study, we set out to fill this gap by quantifying reproductive isolation between the closely related *Drosophila* species, *D. simulans* and *D. sechellia*. These species occur in sympatry in the Seychelles archipelago and are phenotypically quite similar (Matute & Ayroles, 2014), but show evidence for reproductive isolation (Coyne et al., 1994). *D. simulans* and *D. sechellia* most likely speciated in allopatry (Kliman et al., 2000), and have only recently become sympatric as *D. simulans* has invaded the Seychelles archipelago (Lachaise & Silvain, 2004; D. R. Matute et al., 2014). There is the potential for both male and female mate choice in *Drosophila* species, as males collect chemical, tactile and visual information from females before deciding whether to court (Sokolowski, 2001a), and females use male courtship behavior to evaluate the male and decide whether to copulate (Greenspan & Ferveur, 2000). We therefore partitioned the reproductive isolation between *D. simulans* and *D. sechellia* into three distinctive barriers: (1) male mate discrimination, (2) female mate discrimination, and (3) hybrid incompatibility. By comparing the contributions of each barrier, we aim to quantify the role of male discrimination, relative to female discrimination, in reproductively isolating these taxa. We further identify the mechanism by

which males discriminate between conspecific and heterospecific mates, and provide data supporting the hypothesis that male courtship and mating costs may favor the evolution of male mate choice in these species.

Materials and methods

Drosophila stocks and maintenance

We maintained all fly strains in 20 mm vials on standard cornmeal/molasses/yeast medium at 25℃ under a 12h:12h light/dark cycle. Under these conditions, we established non-overlapping two-week lifecycles. Every 14 days, we transferred 20–30 male and female adult flies into vials containing fresh food, where they were allowed to oviposit for 1–3 days before being discarded. We used a single *D. simulans* strain, simC167.4, obtained from the UC San Diego *Drosophila* Stock Center (Stock #: 14021-0251.199). This strain, that we will refer to as "*D. simulans*", was originally collected from Nanyuki, Kenya, and was first described by Davis et al., 1996. Likewise, we used a single *D. sechellia* strain, synA, constructed from lines collected in 1980 at Cousin Island, Seychelles, obtained from the same stock center (Stock #: 14021-0248.28) that we will refer to as "*D. sechellia*". We collected all male and female flies used in the experiments described below from these strains as virgins within 6 hours of eclosion on the eleventh or twelfth day following oviposition.

Two-day courtship assays with conspecific and heterospecific females

We aged virgin males and females from both species in single-sex vials for 4 days at 25℃ at densities of 10 and 20, respectively, before we measured courtship. We used virgin males for all assays because previous mating experience has been shown to increase male mating success (Saleem et al., 2014) and strengthen male mate choice in *D. melanogaster* (Byrne & Rice, 2006). 24 hours before each assay, we gently aspirated individual males into vials sealed with foam plugs, setting up an approximately equal number of vials containing *D. simulans* and *D. sechellia* males. We performed 2–3 courtship assays per day at room temperature between 9 and 11 AM (i.e. between 1 and 3 hours following "lights-on" for these flies). To begin each assay, we aspirated single females into each vial, and pushed the foam plug down into the vial until it was just 1–2 cm from the food surface (the limited space leads to faster interaction between flies). For 30-minute intervals, we collected minute-by-minute courtship data, manually scoring each pairing for three easily detected stages of courtship: singing (single wing extensions and vibration), attempted copulation, and successful copulation. Pairs that exhibited multiple stages within a single minute were scored once for each stage within that minute. Immediately after the observation period, we removed each female and allowed the males to recover for 24 hours at 25℃. The following morning, we repeated the process with a new female. We used a full factorial design for courtship assays, creating 4 different treatments based on female identity: i) conspecific females on the first day, heterospecific females on the second day, ii) heterospecific females on the first day, conspecific on the second, iii) conspecific females on both days, and iv) heterospecific females on both days (N=36–49 per treatment). We collected courtship data over three consecutive weeks. We only considered males that spent 10% or more of the total

assay time (i.e. ≥ 3 mins) in one of the three courtship stages as successfully displaying courtship.

Calculating relative contributions of reproductive barriers

Before initiating courtship, *Drosophila* males encounter and approach females, collecting information in the form of chemical, tactile, and visual cues. If a male likes what he smells, tastes, and sees, he begins an elaborate courtship ritual where he sends a full spectrum of sensory stimuli to the female (Sokolowski, 2001a). The female uses male courtship behavior to evaluate the male and decide whether to copulate (Greenspan et al., 2000). Because males must assess and decide to court a female before a female can gather much information on a male suitor, we treated male and female preferences as sequentially acting reproductive barriers. In addition to male and female choice, we included hybrid incompatibility, totaling three sequentially acting reproductive barriers, two prezygotic and one postzygotic (1: male mate choice, 2: female mate choice, 3: hybrid incompatibility). We used the frequency of male courtship as a proxy for male mate choice, and copulation frequency as a proxy for female choice. We calculated the amount of gene flow limited by male mate choice (RI_1) using the equations suggested by Sobel & Chen (2014):

$$RI_1 = 1 - 2\left(\frac{frequency\ heterospecific\ courtship}{frequency\ heterospecific\ courtship + frequency\ conspecific\ courtship}\right).$$

Likewise, we calculated the gene flow limited by female mate choice:

$$RI_2 = 1 - 2\left(\frac{frequency\ heterospecific\ copulation}{frequency\ heterospecific\ copulation + frequency\ conspecific\ copulation}\right).$$

This method provides a linear relationship between RI_n and the amount of gene flow reduced (Sobel & Chen, 2014). Using proportional values for courtship and copulation frequency also accounts for slight variations in sample size. We used a value of 0.5 for hybrid incompatibility (RI_3) because all male hybrid offspring (i.e., half the offspring) are sterile, whereas all female offspring are fertile. To estimate the relative contribution of male and female behavior to the total reproductive isolation between *D. simulans* and *D. sechellia*, we followed the methods of Ramsey et al (2003). Following this method, the potential gene flow restricted by each barrier (RI_n) is calculated individually (as described above), and then used in a sequential model such that any barrier can only limit the gene flow that remains after the barrier acting before it, yielding the actual contribution of each barrier to gene flow restriction (AC_n):

$$\mathrm{AC}_1 = \mathrm{RI}_1$$

$$\mathrm{AC}_2 = \mathrm{RI}_2\,(1 - \mathrm{AC}_1)$$

$$AC_3 = RI_3 [1 - (AC_1 + AC_2)] .$$

These values are summed to calculate the total isolation (T). The relative contribution of each barrier (RC_n), is then calculated by dividing each AC_n value by the total isolation, T. We calculated RI_n, AC_n, T, and RC_n for each male and female heterospecific pairing individually (*D. simulans* males courting *D. sechellia* females, and vice versa). We also calculated these values for the combined data set to compare and assess the relative contribution of males and females from both species to the total calculated isolation between the species. Because we found no effect of assay day or female order on any of our reproductive barriers (see Data analysis), we used unpaired, pooled data for this analysis, discarding the second day of data from males that received females of the same species twice to avoid pseudoreplication.

Fore-tarsi removal and courtship assay

Cuticular hydrocarbon pheromones (CHCs) are contact gustatory signals that differ between female *D. simulans* and *D. sechellia*, and are believed to contribute to reproductive isolation between the species (Coyne & Charlesworth, 1997). To test for the role of gustation in male mate discrimination, we removed the tarsal segment of the forelegs from adult virgin *D. simulans* and *D. sechellia* males. This structure is the primary peripheral gustatory pheromone detection structure (Manning, 1959; Montell, 2009). After collection, we aged males for 3 days. Then, under light anesthesia, we removed the tarsal segment of each foreleg using a scalpel. Immediately after tarsi removal, we sorted males into individual vials, where they recovered for 24 hours at 25℃. After 24 hours, we conducted single day courtship assays, repeating the courtship assay procedure described above with the exception that both males and females were discarded after a single 30-minute observation period. Similar to our previous courtship assays, we only considered males that courted more than 10% of the time as displaying courtship. We compared these data to the data we collected previously using intact animals to test for differences in male mate choice.

Cost of male courtship assay

To quantify the costs of male courtship, we designed a longevity assay following the methods of Partridge and Andrews (1985). The males and females of both species used in this experiment were collected as virgins and aged 3–4 days in vials at a density of 10 and 20, respectively, before each assay. For each species, we divided males into three experimental groups: controls, conspecific courtship groups, and heterospecific courtship groups, each consisting of 60 males. Males in the control treatment were held individually in vials with standard media. We transferred control males to new vials with fresh media under light anesthesia every seven days. Conspecific courtship males were initially combined with four 4-day old conspecific virgin females. Under light anesthesia, we transferred conspecific courtship males to new vials with fresh media and four new 4-day old virgin females every 7 days. We continued to use young females to standardize both the female age and the amount of courtship per vial each week. Heterospecific courtship males received the same treatment as conspecific courtship males, but with 4 heterospecific virgin females. We counted the

number of dead males in each treatment every 22–26 hours until over 95% (at least 58 of 60) of the flies from each treatment died. We also recorded the number of vials that had larvae each week when the flies were transferred to new vials to approximate the percent of males that successfully mated during this time.

Data analysis

For all of the courtship assays, we calculated courtship frequency as the number of males that courted a particular female divided by the total number of potentially courting pairs. We calculated copulation frequency as the number of males that successfully copulated divided by the total number of males that displayed courtship. We compared courtship and copulation frequencies using Fisher's exact tests followed by posthoc analysis with sequential Bonferroni tests (Holm, 1979) to correct for multiple comparisons. We used a full-factorial design to test for a difference in courtship frequencies for all male-female pairings. We performed the same comparisons for females using copulation frequency. We bootstrapped the data for courtship and copulation frequency 100,000 times to establish 95% confidence intervals around our estimates.

We wished to present every male with females of both species to reduce variation caused by stochastic effects among males. We therefore needed to first test whether presenting a male with a female on one day would affect his courtship of a different female the next day. We tested for effects of female order in our two-day courtship assays by comparing courtship and copulation frequencies for each species across three scenarios: the courtship of a male when presented with a female before any other female vs. when presented with that female for a second time, vs. when presented with that female after the presentation of the other type of female. For these comparisons, we used Fisher's exact tests followed by posthoc sequential Bonferroni tests (Holm, 1979) to correct for multiple comparisons. We found no evidence for an effect of female order in any comparison (all $p > 0.75$). Additionally, no individual test was significant before correction. We also tested for an effect of day and we again found no evidence for an effect of observation day on any comparison (all $p = 1$), and no individual test was significant before correction. Because we found no effect of assay day or female order, we use pooled, unpaired data throughout this study. For males that received the same female genotype on each day, we discarded data from the second day to avoid pseudoreplication.

To determine whether there were significant differences in the relative contribution of male mate choice, female mate choice and hybrid incompatibility to the overall reproductive isolation between these species, we calculated bootstrapped 95% confidence intervals (CI) for all RC_n estimates using 100,000 bootstrap replicates. Confidence intervals that do not overlap indicate RC_n values that are significantly different.

For the cost of male courtship assay, we calculated a daily cumulative survival probability by dividing the number of flies alive at the end of each week by the total number of flies at the start of the experiment. We analyzed the survivorship data using the Log-rank test (Miller, 1981), a nonparametric test for right-skewed data. It produces a chi-squared statistic with n-1 degrees of freedom, where n is the number of groups being compared.

Results

Courtship and copulation frequencies for conspecific and heterospecific pairings

Both *D. simulans* and *D. sechellia* males were far more likely to court females of their own species than females of the other species. In both cases, over 85% of males displayed sustained courtship toward their own females (Figure 1A), and there was no significant difference in the frequency of conspecific courtship between *D. simulans* and *D. sechellia* males ($p = 0.10$). In contrast, there were significant differences in male behavior when exposed to females of the other species. Isolation between *D. simulans* males and *D. sechellia* females was so strong that we did not observe a single male courting in this combination (N=97, Figure 1A). In contrast, the isolation between *D. sechellia* males and *D. simulans* females was strong but not absolute: ~40% of *D. sechellia* males courted *D. simulans* females (a reduction of 54% compared to *D. sechellia* females, N=78).

To assess reproductive isolation driven by female behavior, we determined the proportion of courting males that achieved copulation. In *Drosophila*, adult females must accept male advances in order for copulation to occur, and thus can also exercise choice. As we saw with male courtship, *D. simulans* and *D. sechellia* females displayed equivalent copulation frequencies when paired with males of their own species ($p=1.00$). *D. simulans* females were much less likely to mate with *D. sechellia* males: *D. simulans* females copulated with over 85% of conspecific courting males, but just 19% of *D. sechellia* males ($p<0.0001$; Figure 1B). In the reverse cross, we were unable to measure the willingness of *D. sechellia* females to accept the courtship of *D. simulans* males, because no *D. simulans* males displayed any courtship toward *D. sechellia* females, so copulation never occurred.

Contributions of male and female mate choice to reproductive isolation

Our above results highlight the importance of the sequential nature of *Drosophila* mating preferences: if male mate discrimination is strong, then female mate choice could be rendered irrelevant. Because *D. simulans* males never courted *D. sechellia* females, we were unable to measure conspecific female mate choice in the form of copulation frequency for *D. sechellia* females. In this direction of the hybrid cross, gene flow is completely restricted by male courtship behavior.

To test the importance of male mate choice in the total reproductive isolation between these species, we calculated the potential gene flow limited by each reproductive barrier: male mate choice (courtship frequency), female mate choice (copulation frequency) and postzygotic isolation (hybrid incompatibility) using the pooled heterospecific and conspecific data. The total reduction in gene flow between these species is 94.1%. We found that male and female mate choice have the potential to limit approximately equal amounts of gene flow when considered independently of one another (Table 1, RC_1 and RC_2 respectively). However, when we account for the sequential nature of courtship (AC_n), such that each barrier can only limit gene flow not limited by the previously acting barrier, we find that male mate choice accounts for 71.5% of the total reduction in gene flow (RC_N) between *D. simulans* and *D. sechellia*. This value is over three times greater than the 22.2%

of gene flow restricted by female mate choice. This significant difference results entirely from the fact that male choice acts before female choice.

We also calculated these values separately for each direction of the potential hybrid cross (*D. simulans* males with *D. sechellia* females, and vice versa). Unsurprisingly, because *D. simulans* males do not court *D. sechellia* females, male mate choice accounts for 100% of the total isolation in this direction, and constitutes a complete restriction of gene flow (Table S1). For *D. sechellia* males paired with *D. simulans* females, male mate choice had less potential to constrict gene flow (41%) than female mate choice (64%). However, when adjusted sequentially, male and female mate choice account for equal restrictions of gene flow (45.8% and 42.3% respectively, with overlapping 95% confidence intervals). The total isolation for this direction of the cross was an 89.4% restriction of gene flow (Table S2). When we consider each hybrid cross individually, we find an asymmetric pattern of isolation, where *D. simulans* males are far more discriminating than *D. sechellia* males, and contribute significantly more to reproductive isolation.

Cuticular hydrocarbon pheromones: a mechanism for male mate discrimination

We next sought to identify the cues by which males discriminate between females. Species-specific female cuticular hydrocarbon pheromones (CHCs) are involved in reproductively isolating many pairs of *Drosophila* species (Billeter et al., 2009; Coyne & Charlesworth, 1997), and differ between female *D. simulans* and *D. sechellia* (Cobb & Jallon, 1990). To test whether CHCs also mediate male mate choice between our focal species, we compared intact adult males to tarsi-less adult males, as the male tarsi is the primary structure for peripheral gustatory pheromone detection (Montell, 2009). Overall, we found that CHCs do indeed cause male-mediated reproductive isolation in *D. simulans* and *D. sechellia* (Figure 2). Compared to intact *D. simulans* males, which never courted *D. sechellia* females, tarsi-less *D. simulans* males courted *D. sechellia* females 57.1% of the time ($p<0.0001$). These tarsi-less *D. simulans* males were also equally likely to court *D. simulans* and *D. sechellia* females ($p=0.706$), albeit only about half as frequently as intact males courted *D. simulans* females (Figure 1A). Tarsi-less *D. sechellia* males also courted *D. simulans* and *D. sechellia* females indiscriminately ($p=1.00$), and with statistically equivalent frequencies to intact *D. sechellia* males with *D. sechellia* females ($p = 0.910$ and $p= 0.985$ respectively, Figure 1B).

Costs of male courtship

One reason males might evolve to be choosy is because courtship could be costly. We identified a significant cost to male courtship and mating in *D. simulans* (Figure 3A): males held in vials with *D. simulans* females died significantly faster than *D. simulans* males held singly ($p < 0.0001$, $df = 1$, $\chi^2 = 59.6$). For *D. simulans* males held with *D. simulans* females, we observed larvae in 73.9-100% of vials each week, indicating high levels of courtship and mating throughout the study (Table S3). The male-associated costs of courtship, however, were entirely alleviated by male mate choice: *D. simulans* males held in vials with *D. sechellia* females died at a rate equivalent to males held singly ($p = 0.157$, $df = 1$, $\chi^2 = 2$), and lived significantly longer than *D. simulans* males held with their own females ($p = < 0.0001$, $df = 1$, $\chi^2 = 65.9$). Only 0 –1.9% of vials containing *D. simulans* males with *D.*

sechellia females had larvae each week, indicating that courtship and mating were extremely rare in this treatment (Table S3).

Conversely, we found no evidence of a cost to male courtship or mating in *D. sechellia* (Figure 3B). *D. sechellia* males held with *D. sechellia* females died at the same rate as males held in isolation ($p = 0.246$, $df = 1$, $\chi^2 = 2.4$). Throughout the experiment, the number of vials containing *D. sechellia* males with *D. sechellia* females with larvae each week ranged from 25.0–98.2%, with numbers declining as the experiment progressed (Table S3). Likewise, *D. sechellia* males held with *D. simulans* females died at statistically equivalent rates to males held singly ($p = 0.057$, $df = 1$, $\chi^2 = 5.5$) and males held with *D. sechellia* females ($p = 0.604$, $df = 1$, $\chi^2 = 0.3$). For vials containing *D. sechellia* males and *D. simulans* females, we observed larvae in 29.4–78.9% of vials each week, again, with percentages declining steadily as the experiment progressed (Table S3).

Discussion

In this study, we demonstrate that male mate choice is the largest contributor to the total existing reproductive isolation between the sympatric species *D. simulans* and *D. sechellia*—far outweighing the effects of female mate choice. This finding is significant, because it contrasts traditional sexual selection theory, which posits strong female choice driven by greater reproductive investments and costs, with males being much less discriminating (Trivers, 1972). Our results are particularly relevant for other systems with sequential courtship, in which males assess females before courting and/or mating. Because male mate choice is the first barrier to reproduction in these species, it has the potential to disproportionately limit gene flow relative to female choice. It is important to note, however, that even if we did not evaluate reproductive barriers sequentially in our study, male mate choice would still limit an equal amount of gene flow as female mate choice (Table 1). This implies that male mate choice may continue to act as an important reproductive barrier between species with non-sequential mutual mate choice. Below, we describe these results in more detail, and discuss potential scenarios that could favor the evolution of isolation by male mate choice. Our study underscores the potential significance of male mating preferences in reproductively isolating species.

Contributions of male mate choice to reproductive isolation

When we consider the sequential nature of *Drosophila* courtship, male mate choice accounts for 71.5% of the total gene flow restriction between *D. simulans* and *D. sechellia*, far outweighing the 22.2% restricted by female mate choice. Although we used a single strain as a representative for each species, the calculated value for the total reproductive isolation (T), 94.1% restriction of gene flow, is comparable with previous field estimates from the Seychelles archipelago (~96.7%), where these species occur in sympatry (Matute et al., 2014). Further, the unidirectional pattern of gene flow we observed (*D. sechellia* males courting and mating with *D. simulans* females) is consistent with molecular genotyping results of all wild-caught hybrids to date (Matute et al., 2014). The similarities between our findings and those reported in nature suggest that the behaviors exhibited by our experimental strains are characteristic for these species. Despite these consistencies, there is

a possibility that inbreeding and/or laboratory adaptation may have influenced our results. Future work using multiple lines or wild-caught flies for each species will more conclusively demonstrate the generality of our findings.

We used the proportion of males that court as a proxy for male mate choice when calculating its relative contribution to the total reproductive isolation. This may be a lower bound for the overall strength of male mate choice, which can also act after courtship initiation via differential investment in courtship (Eddy et al., 2016; Edward et al., 2011). We did find that *D. sechellia* males courted *D. simulans* females with significantly less effort relative to their own females (with courtship effort measured as the proportion of time a male spent courting; Figure S1). Although male courtship effort was positively associated with copulation success in our pooled data set (logistic regression, $p < 0.001$), the accuracy of prediction was poor (Table S4), so we chose not to include courtship effort as an additional reproductive barrier. Male choice can also occur during copulation in the form of differential sperm allocation and ejaculate composition (Lüpold et al., 2011; Reinhold et al., 2002). These cryptic behaviors are difficult to measure, and thus were also not included in our sequential model. Had we included these supplemental forms of male choice in our study, they would likely increase the contribution of male mate choice to the reproductive isolation between these species, further strengthening our result.

For our measurements of courtship and copulation frequency, we used “no-choice” style assays, where males are presented with a single female at a time. This was a conservative decision, as the inclusion of choice increases sexual isolation in sister taxa (Coyne et al., 2005), and would thus likely increase our relative calculations for male mate choice. We also chose this assay to best simulate courtship in nature. There is evidence from closely related *Drosophila* species that males encounter mates sequentially in the field, and females are more likely to copulate when approached singly (Noor & Ortiz-Barrientos, 2006).

We cannot completely discount the possibility that there are cryptic female rejection behaviors that cause males to avoid courtship. If so, female preferences could play a role in determining the proportion of males that court. During our observation periods, however, we did not observe any female rejection behaviors. In addition, these behaviors do little to deter prolonged male interest in closely related species (Connolly & Cook, 1973). Finally, we think cryptic female rejection behaviors are unlikely because heterospecific courtship increased in both directions when tarsi were removed. If females were rejecting males, this behavior should still occur (or even increase) in our tarsi-less assay. The only exception would be if the rejection behavior were an extremely transient change in CHCs, which, to our knowledge, has never been demonstrated.

The importance of intrinsic prezygotic reproductive barriers

Our results also reaffirm the importance of prezygotic barriers in reproductive isolation. In total, the prezygotic barriers we measured (male and female mate choice) together account for 93.7% of the total isolation, while postzygotic isolation accounts for just 6.3%. Although there are significant hybrid incompatibilities between these taxa, prezygotic barriers act first, and thus account for a majority of the total isolation. This is consistent with previous findings in *Drosophila* showing that intrinsic prezygotic barriers, particularly differences in

mating behavior, are highly important for sympatric taxa, and evolve quickly (Coyne & Orr, 1997). In *Drosophila*, sympatry with closely related species is also strongly correlated with increased sexual isolation and is common across the phylogeny (Noor, 1997). Our results underscore the necessity for in-depth analysis of prezygotic reproductive barriers in understanding the evolution of reproductive isolation.

Following the methods of Coyne and Orr (1989), we used a value of 0.5 for postzygotic isolation (RI_3) in our calculations to represent hybrid male sterility. This is likely an underestimate, however, because it does not include potential differences in hybrid fitness or ability to secure mates. However, if we assume that no hybrid offspring reproduce, which has been disproven in the field (Matute et al., 2009), and use an extreme value of 1 for postzygotic isolation, we find that the relative contribution of postzygotic isolation increases nominally, from 6.3% to 11.8 % of the total isolation. While maximizing this measurement may slightly increase the total isolation and the relative contribution of postzygotic isolation, it has almost no effect on the relative contributions of the barriers that act before it. When we use a value of 1 for postzygotic isolation, male mate choice still accounts for 67.3% of the restricted gene flow, while female choice explains only 20.9%.

Additionally, we did not include any post-mating prezygotic (PMPZ) reproductive barriers in our analysis, as they can be difficult to measure with accuracy (Knowles & Markow, 2001). Such barriers include differences in fertilization rates, female fecundity, or cryptic female choice. For example, *D. simulans* females have reduced fecundity after mating with *D. sechellia* males (Price et al., 2001), and conspecific sperm fertilizes the majority of eggs produced by *D. simulans* females that have mated with both *D. simulans* and *D. sechellia* males (Price, 1997). Although PMPZ barriers are likely present in our system, they would not affect our conclusions about the importance of male mate choice because they act after mating has occurred (i.e. after both male and female mate choice). Including these barriers in our analysis would likely nominally increase the total reproductive isolation (T), while marginally decreasing the already minimal contribution of postzygotic barriers.

Male mate discrimination is driven by cuticular hydrocarbon pheromones

Our results imply that species-specific female cuticular hydrocarbon pheromones are responsible for male mate discrimination between *D. simulans* and *D. sechellia.* When the fore-tarsi are ablated, males completely lose the ability to selectively court females of their own species. Thus, male mate choice via cuticular hydrocarbon pheromones explains the entirety of gene flow restricted by male mating preferences. This is congruent with previous work between *D. melanogaster* and *D. simulans*; the *D. melanogaster* female CHC 7,11-HD acts as an aphrodisiac to *D. melanogaster* males while entirely suppressing *D. simulans* male courtship (Billeter et al., 2009). *D. sechellia* females also express this pheromone, and are remarkably similar to *D. melanogaster* in complete pheromone bouquet (Jallon & David, 1987). Therefore, it is likely that 7,11-HD is a signal that *D. simulans* and *D. sechellia* males are responding to in our own assays as well.

Our results also indicate a potentially different response to conspecific female CHCs between the species. While removing pheromone-specific gustation caused males from both species to court indiscriminately, it had contrasting effects on the overall amount of

courtship we observed. When *D. simulans* males had their fore-tarsi removed, the amount of courtship toward conspecific females decreased by over 40% (Figure 1A, Figure 2A). This implies that CHCs are a necessary signal to induce normal levels of courtship, and suggests the presence of aphrodisiac pheromone(s) on the *D. simulans* cuticle. This is surprising for *D. simulans*, however, because males and females do not differ with respect to CHC pheromones (Pechine et al., 1985). Nonetheless, 7-Tricosene (7T), the most abundant of these CHCs, generally stimulates courtship from *D. simulans* males (Cobb et al., 1990). Males likely use an additional, undescribed cue to discriminate between the sexes. Conversely, the removal of the fore-tarsi had no effect on the amount of conspecific courtship observed in *D. sechellia* males (Figure 1A, Figure 2B). It appears that CHCs are not essential to stimulate courtship in this species, and may primarily be used as a species identification signal.

We cannot rule out the possibility that this finding is unique to our strains, as significant variation in male CHC preference response has been observed across strains of *D. melanogaster* (Pischedda et al., 2014). We think this is unlikely however, because our results mirror the pattern of CHC preferences between *D. melanogaster* and *D. simulans* (Billeter et al., 2009), and our results are comparable with male courtship patterns observed using different strains of *D. simulans* and *D. sechellia* (Cobb et al., 1990). It appears that CHC preferences may be less variable between species than within. Still, a more fine-scale dissection of the nuanced relationships between various pheromones and the valence and variation of male response is necessary to identify the precise mechanisms by which CHCs isolate these taxa. Recently, the 7,11-HD response circuit was mapped in *D. melanogaster* (Clowney et al., 2015). Similar efforts in *D. simulans* and *D. sechellia* could prove illuminating with respect to differences in CHC responses and the proximate mechanisms by which male mate preferences diverge.

The evolution of male mate choice and its role in reproductive isolation

Theory predicts that reproductive isolation is primarily determined by the choosier sex (Wirtz, 1999). How, then, does male choice evolve? Female mate choice has been proven to evolve via both direct and indirect selection (Andersson & Simmons, 2006), and the same can be true of male mate choice. With respect to reproductive isolation, direct selection could favor divergence in male traits to alleviate significant costs of courtship and/or mating for males. In *Drosophila*, the cost of courtship for males has been well demonstrated. Males engage in an elaborate ritual with potential mates (Sokolowski, 2001b); these displays can take considerable time and energy, and have been shown to shorten male lifespan (Cordts & Partridge, 1996; Partridge et al., 1985). These costs could be enhanced if males spend substantial time and energy courting heterospecific females that will rarely copulate. When these costs are great enough, selection will directly favor mechanisms that provide males with enhanced mate discrimination, ensuring they only court and mate with the most beneficial partners (Noor, 1995). Under this scenario, female choice may have initially played a stronger role in the isolation between these species, with strong male mate choice evolving secondarily. Indeed, male mate recognition has evolved in species where there is a high cost to courtship or mating, particularly with sympatric heterospecific females (Albert et al., 2004; Peterson et al., 2005).

This is a plausible explanation for the evolution of male mate choice in *D. simulans* and *D. sechellia.* Our results show that there is a significant cost of courtship/mating for *D. simulans* males, and that mate discrimination completely alleviates these costs. Similar costs of courtship and mating have been documented for the closest outgroup, *D. melanogaster* (Cordts et al., 1996). Our inability to detect a cost of courtship and mating for *D. sechellia* males might be explained by the reduced effort with which *D. sechellia* males court conspecific females compared to *D. simulans* males (Figure S1). The speciation of *D. simulans* and *D. sechellia* from a common ancestor likely occurred in allopatry (Kliman et al., 2000). Once geographically separated, hybrid incompatibilities can evolve simply as a byproduct of restricted gene flow (Brideau et al., 2006), further augmenting the costs to heterospecific courtship. When the species recently resumed contact, the existing male preferences restricted the largest amount of gene flow between them, and for *D. simulans*, alleviated these costs. In this case, male mate choice is essential to maintaining species boundaries, and thus the speciation process.

Alternatively, it is possible that selection favored the divergence of female-specific secondary sex characteristics to alleviate the costs of heterospecific male courtship. In this scenario, males have pre-existing and divergent biases that arose in allopatry. Upon secondary contact, females of one species evolve a novel signal that exploits these pre-existing biases, thereby minimizing the female costs associated with heterospecific courtship and/or mating. Pre-existing preference for a novel secondary sex characteristic has been described in other insects (Gray et al., 2016). With respect to differences in female CHCs, the evolution of the precise enzymes involved in the divergence of female-specific 7,11-HD expression is well described (Chertemps et al., 2006; Chertemps et al., 2007), with one enzyme in particular, *desatF*, showing a dynamic evolutionary history of gains and losses (Shirangi et al., 2009). An additional fine-scale genetic dissection of male mate choice behavior could be illuminating with respect to the ultimate causes of the evolution of reproductive isolation by male choice.

Male mate discrimination can also evolve indirectly, as a byproduct of ecological adaptation. In this scenario, adaptation to differences in environment during allopatry drives divergence of the mate recognition mechanism. CHCs protect insects from desiccation (Gibbs, 1998); in *Drosophila*, they have been shown to vary with climate (Rouault et al., 2001) and respond plastically to increased rearing temperatures (Rouault et al., 2004). QTL analysis of *D. melanogaster* strains shows genetic overlap between CHC profiles and desiccation resistance (Foley & Telonis-Scott, 2011), so is possible that female CHCs originally diverged between *D. simulans* and *D. sechellia* during allopatry as a result of climatic adaptation. Male preferences for these novel female CHCs could have then evolved either in allopatry or at secondary contact as a mechanism of species identification.

D. simulans and *D. sechellia* also differ in their host preferences. In the Seychelles, *D. sechellia* are found almost exclusively on the noni fruit, *Morinda citrifolia*, which is toxic to most other *Drosophila* species, including *D. simulans* (Matute et al., 2014). Host differences affect pheromone expression and perception in many insects (Reddy & Guerrero, 2004), including *Drosophila* species (Stennett & Etges, 1997), so it is possible that CHCs and/or male preferences diverged during *D. sechellia*'s host specialization. Again, identifying the

loci underlying differences in male CHC preference may allow us to identify the selective pressures responsible for the evolution of male mate discrimination as a reproductive barrier. Despite their utility, we have only a few examples of mechanisms that can promote the evolution of male mate choice (Gregorio et al., 2012; Peterson et al., 2005), which may be a widespread component of speciation in the natural world (Shaw & Parsons, 2002).

Conclusions

Here we documented strong male mate choice based on female cuticular hydrocarbon pheromones between two sympatric species of *Drosophila*. We showed that male choice accounts for the largest restriction of gene flow between these species when compared to other reproductive barriers. We further documented a significant cost of courtship and mating for males of one species, and showed that this cost is totally alleviated by male mate discrimination. Taken together, these results provide insight into the evolutionary mechanisms by which male choice may have evolved. Regardless of the mode of selection acting on male mate choice, our results highlight the potentially significant and often overlooked role of male choice in reproductive isolation and speciation, adding to recent findings in other systems (Moran et al., 2017). We suggest that further study of the mechanisms by which male mate discrimination evolves would provide valuable insight into the selection pressures driving the formation of reproductively isolating barriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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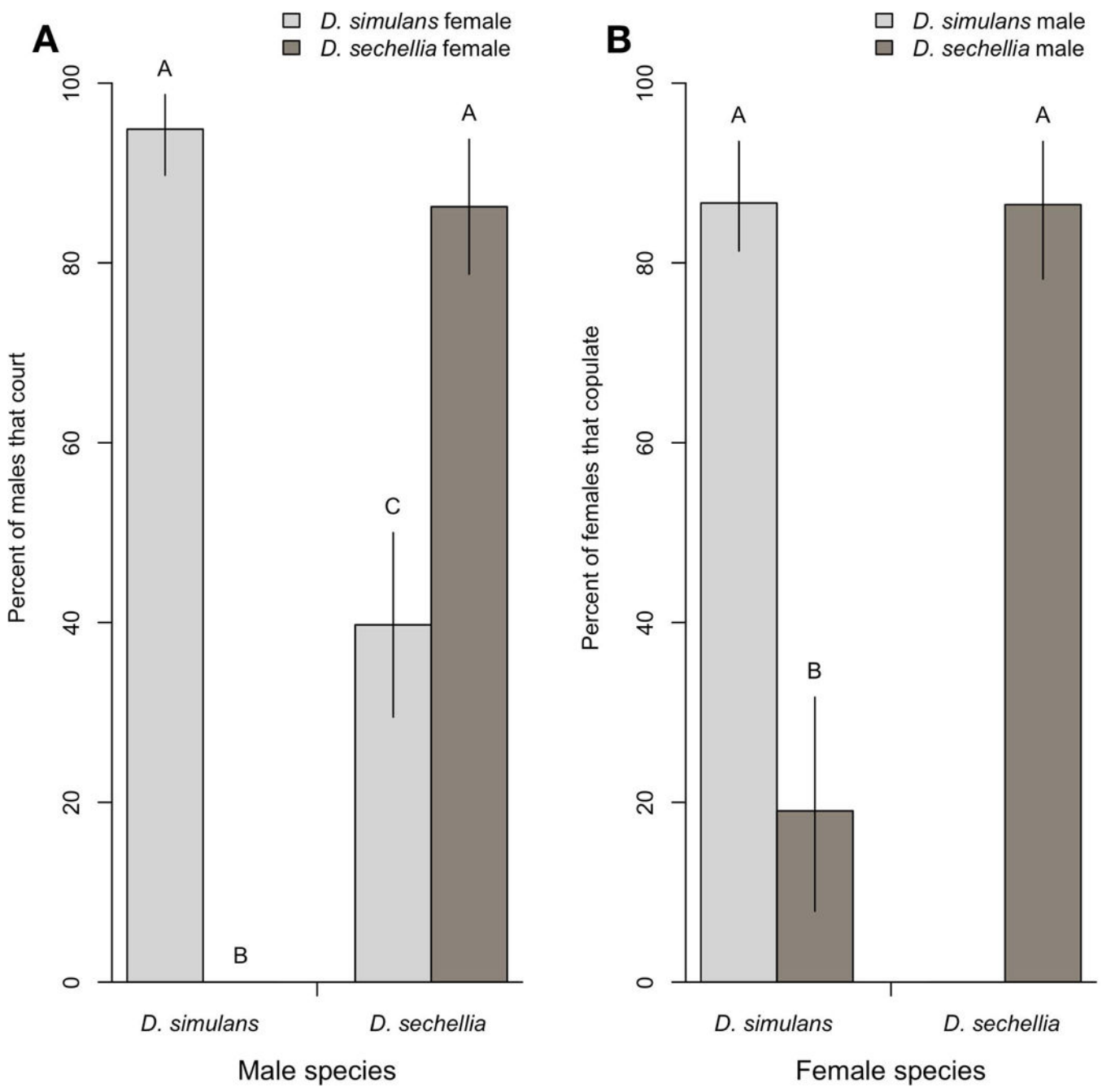

Figure 1.

Courtship frequencies (A) and copulation frequencies (B) for conspecific and heterospecific pairings of *D. simulans* and *D. sechellia*. For each panel, columns labeled with different letters are significantly different from one another (calculated using Fisher's exact tests corrected for multiple comparisons; N = 78–97). Error bars represent bootstrapped 95% confidence intervals.

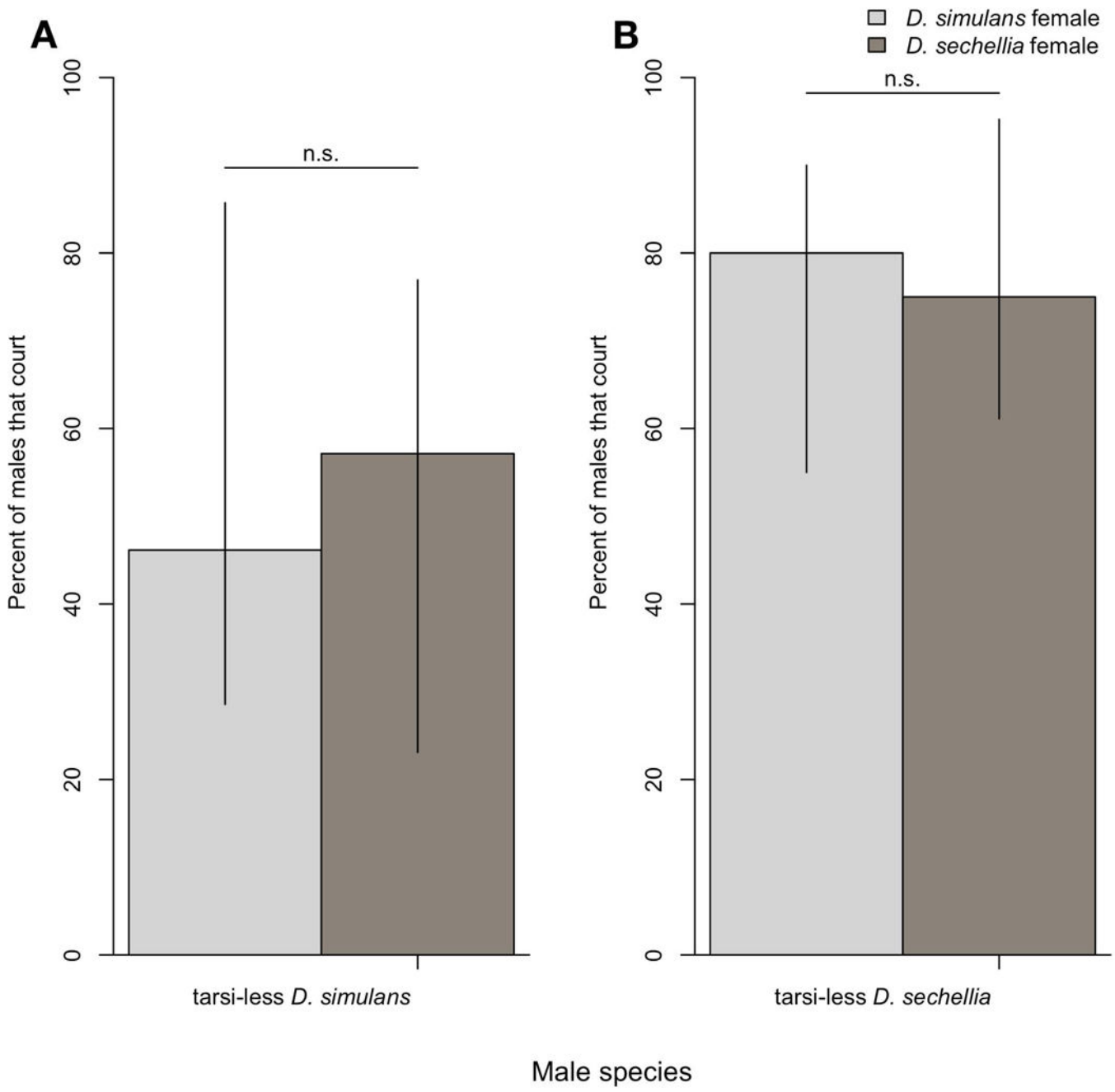

Figure 2.

Courtship frequencies for tarsi-less (N = 13–20) conspecific and heterospecific pairings of *D. simulans* (A) and *D. sechellia* (B). Error bars represent bootstrapped 95% confidence intervals.

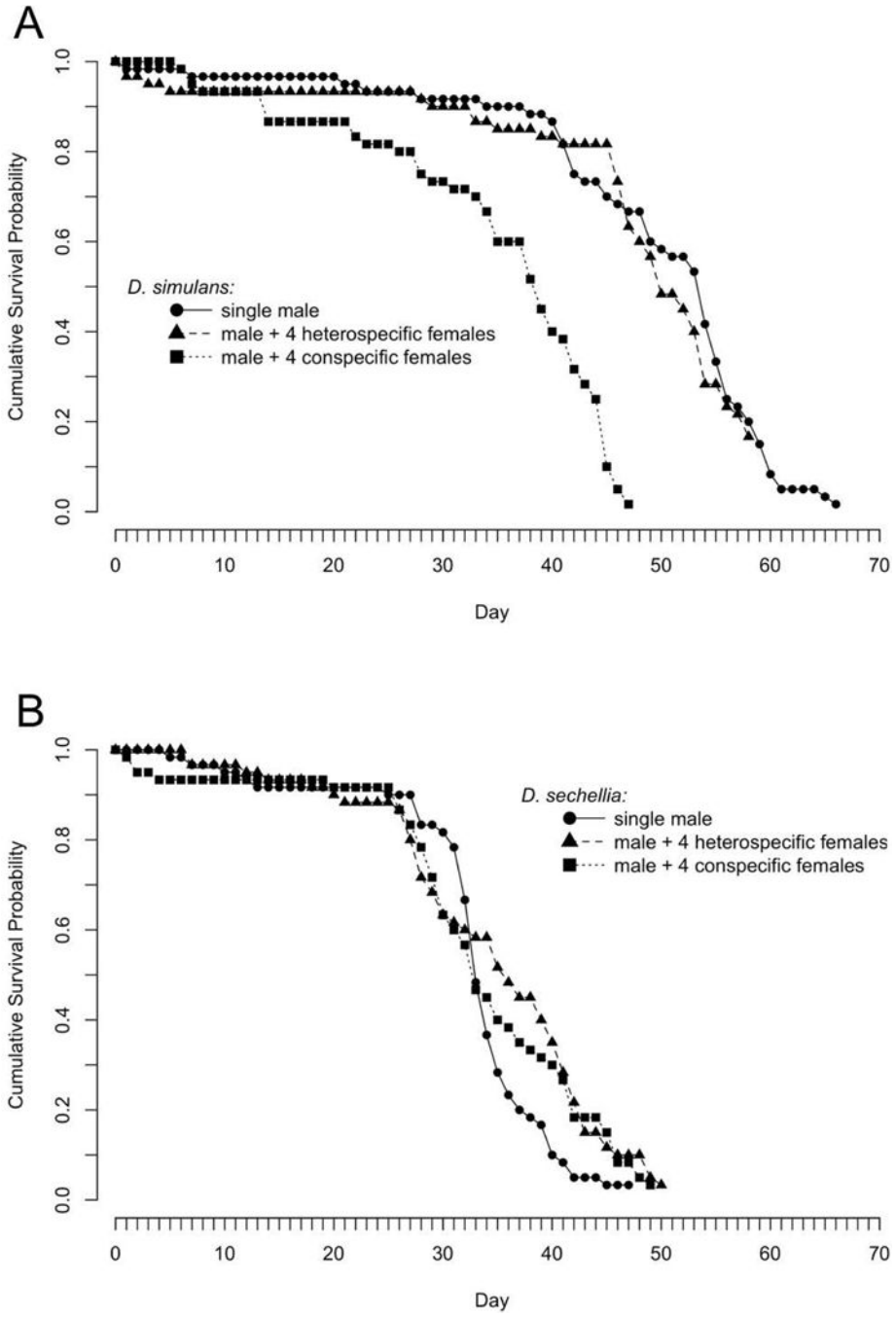

Figure 3.

A. Cumulative survival probability curves for *D. simulans* males held singly (circles), with 4 heterospecific *D. sechellia* females (triangles), or with 4 conspecific *D. simulans* females (squares). **B.** Cumulative survival probability curves for *D. sechellia* males held singly (circles), with 4 heterospecific *D. simulans* females (triangles), and males held with 4 conspecific *D. sechellia* females (squares). Cumulative survival probability was calculated as the proportion of surviving males (from initial N=60 for all) on each day.

Table 1

Strength of isolation resulting from individual reproductive barriers occurring between *D. simulans* and *D. sechellia*. Values indicate the proportion of gene flow limited by each barrier without sequential consideration (RI_n), after being considered sequentially (AC_n), and the relative contribution (RC_n) of each barrier (including bootstrapped 95% confidence intervals) to the total gene flow restricted (T).

Reproductive barriers	**RI_n**	**AC_n**	**RC_n (95% CI)**
RI_1: ♂ Courtship frequency	0.673	0.673	0.715 (0.642–0.786)
RI_2: ♀ Copulation frequency	0.639	0.209	0.222 (0.184–0.262)
RI_3: Postzygotic isolation	0.5	0.059	0.063 (0.030–0.097)
T: Total RI		0.941	