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Research Paper

Reproductive Tract Interactions Contribute to Isolation in *Drosophila*

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KEY WORDS

sperm, sperm storage, female reproductive tract, speciation, reproductive isolation, geographically isolated subpopulations

ABBREVIATIONS

PCPZ	postcopulatory-prezygotic
MY	million years
AB	Anza Borrego Desert, California
CI	Santa Catalina Island, California
EN	Ensenada de los Muertos, Mexico

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NOTE

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ABSTRACT

The process of speciation requires the development of isolating mechanisms that act as barriers to gene flow between incipient species. Such mechanisms can occur at three different levels: precopulatory or behavioral isolation, postcopulatory-prezygotic isolation occurring in the female reproductive tract, or postzygotic isolation resulting in hybrid sterility or inviability. Only by extensively studying all three types of barriers in young species pairs can we begin to understand the evolution of early reproductive incompatibilities, which may be important to the speciation process. Although precopulatory and postzygotic isolation have been well described it is only recently that the female reproductive tract has been intensely examined for possible mechanisms of reproductive isolation (reviewed in refs 1and 2). The types of isolating mechanisms that develop at this level and their role in speciation, therefore, remain poorly understood.

Polyandry, internal fertilization, and sperm storage have made *Drosophila* a popular system for the study of reproductive tract interactions, and there is a range of points along the postcopulatory-prezygotic (PCPZ) trajectory at which incompatibilities could arise. Males must transfer sperm successfully and the sperm must enter sperm storage organs, remain viable, and be able to fertilize eggs. Additionally, in many species of *Drosophila* females must be stimulated by mating to oviposit.[3] These postcopulatory processes rely on functional interactions between male and female morphology[4] and molecular biochemistry.[5-7] Such interactions are determinants of reproductive success, and therefore sexual selection and intersexual coevolution have caused them to become extremely divergent between species.[8-10] The morphology of sperm and sperm storage organs and the patterns of sperm transfer and storage show extreme variation across the genus.[8] Additionally, male seminal or accessory gland proteins, and female reproductive molecules are highly divergent between species and many show signatures of adaptive evolution at the molecular level.[11-17] Such coadapated divergence predicts failures of morphological and molecular interactions in heterospecific crosses.

In this study, we examined the role of reproductive tract interactions as isolating mechanisms between the cactophilic *Drosophila*, *D. mojavensis*, and its sister species *D. arizonae* (distributions shown in Fig. 1). Because this species pair is young (~0.8 MY[17]), partially sympatric, and will hybridize in the laboratory, it provides an excellent opportunity for identifying early-acting barriers. Additionally, both precopulatory[19-22] and postzygotic isolation[23,24] have been examined extensively. Several clues suggest that PCPZ isolation may also play an important role in restricting gene flow between these two species. First, there is a marked reduction in the proportion of heterospecifically mated *D. mojavensis* females that produce offspring.[25] Additionally, fertile heterospecific crosses produce very few hybrids, although the level of oviposition is normal.[23] Finally, the insemination reaction, a large white mass that forms in the uterus after mating in many *Drosophila*,[26] is reportedly more severe in heterospecific crosses.[25] Although the function of the reaction mass remains unknown, it may serve to delay female remating[27,28,29] and therefore be coevolving antagonistically between the sexes due to sexual conflict.[29]

We first examined both the fecundity and fertility of homospecifically and heterospecifically mated *D. mojavensis* females from three geographically isolated populations: Anza Borrego Desert, California (AB), Santa Catalina Island, California (CI), and Ensenada de los Muertos, Mexico (EN). Upon finding evidence that productivity of heterospecific crosses was severely reduced, we examined the reproductive tracts of mated females to identify specific incompatibilities. Evidence for incompatibilities in four distinct PCPZ processes was found: sperm storage, sperm viability, fertilization, and oviposition.

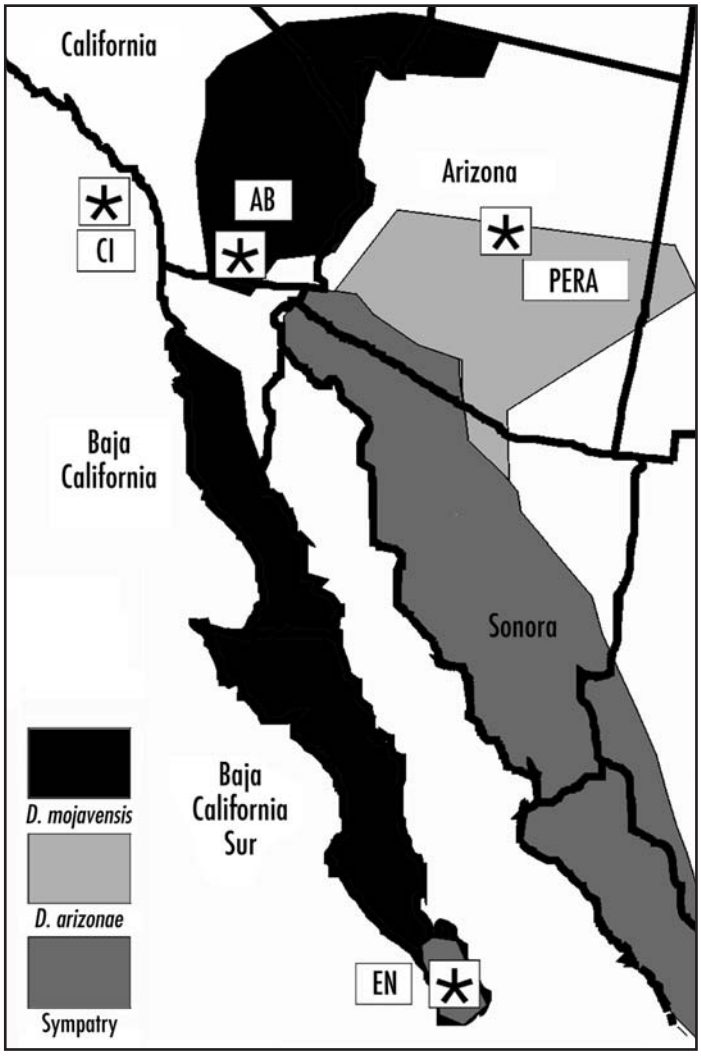

Figure 1. Species distributions of *D. mojavensis* and *D.arizonae*. Three allopatric and one sympatric population of *D. mojavensis* are indicated. One continuous population of *D. arizonae* is indicated.

MATERIALS AND METHODS

Collection and rearing. *D. mojavensis* was collected from Ensenada de los Muertos, Mexico, in January 2001, Catalina Island, California, in April 2001, and Anza Borrego Desert, California, in March 1995 and April 2002. *D. arizonae* was collected from Peralta Canyon, Arizona, in April 1997 (Fig. 1). For the strains collected in Anza Borrego, the March 1995 strain was used in the offspring viability and fertilization studies, while the 2002 strain was used in the microscopy study. Both species were reared on standard opuntia-banana medium (for recipe see http://stockcenter.arl.arizona.edu/), and have similar generation times of ~19 days.[30]

Offspring viability measures. Sexually mature flies no older than nine days post-eclosion were paired in individual vials and observed until copulation. Females were then isolated and transferred daily to fresh vials of opuntia banana medium. Daily oviposition and emerging adults were quantified. Two replicates were performed.

Percentage eggs fertilized. Flies were mass-mated and the resulting eggs were collected on agar plates. Although it was not possible to verify all eggs were oviposited by mated females for this portion of the study, *D. mojavensis* females require mating for oviposition.[3] Eggs were dechorionated in 2% hypochlorite, and their nucleic acid stained with 4',6-diamidino-2-phenylindole (DAPI). Prepared eggs were examined under a fluorescent microscope (200x) to determine if they were fertilized. Fertilized eggs are easily identified by the wiry appearance of the male pronucleus, adjacent to the micropyle.[31]

Microscopy of mated uteri. Sexually mature females no older than 12 days post-eclosion were observed to mate and then isolated on opuntia-banana medium for five days. Oviposition was quantified, as was total number of emerging adults from deposited oocytes. At five days post mating, whole lower female reproductive tracts, including the uterus, seminal receptacle, spermathecae, parovaria and common oviduct were removed in PBS and mounted on a glass slide. Slides were observed with a Nikon E800 upright microscope under dark-field (200x). Digital images were taken with an attached camera and SPOT image software (www.diaginc.com/supdownloads.asp).

Scoring of phenotypes. Females dissected five days post-mating were scored for three different phenotypes: sperm storage, sperm viability, and severity of the insemination reaction mass. Sperm storage and viability refer only to the seminal receptacle, as *D. mojavensis* females do not store sperm in the spermathecae.[8] We chose to dissect flies five days post-mating because qualitative preliminary data indicated there were clear differences in the reproductive tracts of homospecifically and heterospecifically mated females at this time point. Females with one or more sperm in the seminal receptacle were scored as storing sperm. Females with one or more motile sperm were scored as having motile sperm. Females with any evidence of a reaction mass were scored as exhibiting a mass, while females with no evidence of a reaction mass were scored as no mass. We further scored the severity of the insemination reaction was from 1 to 6:1—clear uterus, 2—fluid or debris present, 3—small mass, 4—large mass, 5—condensed clog-like mass, 6—clog-like mass with decomposing oocyte.

Statistical analysis. For offspring oviposition and adult hatchability: A model that included female population, cross type, population x cross type, and replicate found no evidence for a replicate effect ($F_{6,231}$ = 0.0239, p = 0.88). Therefore the two replicates were pooled. Descriptive statistics of pooled data are represented in (Fig. 2).

For dissected reproductive tracts: Chi-squared and Fisher's exact test were applied to 2 x 2 contingency tables to determine if the proportion of females who exhibited a given postcopulatory trait was independent of whether the female was mated to a *D. mojavensis* male or a *D. arizonae* male. Specifically, for each *D. mojavensis* population, proportions of females for a bivariate phenotype (for example, sperm and no sperm) were compared between homospecific and heterospecific crosses.

RESULTS

We assessed fecundity and fertility of heterospecific and homospecific crosses by quantifying oviposition and offspring production over a seven-day period. Approximately 50% of heterospecifically-mated females failed to oviposit and were excluded from further analysis as possible instances of pseudocopulation. Heterospecifically mated *D. mojavensis* females from CI and EN that did oviposit laid significantly fewer eggs than homospecifically mated females, while AB females laid significantly more (Fig. 2). The more striking pattern, however, is that fertility, as measured by the ratio of viable adults to oviposited eggs, is reduced from 60–70% in homospecific to 4–16% in heterospecific matings (Fig. 2). When fertilization success

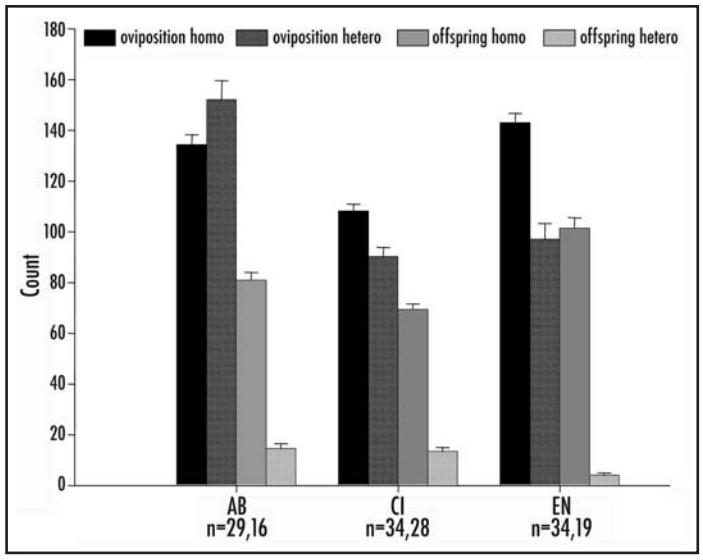

Figure 2. Reproductive output of homospecific and heterospecific crosses. Oviposition (average number of fertilized eggs) and offspring production (average number of viable adults) for homospecifically and heterospecifically mated *D. mojavensis* females from Anza Borrego Desert (AB), Santa Catalina Island (CI), and Ensenada de los Muertos (EN). *D. arizonae* males denoted by (A). Samples sizes for the homospecific and heterospecific cross are indicated. Error bars indicate standard error (SE).

was examined by staining eggs for the presence of sperm heads, the low fertility of heterospecific crosses having normal levels of oviposition was found to result from fertilization failure rather than hybrid inviability (Supplementary Material). These data clearly indicate the existence of isolating mechanisms that occur in the reproductive tracts of heterospecifically mated *D. mojavensis* females.

To identify the physical basis of the observed reductions in oviposition and fertilization, we examined the reproductive tracts of mated *D. mojavensis* females five days after copulation. Specifically, the presence and motility of sperm in the seminal receptacle and the presence and appearance of the insemination reaction were scored. Oviposition and offspring production were also quantified for each dissected female. Strong evidence for mismatches between several reproductive traits of the two species was found (Table 1).

Although all homospecifically-mated females contained stored sperm, no sperm were seen in a significant portion of heterospecifically-mated females. Since every female who failed to store sperm produced no offspring, this incompatibility resulted in a completely infertile cross. Additionally, only a small proportion of eggs oviposited by those heterospecifically mated females with sperm ever produced offspring. Clearly, problems in sperm storage alone cannot explain the low fertility of heterospecific crosses: an additional incompatibility must occur later. The nature of this incompatibility remains unclear, but failures in sperm release from the receptacle, or in the timing or chemistry of the fertilization process, seem probable.

For every mating type, complete sperm mortality, as evidenced by a lack of motile sperm, occurred in some proportion of females examined (Table 1). Significant population variation in this proportion suggests different populations may experience different selective pressures for sperm longevity. Additionally, females from AB show a significant increase in mortality of stored heterospecific sperm. The increase in sperm death could result from two separate processes. First, the seminal receptacle could fail to provide a hospitable environment to *D. arizonae* sperm due to an intrinsic incompatibility in the environment provided and the metabolic requirements of the sperm. Alternatively, cryptic female choice could cause females to either under nourish undesired sperm or actively release spermicidal compounds.

All populations showed a significant increase in the presence of the insemination reaction in heterospecifically-mated females (Table 1). Indeed, the proportion of heterospecifically-mated females that still exhibited a reaction mass five days post-mating is strikingly high. The difference in appearance and location of the reaction mass between homospecific and heterospecific crosses, furthermore, is a compelling demonstration of PCPZ incompatibility. Five days postmating in homospecific crosses the mass was either absent, implying it had already been degraded by the female, or it appeared as an opaque fluid in the pocketed area of the uterus adjacent to the common oviduct (Fig. 3A). In contrast, the reaction mass in many heterospecifically mated females appeared as a dense gelatinous clog, implying that *D. mojavensis* females are inefficient at degrading the reaction mass induced by the seminal fluid of *D. arizonae* males. When this clog was observed to settle near the exit of the uterus, oviposition was blocked, as evidenced by the high incidence of decaying eggs in the uteri of these females (Fig. 3B).

To quantify the relationship between the reaction mass and oviposition, we used a linear regression between the two variables. The severity of the reaction mass was scored from 1 to 6, in which a ranking of 1 denoted a clear uterus and a ranking of 6 denoted a clogged uterus with a decomposing oocyte. A strong negative correlation was found ($R^2 = 0.22$, $p < 0.001$), which indicates the reduction in oviposition in heterospecific crosses

Table 1 **Incidence of sperm storage, sperm mortality and reaction mass**

	Female population		
	Anza Borrego	**Santa Catalina Island**	**Ensenada de los Muertos**
N (homo)	23	21	20
N (hetero)	26	44	20
Reaction mass (homo)	9 (39%)	1 (5%)	5 (25%)
Reaction mass (hetero)	23 (88%)	23 (52.3%)	18 (90%)
p-value	<0.001 (0.0003)***	<0.001 (0.0001)***	<0.001 (0.00003)***
Sperm storage (homo)	23 (100%)	21 (100%)	20 (100%)
Sperm storage (hetero)	14 (54%)	8 (18%)	11 (55%)
p-value	NA (0.0001)***	NA (7.1e-11)***	NA (0.0006)***
Sperm motility (homo)	11 (48%)	14 (67%)	16 (80%)
Sperm motility (hetero)	1 (7%)	3 (38%)	8 (73%)
p-value	<0.025 (0.01)*	<0.2 (0.15)	<1 (0.5)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **Incidence of the insemination reaction mass, stored sperm in the seminal receptacle, and motile sperm in the seminal receptacle for homospecifically and heterospecifically mated *D. mojavensis* females from Anza Borrego Desert, Santa Catalina Island, and Ensenada de los Muertos. p-values for X^2 and Fisher's exact test (parentheses) for differences between homospecific and heterospecific crosses. NA indicates X^2 was inappropriate to the data.**

can be partially explained by the formation of more severe reaction masses in these females (Fig. 4).

DISCUSSION

We present clear evidence that mismatches in reproductive tract interactions contribute to isolation in *Drosophila*. The identification of isolating mechanisms in the female reproductive tract that affect sperm storage, sperm viability, oviposition, and fertilization, in two closely related sister species with partially overlapping ranges indicate that PCPZ incompatibilities potentially play an important role in speciation. The multitude of processes that are perturbed in the reproductive tracts of heterospecifically mated females indicates that incompatibilities at this level are extremely complex and likely involve the breakdown of several intersexual epistatic interactions. Although the nature of these interactions remains unidentified, accessory gland proteins and female reproductive molecules are likely to play an integral role due to their function in mediating postcopulatory processes.

We hypothesize that PCPZ incompatibilities result from intersexual coevolution between the male ejaculate and female reproductive tract. Interpopulation differences in sperm mortality and reaction mass size seen here (Table 1) are consistent with ejaculate-female co-evolution. Indeed, there is evidence for coevolution of sperm and seminal receptacle size,[32] and reaction mass induction,[29] within populations of *D. mojavensis*. The insemination reaction mass is of particular interest, as sexually antagonistic coevolution of this trait is thought to result from sexual conflict over female remating.[29] The interference of the insemination reaction with oviposition (Fig. 4) therefore points to a role for sexual conflict in the evolution of reproductive isolation between species.

Differences in severity and presence of isolating mechanisms between populations shown here indicate that interpopulation variability within *D. mojavensis* is relevant to reproductive isolation from *D. arizonae*. An incompatibility that affected sperm longevity was found only in females from AB, which implies that some co-evolutionary trajectories may result in incompatibilities, while others may not. Additionally, although all the populations showed a reduction in stored sperm and an increase in the incidence of a persistent insemination reaction in heterospecific crosses, significant variation between populations was found in the severity of these traits.

The incompatibilities we describe do not simply result in low productivity of heterospecific matings; they are extremely costly to females. Oviposition of unfertilized eggs is a poor use of female resources invested in gamete production. Additionally, clogged uteri are likely to permanently sterilize females, having a severe effect on their lifetime reproductive output. Although we did not explicitly address this question, it follows that these costs would select for *D. mojavensis* females who discriminate against *D. arizonae* males in terms of mate choice. Intriguingly, there is strong evidence for reinforcement in sympatry when *D. mojavensis* females are mated with *D. arizonae* males[19-21] but not for the reciprocal cross.[22] As postzygotic isolation in this direction is relatively weak,[23,24] these results imply that reproductive tract interactions should be considered a possible driving force in the evolution of sympatric behavioural isolation, in addition to hybrid sterility and inviability. Further research into the relationship between PCPZ isolation and behavioral isolation will clarify relationships between types of isolating mechanisms and the speciation process as a whole.

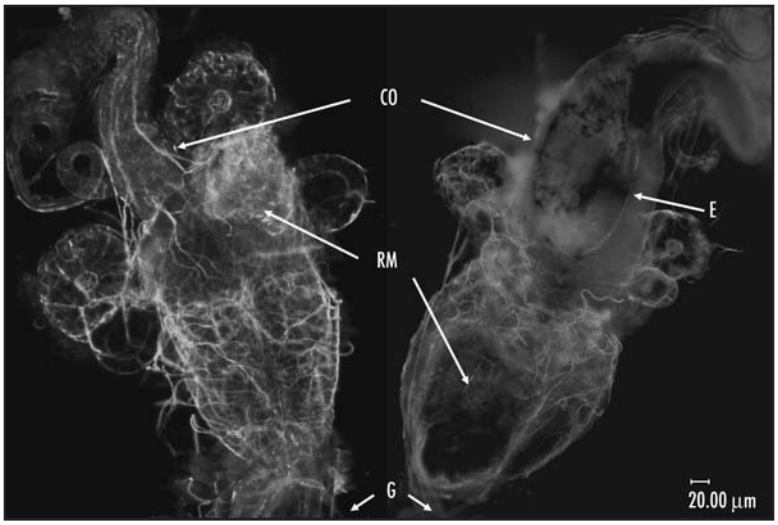

Figure 3. The reaction mass of a homospecifically and heterospecifically mated female. Reproductive tracts of homospecifically (left-panel) and heterospecifically (right-panel) mated *D. mojavensis* females from Santa Catalina Island five days post-copulation. Common oviducts (CO), reaction masses (RM), external genitalia (G), and eggs (E) are indicated.

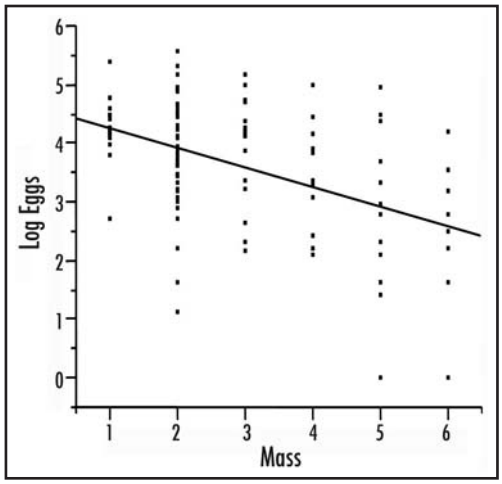

Figure 4. The negative correlation between the reaction mass and oviposition. Mass severity was ranked from 1 to 6. Log transformation of oviposition quantity. $F_{1,109} = 29.87$, $p < 0.0001$, $R^2 = 0.22$.

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