



RAPID EVOLUTION OF ASYMMETRIC REPRODUCTIVE INCOMPATIBILITIES IN STALK-EYED FLIES

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The steps by which isolated populations acquire reproductive incompatibilities remain poorly understood. One potentially important process is postcopulatory sexual selection because it can generate divergence between populations in traits that influence fertilization success after copulation. Here we present a comprehensive analysis of this form of reproductive isolation by conducting reciprocal crosses between variably diverged populations of stalk-eyed flies (*Teleopsis dalmanni*). First, we measure seven types of reproductive incompatibility between copulation and fertilization. We then compare fertilization success to hatching success to quantify hybrid inviability. Finally, we determine if sperm competition acts to reinforce or counteract any incompatibilities. We find evidence for multiple incompatibilities in most crosses, including failure to store sperm after mating, failure of sperm to reach the site of fertilization, failure of sperm to fertilize eggs, and failure of embryos to develop. Local sperm have precedence over foreign sperm, but this effect is due mainly to differences in sperm transfer and reduced hatching success. Crosses between recently diverged populations are asymmetrical with regard to the degree and type of incompatibility. Because sexual conflict in these flies is low, postcopulatory sexual selection, rather than antagonistic coevolution, likely causes incompatibilities due to mismatches between male and female reproductive traits.

KEY WORDS: Gametic isolation, hybrid inviability, postcopulatory sexual selection, speciation, conspecific sperm precedence, *Teleopsis dalmanni*.

Dobzhansky (1937) first recognized that incompatibility at the gametic level could contribute to reproductive isolation (RI). However, the potential for postcopulatory interactions to create barriers to gene flow among populations has only become a focus of investigation recently (Eady 2001; Ludlow and Magurran 2006; Martin-Coello et al. 2009). This interest has arisen in conjunction with an increased awareness that sexual selection often involves interactions between the sexes that occur after mating (Parker 1970). In particular, postcopulatory sexual selection is now recognized as an important evolutionary force capable of causing rapid evolution of female reproductive tracts (Eberhard 1996), sperm (Simmons 2001), and seminal products (Howard 1999; Eady 2001; Coyne and Orr 2004). Change in either male

or female reproductive traits can create correlated selection in the opposite sex and lead to male–female coevolution (Pitnick et al. 1999; Presgraves et al. 1999; Pitnick et al. 2003; Joly and Schiffer 2010; Ronn et al. 2011). Such male–female coevolution can result from antagonistic coevolution in response to sexual conflict (Parker and Partridge 1998; Gavrillets 2000; Gavrillets and Hayashi 2006) or sexual selection (Lande 1981; Panhuis et al. 2001) either of which can potentially lead to reproductive incompatibilities evolving among isolated populations (Arnold et al. 1996; Arnqvist et al. 2000; Knowles and Markow 2001).

Gametic isolation is used to describe any mechanism of RI that occurs between mating and zygote formation. Coyne and Orr (2004) divide gametic isolating barriers into two forms:

noncompetitive and competitive. Noncompetitive gametic isolation impedes fertilization between populations regardless of whether the female has mated with one or multiple males. The most convincing evidence of reproductive barriers evolving by noncompetitive gametic isolation is from sperm–egg incompatibilities in externally spawning organisms. Studies of the evolution of sperm and egg recognition molecules in abalones have found that these molecules are highly species specific and evolve rapidly by positive selection (Kresge et al. 2001). In species with internal fertilization, males must transfer sperm that successfully achieve fertilization and incompatibilities can arise in additional ways. For example, Price et al. (2001) found three separate types of noncompetitive gametic isolation, all involving sperm transfer and storage inefficiencies, among three species in the *Drosophila simulans* complex. Other mechanisms of gametic isolation include decreased oviposition (Brown and Eady 2001) and incomplete fertilization (Alipaz et al. 2001).

Competitive gametic isolation may occur when a female mates with two types of males and their ejaculates overlap in time and space. In this situation, sperm from each male type is physiologically capable of fertilizing the ova, but one male type achieves more fertilizations than the other. Two outcomes are possible: conspecific or heterospecific sperm precedence. Which of these occurs has also been proposed to result from either sexual selection or sexual conflict. If sexual selection leads to coevolution between male and female reproductive traits, a conspecific male should be expected to fertilize more offspring than a heterospecific male. This outcome may be due to sperm competitive advantages of the conspecific male, cryptic female preference for the conspecific male, or a combination of the two (Howard 1999). Such conspecific sperm precedence has been reported in many species (Gregory and Howard 1994; Wade et al. 1994; Rieseberg et al. 1995; Price 1997; Dixon et al. 2003; Chang 2004; Geyer and Palumbi 2005; Yeates et al. 2013) and has potential to create or augment RI. In contrast, if females resist mating efforts by conspecific males due to sexual conflict, males from closely related but different species could have an advantage in sperm competition because females lack appropriate defenses (Rice 1998; Andres and Arnqvist 2001; Hosken et al. 2002). In this case, heterospecific sperm precedence would act against RI because it enhances gene flow (Parker 2006).

Although studies have shown that competitive gametic isolation decreases gene flow in plants (Campbell et al. 2003), marine invertebrates (Geyer and Palumbi 2005), fish (Yeates et al. 2013), and insects (Fricke and Arnqvist 2004), few have accounted for the effects of noncompetitive gametic isolation when examining the importance of competitive gametic isolation as a barrier to gene flow. This is problematic because noncompetitive and competitive gametic isolation occur simultaneously in several systems (Price 1997; Brown and Eady 2001; Price et al. 2001) and can

be confounded. In such cases, differential offspring production may be due to decreased sperm transfer or hatching success by heterospecific males rather than a competitive advantage for conspecific males. For example, apparent conspecific sperm precedence in which heterospecific males produce only 25% of the offspring could be caused by a decrease in sperm transfer or hatching success between the species rather than sperm competition. Conversely, if more sperm are transferred in heterospecific than conspecific crosses, conspecific sperm precedence could be masked.

In this study, we use a series of crosses within and between several allopatric populations of diopsid stalk-eyed flies (*Teleopsis dalmanni*) to determine the manner in which alternative reproductive barriers, especially those that act after mating and before egg hatching, accumulate during lineage isolation and divergence. *Teleopsis dalmanni* is a sexually dimorphic stalk-eyed fly from southeast Asia, which has been used as a model for studies on precopulatory sexual selection as a consequence of either male fighting (Panhuis and Wilkinson 1999; Egge et al. 2011; Egge and Swallow 2011) or female choice (Wilkinson and Reillo 1994; Wilkinson et al. 1998; Hingle et al. 2001a, 2001b). However, postcopulatory sexual selection is also suspected to be intense because females frequently remate (Wilkinson et al. 2003) and store sperm from multiple males for at least a month (Lorch et al. 1993; Wilkinson et al. 2006). Sperm length and female sperm storage organs vary dramatically among species (Presgraves et al. 1999) and are known to be influenced by X-linked factors (Wilkinson et al. 2005; Johns and Wilkinson 2007). These flies are found in riparian habitats and have limited dispersal. Consequently, many populations are genetically isolated (Swallow et al. 2005), which creates an ideal situation for studying the accumulation of reproductive incompatibilities over time.

In a previous study, Christianson et al. (2005) found that postzygotic RI, in the form of male hybrid sterility, could be detected in any *T. dalmanni* population cross that resulted in offspring. They also reported that progeny production decreased in crosses involving flies from different populations as a function of genetic distance but could not determine if this was due to gametic or postzygotic (i.e., embryonic inviability) effects. Here we assess and quantify these two possibilities by comparing the proportion of eggs that hatch to the proportion of eggs that are fertilized between populations with different divergence times. Furthermore, to identify causes of variation in fertilization success, we collect data on mating, sperm transfer, sperm survival, sperm motility, and sperm presence at the site of fertilization. By comparing results within populations to reciprocal crosses between populations, we measure both the presence and magnitude of incompatibilities throughout the reproductive process from mating to egg hatch. In addition, we conduct a sperm competition experiment to determine whether heteropopulation ejaculates would be at a

competitive disadvantage compared to conpopulation males if females mated with both. By using estimates of sperm transferred and hatching success in the absence of sperm competition, we assess the degree to which competitive gametic isolation might reinforce or act in opposition to noncompetitive gametic incompatibilities. Finally, to identify patterns in the evolution of reproductive incompatibilities, we quantify absolute and relative contributions to RI (cf. Ramsey et al. 2003; Mendelson et al. 2007) at different times of genetic divergence.

Materials and Methods

NONCOMPETITIVE GAMETIC ISOLATION

To investigate noncompetitive gametic isolation, we selected four allopatric populations of *T. dalmanni* (synonymized with *Cyrtodiopsis*; Meier and Baker 2002) that interbreed but exhibit varying degrees of RI (Christianson et al. 2005). These populations descend from flies that were collected by hand net near streams from two sites in peninsular Malaysia (Ulu Gombak and Cameron Highlands) and two sites in Sumatra (Bukit Lawang and Soraya) in 1999 or 2000 (Swallow et al. 2005) and are abbreviated G, C, L, and S, respectively. Phylogenetic analysis of mitochondrial and nuclear sequence indicates that these populations form a single clade, (C(G(LS))), with little or no migration. Coalescent estimation of divergence times based on mitochondrial sequences indicates that L and S diverged about 80,000 years ago, G and LS about 470,000 years ago, and C from the others approximately 1 million years ago (Swallow et al. 2005). Thus, flies from these populations can be mated in four divergence categories, that is within population crosses or three types of between population crosses.

Stocks were maintained in large plexiglass cages at 25°C and 70% humidity with a 12-h L:D cycle. Larvae were reared in cups containing 25–50 mL of pureed corn and kept in incubators at 25°C with a 12-h L:D cycle. Within a week of eclosion, females were separated from males to ensure that all flies used in crosses were virgins, as sexual maturity occurs 3 weeks after eclosion (Baker and Wilkinson 2003). All flies were fed pureed corn in disposable cups twice a week unless otherwise indicated.

To identify incompatibilities between populations at multiple stages between mating and hatching, we performed two sets of mating crosses because females dissected to estimate sperm storage, survival, or motility could not be used to measure egg production and eggs scored for fertilization could not be used to score hatching success. In the first set of crosses, we measured mating, sperm transfer, sperm survival, egg production, and hatch success. In the second set of crosses, we determined mating, sperm motility, presence of sperm at the site of fertilization, egg production, and fertilization success. In both sets, the four populations

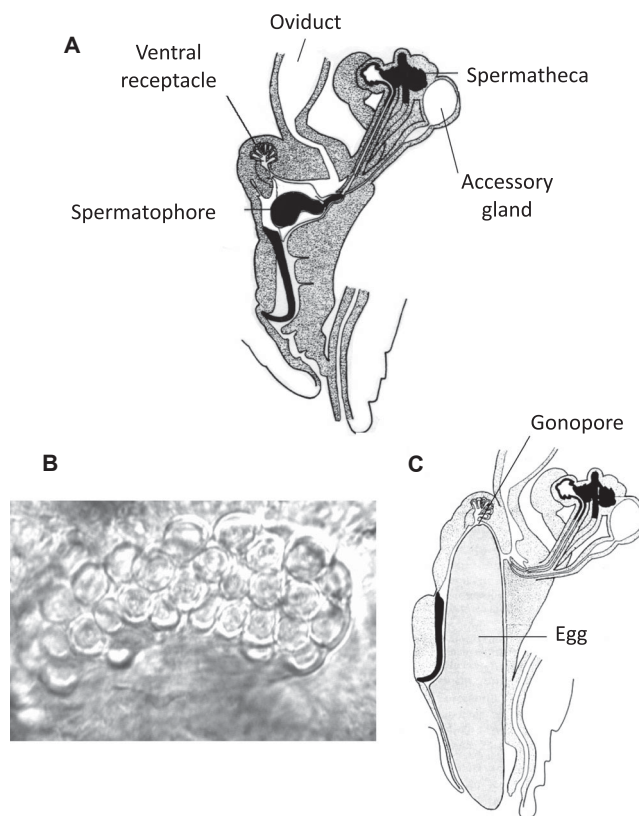


Figure 1. (A) Sketch of a female reproductive tract showing the location of a spermatophore shortly after mating, the female sperm storage organs (spermathecae), and the site of fertilization (ventral receptacle, VR). (B) Oil immersion image (1000 \times) of a VR containing coiled sperm inside each individual chamber. (C) Female reproductive tract prior to oviposition illustrating how the gonopore of the egg is positioned below the VR (A and C adapted from Kotrba 1993).

were crossed in a full factorial design. We conducted a total of 198 crosses in the first set (average 12 replicates per population pair) and 275 crosses in the second set (average 17 replicates per population pair). For all crosses, three virgin females and one virgin male of the appropriate populations were placed in a small cage (16 \times 14 \times 12.5 cm) and allowed to copulate freely for 7 days. We recorded age of the male and female flies, but always used flies at least 6 weeks of age to insure that they were reproductively mature.

MATING, SPERM TRANSFER, AND SPERM SURVIVAL

During a 1–5 min mating, *Teleopsis* males pass a spermatophore containing sperm to females (Kotrba 1996). The spermatophore inserts into a duct (Fig. 1A) through which sperm move into sclerotized spermathecae for storage. Females typically eject the spermatophore 15–60 min after mating, sometimes before all sperm have been transferred (Kotrba 1993). Thus, the ability of sperm

to be transferred and survive inside spermathecae could influence fertilization. We measured sperm number and survival in the first set of crosses by dissecting one female one day after the removal of the male and a second female a week later. These time points were chosen to determine if sperm survival varies depending on length of time in the female storage organ. The female's reproductive tract was removed and placed on a slide with 10 μ L of live/dead stain (Sperm Viability kit, L-7011; Molecular Probes, Eugene, OR). A cover slip was then positioned over the spermathecae and tapped to release sperm (Fry and Wilkinson 2004). The slide was placed on a Nikon Eclipse E600 microscope fitted with two fluorescence filter cubes (B-2E/C and G-2E/C) and examined at 200 \times with each filter to count the number of live (green) and dead (red) sperm stored in each female. The sum of live and dead sperm is the number of sperm transferred. We found no significant difference between the number of sperm present after removal of the male on day 1 versus 7 days previously, so we used the average number of sperm to score sperm stored. Similarly, we found no difference in the proportion of sperm alive on day 1 versus 7 days after male removal, so we used the average proportion of sperm alive to score sperm survival.

Mating was scored as a nominal variable with binomial variance and data were combined for both sets of crosses. In the first set of crosses, we scored mating as having occurred if any sperm were found in the spermathecae or if any eggs hatched for any cross. In the second set of crosses, we inferred mating had occurred if any sperm were found in the spermathecae or if any eggs were fertilized.

SPERM MOTILITY AND PRESENCE AT THE SITE OF FERTILIZATION

For fertilization to occur, sperm must travel down the spermathecal ducts and then move into chambers in the ventral receptacle (VR) at the top of the bursa (cf. Fig. 1). The VR acts as a short-term sperm storage organ and is the site of fertilization (Kotrba 1993). In *Teleopsis* stalk-eyed flies, the VR contains 16–40 small transparent chambers, each of which is capable of holding a single-coiled sperm (Fig. 1B). Fertilization occurs when a sperm leaves a VR chamber and enters an egg through the gonopore (Fig. 1C). Thus, for fertilization to be successful, sperm must be able to survive and move through the female reproductive tract so that they are in the VR when eggs pass through the oviduct. Sperm motility and presence at the site of fertilization were assessed for two females from each cage in the second set of crosses. Females were dissected 3 days after the male was removed from the cage. The reproductive tract was excised, placed on a slide in phosphate-buffered saline (PBS), and sperm released from the spermathecae as described earlier. Sperm were then immediately visualized using differential interference contrast (DIC) microscopy at 400 \times . Differential interference contrast, or Nomarski,

microscopy uses sheared polarized light to produce what looks like a three-dimensional image, which makes unstained sperm highly visible. Motion of live sperm was then recorded for 60 sec using a digital video camera connected to the microscope. Sperm motility was scored for 10 randomly selected sperm as the number of oscillations per 10 sec period using iMovie software.

After recording sperm motility, the VR was visualized using oil immersion and DIC microscopy at 1000 \times . The number of VR chambers with and without sperm was counted to estimate the proportion of chambers containing sperm.

EGG PRODUCTION, FERTILIZATION, AND HATCHING

After a male had been in a cage with three females for 7 days, a folded piece of black construction paper soaked in diluted corn puree was placed in the cage for 3 days as an oviposition site. In the first set of crosses, the paper was subsequently placed in an empty plastic container with damp cotton for 1 week prior to scoring hatching success. For the second set of crosses, the paper was placed in a plastic container for 3 days prior to scoring fertilization success. Egg production was scored in both cases as the number of eggs produced per female per day.

To score hatching success, eggs were inspected and counted under a dissecting microscope 1 week after removal from the cage. Hatched eggs had a distinctive empty chorion that deflated upon probing. Unhatched eggs remained intact upon probing and appeared opaque due to lack of fertilization or development.

To score fertilization success, we removed the chorion and the vitelline membrane and then stained and examined each egg under UV to assess whether cell division had occurred. We removed the chorion by 2 min immersion in 50% commercial bleach. Dechorionated eggs were transferred to a glass vial for removal of the vitelline membrane following the protocol of Weischaus and Nusslein (1986). Eggs were then transferred to a slide, stained with Hoechst 33258, and inspected at 100 \times with UV fluorescence. Eggs with multiple cell nuclei were scored as fertilized.

Egg production was combined for both sets of crosses and scored as the average number of eggs laid by a female in a day. The natural log (+1) was taken to improve normality.

SPERM PRECEDENCE

To test for competitive gametic isolation, we used two pairs of *T. dalmanni* populations (G-S and G-L) that readily produced offspring in single heteropopulation matings to maximize the potential for sperm to interact. To score second male sperm precedence (P2), we mated females to two males, either both from the same population or one from a different population (i.e., AA_A, AA_B, AB_A, BA_A). We performed all 15 possible double-mated crosses (cf. Fig. 5) involving the two pairs of populations. This nested design enabled us to test for effects of cross type and

parental population of origin. Cross type is denoted as [Male 1 population][Male 2 population]_[female population].

Each cross type was replicated 10 times. For each replicate, one mature virgin female and one mature male were placed in a cage and left to copulate for 3 days. The male was then removed and frozen. A second male was then placed in the cage with the female for 3 days and then removed and frozen. The female was allowed to lay eggs for 7 days following removal of the second male.

Eclosed offspring were collected daily and frozen for subsequent parentage analysis. To reduce unnecessary genotyping, we first sampled 10 offspring per female, approximately one-third of the total number of eggs laid—females produce 2–3 eggs per day, on average (Wright et al. 2004; Wilkinson et al. 2006). If all 10 offspring were sired by one male, we inferred that this male sired all offspring. If both paternal genotypes were detected, indicating mixed paternity, then another sample of 10 offspring was genotyped (if enough offspring existed) to better estimate the proportion of offspring sired by each male. Females producing fewer than 10 offspring were excluded from analysis.

All potential parents and offspring were genotyped at three informative autosomal microsatellite loci: 174, 249, and 402a (Wright et al. 2004). Paternity was assigned by the presence of at least one unique PCR product shared between a male parent and offspring. If putative fathers could not be discriminated based on those loci, then up to five additional autosomal loci (402b, 301, 90, 262z, and 39p) were typed. DNA was extracted using Qiagen DNeasy kits. PCR products were separated on an ABI 3730xl DNA analyzer and Genemapper version 4 was used to score fragment length. A total of 1257 progeny from 117 females were collected and genotyped. In total, paternity was successfully assigned in 93% of broods.

STATISTICAL ANALYSIS

Noncompetitive reproductive isolating mechanisms

To identify stages of the reproductive process where incompatibilities may occur, we used nested generalized linear models (GLMs), in which the direction of the cross was nested within each population pair and population pair was nested within the divergence category (one of four as described above) of the cross. In this model, a significant effect of divergence category indicates an incompatibility and a significant effect of population pair means that incompatibilities differ among equally divergent populations. Asymmetric incompatibilities in which the reciprocal crosses differ are detected by the cross-direction effect. Such incompatibilities are expected if male and female reproductive traits coevolve in isolated populations and diverge over time (Knowles and Markow 2001). We included the ages of both the male and female in the model. Data for sperm transfer, motility, and survival were not available for combinations of male and female

populations where mating did not occur. In most cases, these crosses involved the C population of *T. dalmanni*. Therefore, the C population was not included in the GLMs for these sperm variables. In contrast, females readily lay unfertilized eggs, so egg fertilization and hatching success could be measured for all crosses.

Because successful hatching depends on a series of sequential events, reproductive incompatibilities at early stages may influence later stages. In stalk-eyed flies, the sequence of steps are mating, sperm transfer and storage in the spermathecae, sperm survival and motility in the spermathecae, proportion of chambers in the VR containing sperm, egg fertilization, and egg hatch. To identify specific incompatibilities at a stage where a significant effect was detected by the GLM analysis, we used two complementary methods. In the first approach, we conducted an outlier analysis between successive steps in the reproductive process. For example, we compared the average number of sperm stored in spermathecae to the proportion of females that mated for all possible crosses. In such a comparison, an incompatibility associated specifically with sperm storage would score high for mating but low for sperm storage. We conducted similar comparisons between the proportion of VR chambers with sperm and the number of sperm in spermathecae, the proportion of eggs fertilized and the proportion of VR chambers with sperm, and the proportion of eggs hatched and the proportion of eggs fertilized. This last comparison distinguishes between noncompetitive gametic isolation and hybrid inviability. For each of these comparisons, we used least square mean values for each cross type so that data from the two sets of crosses could be compared. We then used variation among crosses to estimate standard errors and determine if the decrease in reproductive performance for any between population cross was a significant outlier, using a generalized extreme Studentized deviate test (Rosner 1983) from a line that passed through the origin and the means of the four within population crosses (cf. Fig. 2).

The second approach involved estimating absolute and relative contributions to RI following the method of Ramsey et al. (2003). This method assumes that components of RI combine multiplicatively and sequentially, with the strength of any particular component ranging from 0 to 1. The absolute contribution (AC) of the first component, that is mating, to RI is $AC_1 = RI_1$. The contribution of the next component, that is sperm stored, is $AC_2 = RI_2(1 - AC_1)$ and the contribution of the third component, sperm in the VR, is $AC_3 = RI_3[1 - (AC_1 + AC_2)]$, etc. The sum of ACs across all reproductive stages measures total RI and can be used to estimate relative contributions at any stage. We estimated the strength of RI for any particular cross at any stage, RI_i , as $1 - (\text{between cross performance})/(\text{average within cross performance})$. We then express the relative contribution of each component as a proportion of egg hatching success for each cross

Table 1. Results from generalized linear models using divergence category, population pair nested in divergence category, and cross direction nested in divergence category and population pair for each stage of reproduction. Likelihood-ratio χ^2 values for the full model and for each effect are shown.

Dependent variable (exptl cross)	Distribution	<i>N</i>	Model	Divergence category	Population pair	Cross direction	Male Age	Female Age
Mating (1,2)	Binomial	471	265.6 ⁴	230.9 ⁴	17.7 ²	32.1 ⁴	0.0	0.4
Log ₁₀ sperm stored (1) ¹	Normal	102	69.3 ⁴	29.5 ⁴	20.3 ³	34.8 ⁴	0.0	4.0
Sperm survival (1) ¹	Normal	93	6.5	0.5	1.1	1.1	0.4	2.7
Sperm motility (2) ¹	Normal	105	13.8	1.4	7.7	4.8	0.2	0.0
Prop. VR with sperm (2) ¹	Normal	124	98.5 ⁴	53.3 ⁴	4.7	45.7 ⁴	4.1	1.5
Prop. eggs fertilized (2)	Normal	250	360.7 ⁴	340.3 ⁴	15.4 ²	42.3 ⁴	1.1	1.1
Prop. eggs hatched (1)	Normal	195	257.1 ⁴	202.6 ⁴	60.4 ⁴	31.8 ⁴	3.4	0.5
Ln(eggs/female/d+1)(1,2)	Normal	453	244.1 ⁴	58.8 ⁴	32.1 ⁴	165.0 ⁴	0.7	2.0

¹Excludes crosses involving population C.

² $P < 0.01$.

³ $P < 0.001$.

⁴ $P < 0.0001$.

to illustrate how reproductive incompatibilities arise over time. To simplify presentation, the few small negative RI estimates were set to 0.

Sperm precedence

To determine if a male from a different population than a female is less successful at producing offspring when his sperm are in competition with sperm from a male from the same population, we carried out a three-way nested analysis of variance (ANOVA) using type of cross (i.e., AA_A, AA_B, AB_A, BA_A), population source of second male, and population source of female as factors. Second male source was nested in cross type and female source was nested in cross type and second male source. Conpopulation sperm precedence predicts a significant effect of cross type with intermediate P2 for the AA_A and AA_B cross types, low P2 for AB_A crosses, and high P2 for BA_A crosses. We used Tukey's HSD test to identify statistical differences among cross types.

To determine if noncompetitive gametic isolation could explain apparent conpopulation sperm precedence, we compared observed to expected P2 for each cross. To estimate expected P2, we utilized counts of sperm transferred and proportion of eggs hatched in noncompetitive single-mated crosses. We assumed sperm are used at random in proportion to the amount passed by a male when singly mated to a female. We then calculated the number of offspring expected to be fathered by each male and multiplied by the proportion of eggs hatched for each male–female population combination to estimate the number of offspring expected to hatch. Expected P2 is then the expected proportion of offspring produced by the second male. We calculated 99% confidence intervals around observed P2 values to determine which deviate from expectation. We used JMP 10.0.2 for all analyses.

Results

NONCOMPETITIVE ISOLATING MECHANISMS

The results of the GLMs for each reproductive stage (Table 1) indicate that multiple noncompetitive gametic incompatibilities would reduce reproduction if secondary contact occurred between these populations. Six variables—mating, number of sperm stored in spermathecae, proportion of VR chambers with sperm, proportion of eggs fertilized, proportion of eggs hatching, and eggs laid—exhibited significant effects of population divergence category indicating that incompatibilities are present. All of these variables except the proportion of the VR with sperm also exhibited differences among population pairs within a divergence category, and all six showed significant differences between the reciprocal crosses. Neither sperm motility nor sperm viability was influenced by any factor in the model. Male and female age also had no effect on any reproductive process (Table 1).

Because reproduction involves sequential events, it is not possible to determine the extent to which events after mating are influenced by prior events using results from each GLM in isolation. Consequently, to determine how each factor influences offspring production, we conducted pairwise comparisons using the significant variables from Table 1 according to the sequential reproductive process. Examination of the number of sperm stored plotted against the proportion of females that mated (Fig. 2A) indicates that all crosses involving the C population exhibit some prezygotic isolation because fewer than 50% of individuals mated. Despite fairly high levels of mating flies from the LS and GS crosses had lower levels of sperm storage than expected suggesting that S females may have difficulty storing sperm from foreign males. In contrast, the reciprocal crosses, SL and SG, had high mating rates and high sperm storage.

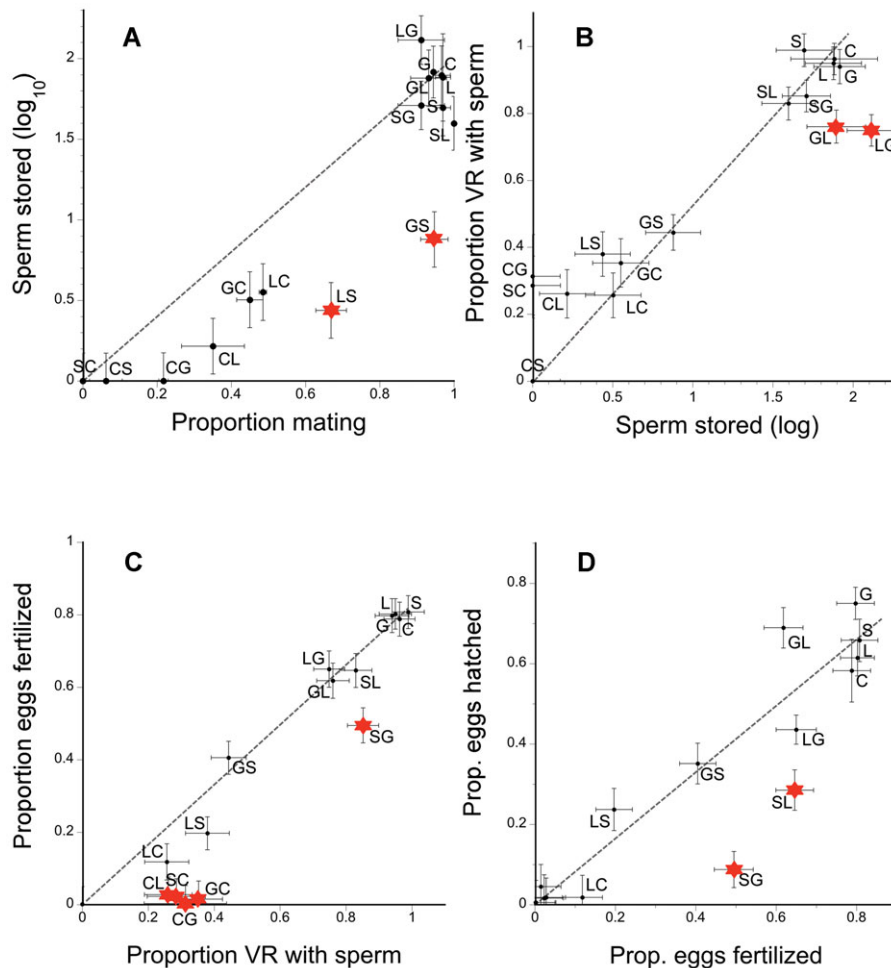


Figure 2. Identification of reproductive incompatibilities by pairwise comparison of sequential reproductive steps between copulation and egg hatch. Least squares means (LSM) and standard error (SE) are shown and labeled by two letters ($m \times f$) for between population crosses or a single letter for within population crosses. In each panel, incompatibilities are indicated by six-point stars for significant outliers from the dashed line, which extends from the origin to the average of the within population crosses, and indicate where (A) sperm are not stored despite evidence of mating, (B) sperm fail to move to the site of fertilization despite being stored, (C) sperm fail to fertilize eggs despite being at the site of fertilization, and (D) eggs fail to hatch despite being fertilized.

The number of sperm stored in the spermathecae was bimodally distributed and strongly related to the proportion of chambers in the VR that contained sperm (Fig. 2B). The four within population crosses and four between population crosses, SL, SG, GL, and LG, exhibited high sperm storage whereas all other crosses showed considerably lower sperm storage and fewer sperm in the VR. The most conspicuous outlier was LG, which had the highest sperm storage of any cross but incomplete sperm movement into the VR. In two crosses, some sperm were found in the VR but not in the spermathecae. This is presumably a consequence of the small number of flies that mated in these crosses and the fact that different individuals were dissected to score each of these variables.

In crosses where sperm was successfully transferred, the proportion of VR chambers containing sperm is highly predictive of

fertilization success except for SG (Fig. 2C). Interestingly, the reciprocal cross, GS, had similar fertilization success, but as shown in Figures 2A and b, this was due to a low number of sperm stored and transferred to the site of fertilization rather than lack of fertilization. In crosses where fewer than 50% of VR chambers contained sperm, fertilization success was low if the populations were no more than 0.5 MY diverged, but essentially zero for more divergent population pairs.

The proportion of eggs fertilized predicted the proportion of eggs hatched (Fig. 2D) with two exceptions. Crosses SG and SL exhibited significant reductions in hatching success compared to fertilization success indicating hybrid inviability. Again, one of the reciprocal crosses, LS, had similar hatching success as SL, but this was due to a lack of sperm transfer (Fig. 2A) rather than hybrid inviability.

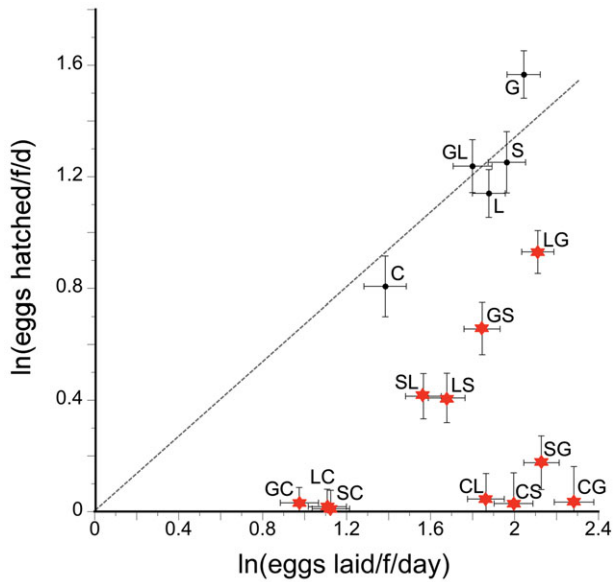


Figure 3. Overall reproductive incompatibility as revealed by the number of eggs hatched plotted against the number of eggs laid per female per day. Least squares means (LSM) and standard error (SE) are shown and labeled by two letters (m × f) for between population crosses or a single letter for within population crosses. Stars indicate significant outliers from the dashed line, which extends from the origin to the average of the within population crosses.

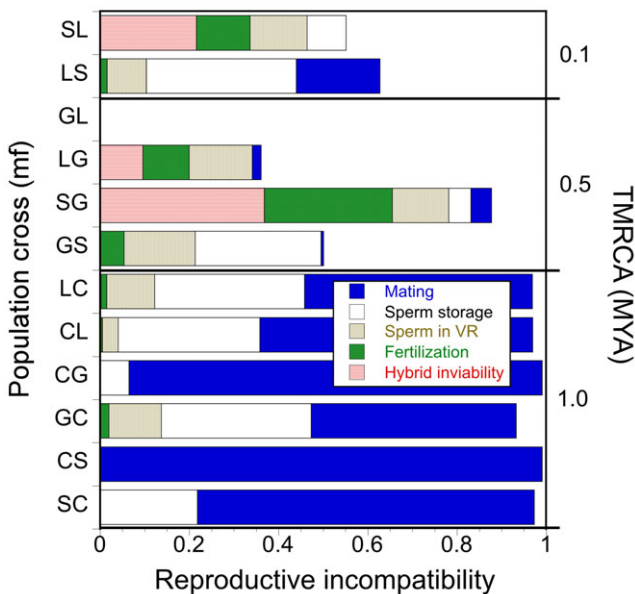


Figure 4. Relative contributions of different reproductive stages to total reproductive incompatibility as measured by proportion of eggs hatching for each population cross. See text for methods. Crosses are grouped and ordered by average estimated time to most recent common ancestor (TMRCA) to the nearest 0.1 MY as reported in Swallow et al. (2005).

Examination of the number of eggs hatched in comparison to the number of eggs laid clearly illustrates that all between population crosses except GL exhibit some amount of reproductive incompatibility (Fig. 3). Furthermore, the number of eggs laid by a female was highly dependent on female population, indicating that source of the male had little effect on female fecundity. However, fecundity of females mated to foreign males was in most cases less than fecundity of females mated to same population males.

Partitioning total RI, as measured by proportion of eggs that hatch, into components (Fig. 4) provides an alternative way of visualizing how incompatibilities accumulate in populations at different times of divergence. The relative strength of the incompatibility is indicated by the length of each bar and matches the patterns revealed in Figure 2. However, inspection of Figure 4 also reveals that premating or sexual isolation has very weak effects on populations that have diverged less than 1 MYA, but very strong effects on more divergent populations. In contrast, more recently diverged populations show strong asymmetry in the effects of individual postcopulatory components, as well as in total RI, with the GxL and SxG crosses providing extreme examples of both patterns.

COMPETITIVE ISOLATING MECHANISMS

The three-way nested ANOVA on second male sperm precedence (P2) revealed a significant effect of cross type ($F = 16.1, P < 0.0001$), source of second male population ($F = 2.5, P = 0.014$), and source of female population ($F = 8.9, P = 0.0003$). Results of Tukey’s HSD post hoc tests (Fig. 5) provide apparent evidence for conpopulation sperm precedence. The crosses in which both males are from the same population (AA_A and AA_B) had intermediate P2 values ($P2_{AA_A} = 0.65, P2_{AA_B} = 0.73$). The crosses in which the first male was from a different population and the second male was from the same population as the female had high second male paternity ($P2_{AB_B} = 0.96$). Finally, the crosses with a conpopulation first male and heteropopulation second male, on average, showed lower second male paternity ($P2_{AB_A} = 0.39$). Therefore, it appears that conpopulation males have a sperm competitive advantage over heteropopulation males regardless of male order. However, the magnitude of this effect clearly depends on which combination of populations is considered. Crosses involving the G and S populations exhibited strong conpopulation sperm precedence with P2 close to one when second male and female were from the same population and P2 close to zero when the second male and female were from different populations. In contrast, the crosses involving the G and L populations showed high P2 when the second male and female were from the same population but also elevated P2 when the second male and female were from different populations (Fig. 5).

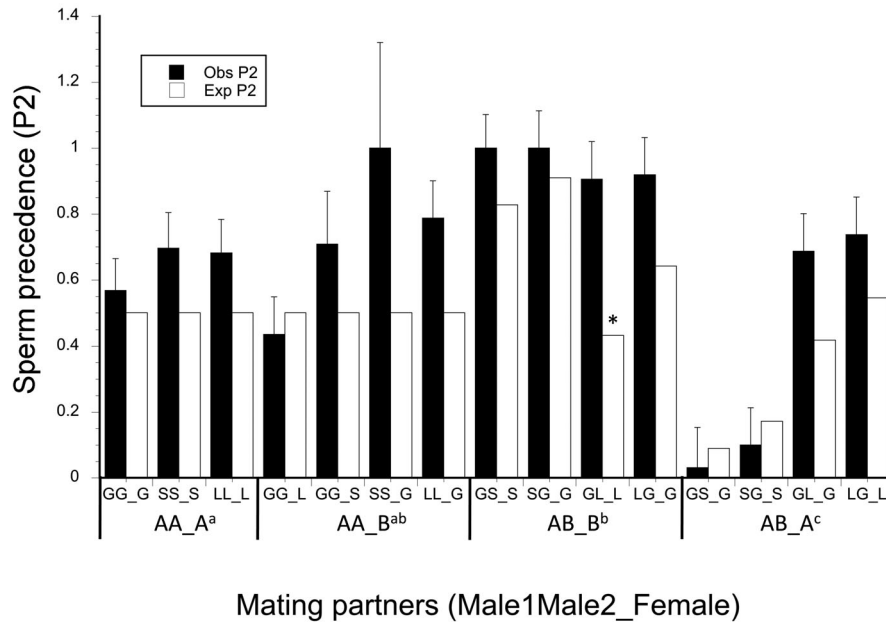


Figure 5. Least squares means (LSM) and standard error (SE) second male paternity (P2) for each cross type involving two pairs of populations. Tukey's HSD significance levels are indicated by superscripts for cross-type and conform to predictions of conspecific sperm precedence, that is the AB_B crosses have high P2 and the AB_A crosses have low P2. Expected P2 bars illustrate patterns of sperm precedence derived from counts of sperm transferred and hatching success in single matings. Asterisk indicates cross-type where expected P2 falls outside the 99% confidence interval for observed P2.

To determine the degree to which noncompetitive gametic isolating factors might contribute to apparent conpopulation sperm precedence, we estimated expected P2 using counts of sperm stored and hatching success from females singly mated to males from each population. Comparison of observed to expected P2 for each mating combination revealed that in only one case, the GL_L cross, did the expected value of P2 fall outside the 99% confidence interval in the direction expected for conpopulation sperm precedence.

Discussion

Sexual selection is often invoked as a mechanism for speciation, either due to rapid divergence of courtship traits (Panhuis et al. 2001) or characteristics of ejaculates and fertilization factors (Panhuis et al. 2003; Birkhead and Brillard 2007; Ramm et al. 2009; Aagaard et al. 2010) but rarely are the two processes considered together. The potential importance of both pre- and post-copulatory sexual selection in stalk-eyed flies makes them ideal for such a comparison. By using genetically isolated populations of *T. dalmanni* with different divergence times, we identified where and when reproductive incompatibilities have arisen. Below we summarize our findings and draw inferences about how RI evolves in these flies.

NONCOMPETITIVE GAMETIC ISOLATION

We measured eight variables potentially related to RI: mating, number of sperm stored, sperm survival, sperm motility, presence of sperm at the site of fertilization, number of eggs laid, proportion of eggs fertilized, and proportion of eggs hatched. Six of these—mating, sperm number, proportion of VR chambers with sperm inside, proportion of eggs fertilized, proportion of eggs hatching, and eggs laid—were significantly affected by the amount of divergence between population pairs and by the direction of the cross and, therefore, are candidates for causing reproductive incompatibilities. In each case, reproductive competency depends on the direction of the cross. Surprisingly, neither sperm viability nor sperm motility was significantly affected by divergence time or cross direction. Furthermore, sperm viability was high (typically 90% or greater) in most crosses. Thus, the internal female reproductive environment appears to be largely benign to foreign sperm at least among these closely related populations. These results suggest that females do not exert cryptic choice by selectively killing stored sperm. Therefore, if female choice exists, it must occur as a consequence of biased sperm movement inside the female reproductive tract.

Pairwise comparison of sequential stages in the reproductive process reveals three potentially generalizable patterns in the evolution of postcopulatory RI in stalk-eyed flies. First, in many cases incompatibilities involve several steps in the reproductive

process (cf. Fig. 4). For example, the SG cross exhibited a reduction in fertilization success and an additional reduction in hatching success. The most genetically divergent population, Cameron, showed nearly complete RI from the other three populations. In most crosses involving the C population, the primary cause of RI was reduced mating and decreased sperm transfer. These results suggest that premating sexual isolation evolves more slowly in these populations of flies than gametic isolation. One possible explanation for this pattern could be that sperm or female reproductive tracts or both are evolving more rapidly than the traits associated with sexual isolation. Prior studies have shown that eyespan-body length allometry does not differ among these populations (Swallow et al. 2005), so the trait that is under precopulatory sexual selection within populations, that is relative eyespan (Wilkinson and Reillo 1994; Wilkinson et al. 1998), does not appear to be important for RI.

Second, both postcopulatory and postzygotic incompatibilities evolve rapidly, that is within the 80,000 years since populations S and L have diverged. The hatching success when these two populations are crossed is less than half that of either parental population. Previous work has shown that hybrid males produced by either possible reciprocal pairing of these populations are also sterile (Christianson et al. 2005), indicating that both hybrid sterility and inviability evolve rapidly.

The S and L populations also provide an example of the third pattern, which is that most incompatibilities are asymmetric, that is they differ in strength and type among reciprocal pairings of the same two populations, an observation sometimes referred to as Darwin's corollary to Haldane's rule (Turelli and Moyle 2007). For example, in the LS cross hatching success is low because relatively few sperm are stored after mating and that effect influences subsequent steps. In contrast, plenty of sperm are stored in the reciprocal SL cross, those sperm make it to the site of fertilization and a high proportion of eggs are fertilized, but only half of the fertilized eggs hatch, indicating the presence of a postzygotic incompatibility. Sperm storage and hybrid viability also differ dramatically between reciprocal crosses involving the G and S as well as the L and G populations.

Differences in the form of incompatibility between reciprocal pairings of the same two populations are expected when there is rapid coevolution of male ejaculates and female reproductive tracts. For example, if sperm length and female sperm storage organs increase in population A but decrease in population B, long A sperm may be incompatible with female sperm storage organs in population B whereas short B sperm may be compatible with A population sperm storage organs. We know that sperm length and sperm storage organs vary greatly in size and exhibit correlated change between the sexes among genera and species in diopsids (Presgraves et al. 1999). Sperm length and female sperm storage organs are also heritable (Wilkinson et al. 2005; Johns and Wilkin-

son 2007), so it is reasonable to assume that isolated populations within species would exhibit variation in these reproductive traits, although this prediction deserves to be tested. Because a sperm must completely enter the egg to cause fertilization and trigger embryonic development (Karr 1991), variation in sperm length might also influence fertilization success. The process causing differences in reproductive traits, such as sperm length or sperm storage organ size, cannot be inferred from the data presented here, but two possibilities can be considered.

Correlated change in male and female traits can potentially occur either by sexual selection or by antagonistic coevolution in response to sexual conflict. In the former case, population divergence is expected if isolated populations are founded by a small number of individuals such that genetic drift can occur (Lande 1981). In the latter case, divergence is expected to be greater in large populations (Gavrilets 2000), which retain more genetic variation in the face of strong selection. Antagonistic coevolution further assumes that females resist mating due to a cost of reproduction. One of the best examples of antagonistic coevolution among reproductive traits involves water striders in which female morphology has evolved in response to male clasping (Arnqvist and Rowe 2002), a male postmating behavior that decreases female survival (Rowe 1994). In contrast, in *T. dalmanni* female fertility increases when females mate multiple times (Baker et al. 2001), females allowed to mate had similar survival to those kept as virgins, and egg production did not differ between virgin and mated females (Reguera et al. 2004). Sexual conflict before or after mating appears, therefore, to be low or absent, which is consistent with a life history in which females mate frequently (Lorch et al. 1993; Wilkinson et al. 2003), receive few sperm in a mating (Lorch et al. 1993), lay few eggs each day, and live for months (Wilkinson et al. 2006). Thus, differences in reproductive traits that give rise to gametic incompatibilities are more likely to result from postcopulatory sexual selection via sperm competition, cryptic female choice, or both than from antagonistic coevolution.

HYBRID INVIABILITY

One goal of these experiments was to discriminate between noncompetitive gametic isolation and postzygotic isolation in heteropopulation crosses where hatching success has been observed to decrease with genetic distance (Christianson et al. 2005). A decrease in hatching success could be a product of noncompetitive gametic isolation (i.e., unhatched eggs were unfertilized) or postzygotic isolation (i.e., unhatched eggs were zygotes that failed to develop due to hybrid inviability). To distinguish between these types of incompatibilities, we compared fertilization success to hatching success and found that two of 12 heteropopulation crosses examined have significantly lower hatching success than expected given their fertilization success. An obvious hypothesis is that hybrid inviability in these two cases (SG

and SL) results from the accumulation of deleterious epistatic Dobzhansky–Muller incompatibilities (Orr and Turelli 2001). Interestingly, both of these cases involve the same paternal population and could, therefore, be due to incompatibilities associated with Y-linked genes that interact with either X or autosomal loci. Because Y-linked incompatibilities would only affect male offspring, they could result in female-biased sex ratios, a potential outcome that deserves further study, especially given the presence of sex chromosome meiotic drive in these flies (Presgraves et al. 1997; Wilkinson et al. 2003). Incompatibilities arising from sex-linked genetic interactions are a common cause of asymmetries in postzygotic RI (Turelli and Moyle 2007). Sex-linked genetic interactions could be expected to evolve rapidly in stalk-eyed flies given that these flies contain a neo-X chromosome (Baker and Wilkinson 2010) and both gene duplication and gene movement on and off the sex chromosomes occurs frequently (Baker et al. 2012).

COMPETITIVE GAMETIC ISOLATION

Analysis of sperm precedence patterns appears to indicate that sperm competition favors the conpopulation male. However, most of the variation in P2 can be explained once the number of sperm transferred and proportion of eggs hatched for each cross is considered. Examination of expected and observed P2 for the 15 different crosses reveals only one case, GL_G, in which observed P2 is greater than expectation, and in the direction expected if conpopulation sperm precedence occurs. In all other mating combinations, expected and observed P2 do not differ indicating that sperm mixing alone is sufficient to predict the pattern of second male sperm precedence. These results do not rule out the possibility that competitive gametic isolation exists, they simply indicate that most of the variation in P2 can be explained without invoking additional sperm competitive interactions.

EVOLUTION OF MULTIPLE REPRODUCTIVE ISOLATING BARRIERS

Reproductive isolation among populations of stalk-eyed flies arises via accumulation of multiple reproductive isolating barriers over time. Among the most distantly related populations, premating isolation is the primary barrier observed. Given that these populations are allopatric, reinforcement is not currently occurring, although we cannot discount the possibility that these populations have had some contact in the past given that sea level has fluctuated in this region over the past million years (Hanebuth et al. 2000; Miller et al. 2005). However, among closely related populations of stalk-eyed flies, prezygotic isolation is largely absent but both gametic and postzygotic isolation exist. These results appear to be in contrast to *Drosophila*, where prezygotic and postzygotic isolation have been inferred to evolve at similar rates among allopatric populations (Coyne and Orr 1989, 1997).

One obvious difference between many species of *Drosophila* and most diopside is that *Drosophila* males often exhibit elaborate multimodal courtship behaviors that females use for mate recognition (Spieth 1952; Markow 1987; Cobb and Ferveur 1995). The absence of obvious courtship behaviors in *Teleopsis* may reduce opportunities for prezygotic isolation to operate (Wilkinson and Johns 2005).

These results, in combination with the findings of Christianson et al. (2005), provide evidence that RI evolves rapidly among populations of *Teleopsis* stalk-eyed flies. This conclusion is consistent with an emerging trend in the study of RI: that multiple reproductive isolating barriers act among populations (Coyne and Orr 1997; Malone and Fontenot 2008; Matsubayashi and Katakura 2009; Dopman et al. 2010; Stelkens et al. 2010). Even if each barrier is incomplete, in combination, multiple barriers could prevent gene flow (Matsubayashi and Katakura 2009; Sobel et al. 2010; Jennings et al. 2011). If concurrent evolution of numerous incomplete reproductive isolating barriers is commonplace, then conclusions about the importance of a given form of RI should be tentative until all types of RI have been studied. Furthermore, studies aiming to identify the genetic changes responsible for RI need to focus on recently diverged population pairs with few well-characterized isolating factors.

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DATA ARCHIVING

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